

Sox4 functions as a positive regulator of β -catenin signaling through upregulation of *TCF4* during morular differentiation of endometrial carcinomas

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Sox factors function as either activators or repressors of β -catenin/TCF transcription depending on the cellular context and associated interacting proteins. Our previous study provided evidence that alteration in β -catenin signaling is an essential event during transdifferentiation toward the morular phenotype of endometrial carcinomas (Em Cas). Here, we focused on related functional roles of Sox factors. Of eight Sox factors investigated, Sox4 could enhance β -catenin/TCF4 transcription, through upregulation of *TCF4* at the transcription level, without any direct β -catenin association. Cells stably overexpressing Sox4 showed significant decreases in proliferation rate, along with increases in expression of p21^{WAF1}, as well as TCF4, in contrast to increased cell growth observed with knockdown. Of these factors, only Sox7 could transcriptionally upregulate Sox4 expression, but it also resulted in not only inhibition of Sox4-mediated activation of β -catenin/TCF4-driven transcription, but also repression of its own promoter activity, indicating the existence of very complex feedback loop for Sox-mediated signal cascades. Finally, Sox4 immunoreactivity was frequently pronounced in morular lesions of Em Cas, the expression being positively correlated with status of β -catenin, TCF4, and Sox7, and inversely with cell proliferation. These data therefore suggest that Sox4 may serve as a positive regulator of β -catenin signaling through alteration in TCF4 expression during morular differentiation of Em Ca cells, leading to inhibition of cell proliferation. In addition, Sox7 may also participate in the process, having complex roles in modulation of signaling.

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The sex-determining region Y (Sry) box or Sox factors, comprising 20 highly conserved transcription molecules that have important roles in diverse developmental process in most vertebrates, feature two functional domains: an Sry-related high-mobility group (HMG) box, located in the N-terminal half of the protein, and a transactivation domain, located at the C terminus.^{1–3} On the basis of protein sequence comparisons, Sox factors are divided into eight groups, A–H, and those within each subgroup share conserved structural domains outside the HMG domain, functioning as either transcriptional activators or repressors depending on the cellular context and associated interacting proteins.^{4,5}

Sox4, a member of the SoxC group, preferentially binds to the A/T A/T CAAA T/G DNA motif and induces bending of

DNA, thus facilitating protein–protein or protein–DNA interactions.^{6,7} In general, its expression is high in embryonic neural progenitors and mesenchymal cells in many developing organs, but becomes more restricted after birth, as cells reach their final differentiation state.⁸ However, upregulation is observed in response to progestin through progesterone receptor binding to the gene in mammary glands and uterus of mice,^{9,10} and overexpression has been reported in a variety of human malignancies, such as leukemias, melanomas, glioblastomas, and cancers of the prostate, bladder, and lung,^{11–13} although it remains to be clarified what roles the factor has *in vivo* and during tumorigenesis.

Focal squamous differentiation into a morular phenotype in endometrial carcinomas (Em Cas) is considered to be linked with an increased probability of survival, implying

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that the elucidation of the mechanisms may provide clues in understanding of biological behavior.^{14–16} Our previous studies revealed that accumulation of β -catenin in nuclei may have an essential role in an induction of transdifferentiation toward morular phenotype of Em Ca cells, leading to inhibition of cell proliferation through cooperation with other many signaling pathways.^{17–20} Given the documented possible roles of Sox4, as well as other Sox factors, in β -catenin/TCF-driven transcription,²¹ we hypothesized that the factor may also contribute to the morular differentiation processes. To test this, we investigated here Sox4 expression, with reference to status of other Sox factors, β -catenin, TCF4, and cell proliferation, using Em Ca tissues and cell lines.

MATERIALS AND METHODS

Plasmids, Cell Lines, and Antibodies

Full-length cDNAs of human *Sox3*, *Sox4*, *Sox5*, *Sox6*, *Sox7*, *Sox9*, and mouse *Sox11* (Open Biosystems, Huntsville, AL, USA) with or without an HA tag were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pEGFP vectors (BD Biosciences Clontech, Worcester, MA, USA). A full-length cDNA for human *Sox17* (GenBank accession number NM022454) was also generated by PCR. The human *Sox4* promoter (AL136179) between -2647 and $+11$ bp from transcription start site was amplified by PCR, and subcloned into the pGL-3B vector (Promega, Madison, WI, USA). The human *Sox7* promoter (NM031439) between -1633 and -462 bp from translation start site was also generated using similar procedures, and a series of 5'-truncated promoter constructs of *TCF4* and *Sox7* genes were generated by PCR- and enzyme-digestion-based methods, as described previously.¹⁹ Site-directed mutagenesis in putative Sox-binding sites in *TCF4* gene was also carried out using

Mega-PCR methods.²² The identity of all constructs was confirmed by sequencing before use. The sequences of PCR primers employed in this study are listed in Supplementary Table S1. pcDNA3.1-HA- β -catenin Δ S45, pM- β -catenin, pG5-luc, pUSE-Myc-TCF4, pCI-Flag-p300, p21^{WAF1}-luc, and Top reporter constructs were the same as described previously.^{17–20}

Table 1 Correlations among mRNA expression of Sox factors in Em Ca cells

	Sox3 <i>r</i> (<i>P</i>)	Sox4 <i>r</i> (<i>P</i>)	Sox5 <i>r</i> (<i>P</i>)	Sox6 <i>r</i> (<i>P</i>)	Sox7 <i>r</i> (<i>P</i>)	Sox9 <i>r</i> (<i>P</i>)	Sox11 <i>r</i> (<i>P</i>)
Sox4	0.66 0.04	* *	* *	* *	* *	* *	* *
Sox5	0.53 0.1	0.73 0.02	* *	* *	* *	* *	* *
Sox6	0.4 0.2	0.64 0.04	0.52 0.09	* *	* *	* *	* *
Sox7	0.45 0.2	0.66 0.038	0.65 0.04	0.78 0.01	* *	* *	* *
Sox9	-0.64 0.8	-0.35 0.27	0.28 0.38	-0.37 0.24	-0.25 0.43	* *	* *
Sox11	0.74 0.02	0.56 0.08	0.63 0.04	0.29 0.36	0.42 0.18	-0.14 0.65	* *
Sox17	0.46 0.14	0.48 0.12	0.43 0.17	0.27 0.38	0.01 0.98	-0.38 0.22	0.68 0.03

r: Spearman's correlation coefficient.

*Not examined.

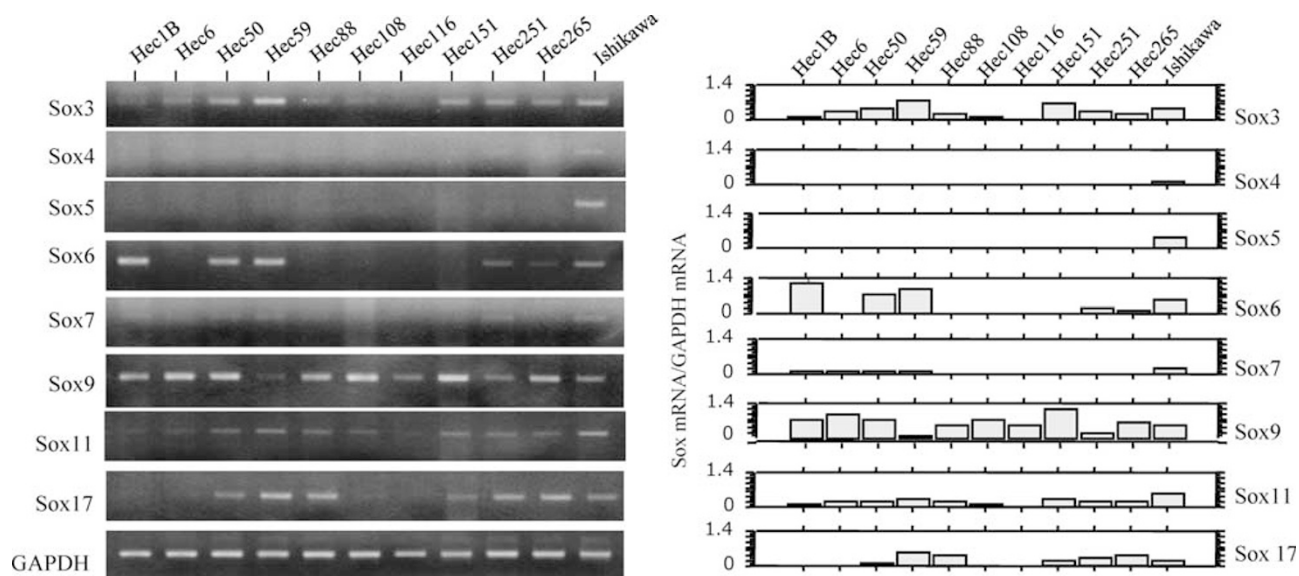


Figure 1 mRNA expression of Sox factors in Em Ca cells. Left: mRNA expression of eight Sox factors in 11 Em Ca cell lines detected by RT-PCR assay. Right: relative amounts of the Sox mRNA expression levels calculated by normalization to signals for the *GAPDH* gene using NIH Image.

Eleven endometrial cancer cell lines, Hec1B, Hec6, Hec50, Hec59, Hec88, Hec108, Hec116, Hec151, Hec251, Hec265, and Ishikawa cells, were maintained in Eagle's MEM with 10% bovine calf serum.^{23,24} To establish cells stably over-expressing HA-Sox4, expression plasmids or empty vectors were transfected into Ishikawa cells, and two independent clones were established, as described previously.^{17,20}

Anti-Sox4 (ab52043) was supplied by Abcam (Cambridge, MA, USA). Anti-Sox7 (HPA009065), anti-TCF4 (HPA025958), anti-Flag M2, and anti- β -actin were from Sigma Chemical (St Louis, MO, USA). Anti- β -catenin was from Transduction Laboratories (Lexington, KY, USA), anti-p21^{WAF1} and anti-Ki-67 were from Dako (Copenhagen, Denmark), and anti-HA and anti-Myc were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transfection

Transfection was carried out using Lipofectamine PLUS (Invitrogen) in duplicate or triplicate. Luciferase activity was assayed, as described previously.^{17–20} In addition, siRNAs for Sox4, as well as the negative controls, were transfected using siPORT NeoFX transfection agent (Ambion, Austin, TX) according to the manufacturers' instructions.²⁵

RT-PCR

Amplification was carried out in the exponential phase (20–28 cycles) to allow comparison among cDNAs synthesized from identical reactions, using specific primers (Supplementary Table S1). Primers for the *GAPDH* gene were also applied as described previously.^{17–20} The intensity of

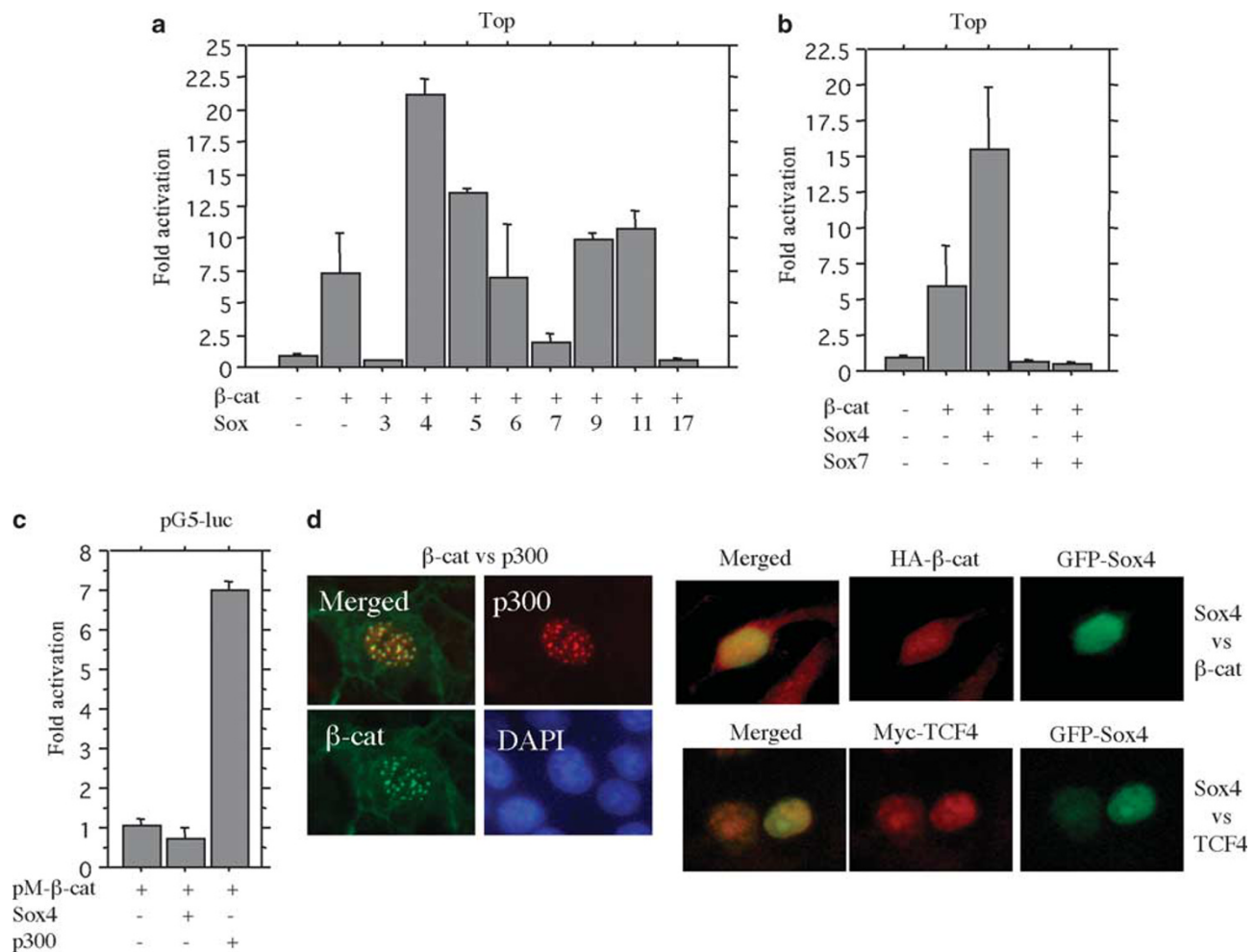


Figure 2 Activation of β -catenin/TCF4-driven transcription by Sox4. **(a)** Ishikawa cells were transfected with a Top reporter construct, along with expression plasmids for β -catenin lacking Ser45 and eight Sox factors. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means \pm s.d.s. The experiment was performed in triplicate. **(b)** Ishikawa cells were transfected with Top reporter constructs, together with β -catenin lacking Ser45, Sox4, and Sox7. **(c)** Results of one hybrid assay. Ishikawa cells were transfected with pGL5-luc, along with pM- β -catenin, Sox4, and p300. **(d)** (left) After co-transfection of β -catenin and Flag-p300, staining with anti- β -catenin and anti-Flag was performed. Right: after co-transfection of GFP-Sox4 and either β -catenin or TCF4 into Ishikawa cells, staining with anti-HA or anti-Myc antibodies was performed.

individual signals was measured with the NIH Image software, as detailed earlier.²⁵

Immunofluorescence

After transfection of GFP-Sox4, together with HA- β -catenin, Myc-TCF4, or Flag-p300, the cells were incubated with anti- β -catenin, anti-TCF4, anti-HA, anti-Myc, and anti-Flag antibodies, respectively. FITC- or rhodamine-labeled anti-rabbit IgGs (Molecular Probes, Leiden, The Netherlands) were used as secondary antibodies, as described previously.^{17–20}

Western Blot Assays

Total cellular proteins were prepared using RIPA buffer (50 mM Tris-HCl (pH 7.2), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and western blot assays were carried out as described previously.^{17–20}

Clinical Cases

Histological findings were reviewed for hysterectomy specimens of endometrioid-type Em Cas in the case records of Kitasato University Hospital during the period from 2000 to 2008, according to the criteria of the 2003 World Health Organization classification.²⁶ Areas consisting of spindle-shaped cells forming growth sheets were defined as morules, as described previously.^{17–20} Each Em Ca was also staged according to the 1988 International Federation of Gynecology and Obstetrics (FIGO) staging system.²⁷ A total of 48 cases of Em Cas without morules, including 29 of grade 1 (G1) and 19 of G2 or G3, as well as 25 of atypical hyperplasia (AH), were investigated. Of those available, 30 were subcategorized as stage I and 18 as stage II–IV, whereas 28 demonstrated upper invasion (less than half of the myometrial depth) and 20 lower invasion, as well as 10 for positive and 35 for negative nodal metastasis. A total of 38 cases of G1 tumors

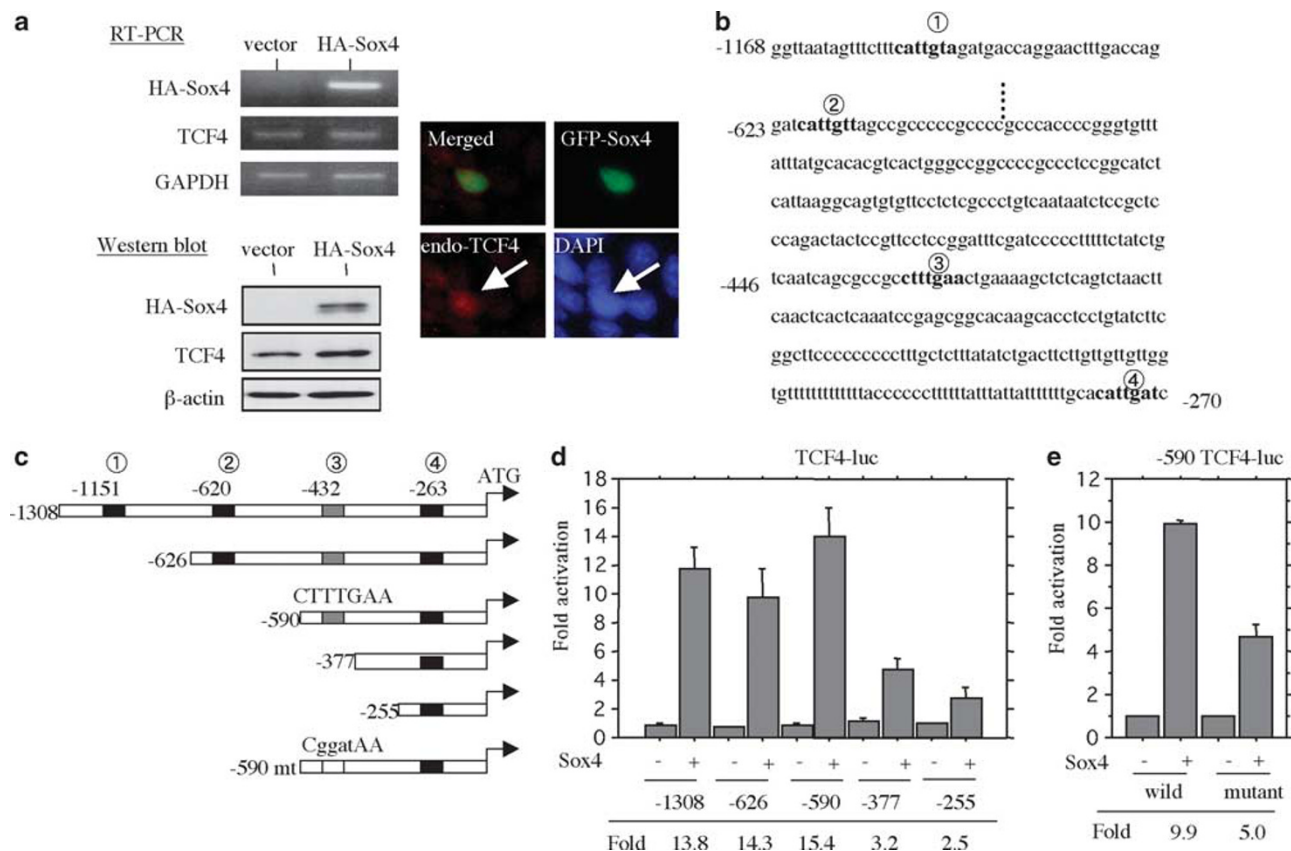


Figure 3 Identification of Sox4-responsive elements in the *TCF4* promoter. (a, left) Analysis of mRNA and protein expression levels for the *TCF4* gene with total RNA or protein extracted from HA-Sox4-transfected Ishikawa cells using RT-PCR (upper) and western blot (lower) assays, respectively. HA-5' forward (5'-TACCATAACGATGTTCCAGATTACGC-3') and Sox4-reverse (Supplementary Table S1) primers were used. Right: after transfection of GFP-Sox4, staining with anti-TCF4 was performed. Note the increased expression of endogenous TCF4 in cells expressing GFP-Sox4 (indicated by arrows). (b) The *TCF4* promoter sequence containing four putative Sox-binding sites. (c) Various promoter deletion constructs used for evaluating transcriptional regulation of the *TCF4* promoter by Sox4. Results of introduction of a mutation into pGL3(-590)-*TCF4*-luc are also demonstrated. (d) Ishikawa cells were transfected with various 5'-truncated constructs of the *TCF4* promoter, along with Sox4 expression plasmids. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means \pm s.d.s. The experiment was performed in triplicate. (e) Ishikawa cells were transfected with mutant constructs of the *TCF4* promoter, together with Sox4 expression plasmids.

with morules were also selected. In addition, 40 biopsy specimens of normal endometrial tissues, including 10 in the proliferative and 30 in secretory phases, were investigated, the latter being subclassified into 10 early and 20 middle and late phases, according to the criteria of Mutter *et al.*²⁸ All tissues were routinely fixed in 10% formalin and processed for embedding in paraffin wax. Approval for this study was given by the Ethics Committee of the Kitasato University School of Medicine (B07-16).

Immunohistochemistry

Immunohistochemistry was performed using a combination of the microwave-oven heating and polymer immunocomplex (Envision, Dako) methods, followed by evaluation, in terms of immunopositive nuclei counted, to allow calculation of labeling indices (LIs), as described previously.^{17–20}

Sox7 Promoter Methylation Assay

Genomic DNAs were treated by bisulfate using the EZ DNA Methylation-Gold Kit (ZYMO Research, Orange, CA, USA). Methylation-specific PCR for *Sox7* promoter was carried out, as described by Guo *et al.*²⁹

Statistics

Comparative data were analyzed using the Mann–Whitney *U*-test and the Spearman's correlation coefficient. The cutoff for statistical significance was set as $P < 0.05$.

RESULTS

Activation of β -Catenin/TCF4-Driven Transcription by Sox4 in Em Ca Cells

As Sox factors are frequently co-expressed,² we first examined these mRNA expression levels in 11 Em Ca cell lines. Relatively high mRNA expression for Sox3, Sox6, Sox9, Sox11,

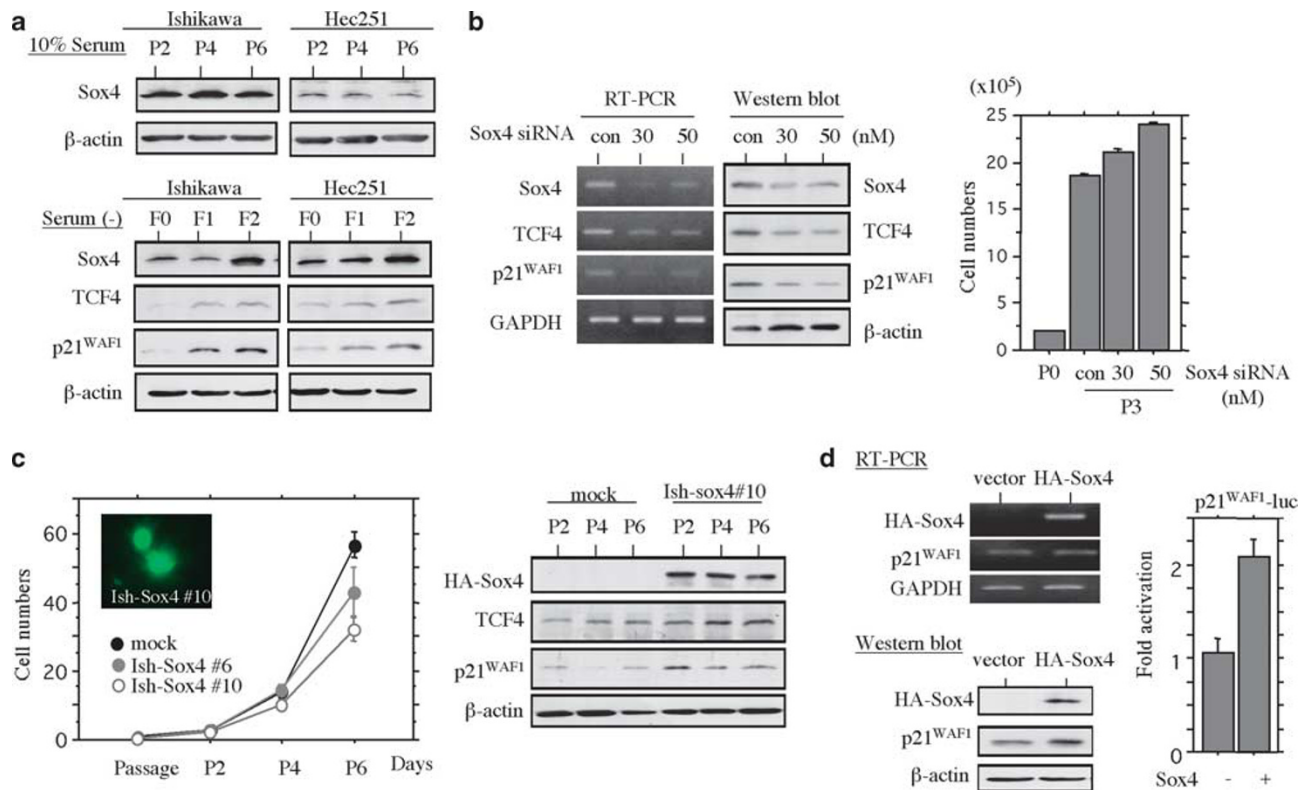


Figure 4 Relationship between Sox4 expression and cell kinetics in Em Ca cells. **(a)** Western blot analysis of Sox4 expression at different days of cell growth in the presence (upper) or absence (lower) of serum in Ishikawa and Hec251 cells. P2, P4, and P6: 2, 4, and 6 days after cell passage; F0, F1, and F3: 0, 1, and 2 days after serum starvation. **(b, left)** Analysis of mRNA and total protein (at 48 h after transfection) in Ishikawa cells transfected with 30 and 50 nM siRNAs for Sox4. Right: at 48 hours after Sox4 siRNA transfection, cell growth was monitored. The cell numbers presented are means \pm s.d.s. Con, negative control; P0 and P3, 0 and 3 days after cell passage. **(c, upper)** Two independent Ishikawa cells stably overexpressing HA-Sox4 (no. 6 and 10), as well as the mock cells, were seeded at low density and monitored for growth. The cell numbers presented are means \pm s.d.s. Note the HA-Sox4 expression in nuclei. Lower: western blot analysis of expression of HA-Sox4, TCF4, and p21^{WAF1} at different days of cell growth in a Sox4-stable cell line (no. 10). P2, P4, and P6, 2, 4, and 6 days after cell passage. **(d, upper and middle)** Analysis of mRNA and protein expression levels for the *TCF4* gene with total RNA or protein extracted from HA-Sox4-transfected Ishikawa cells using RT-PCR (upper) and western blot (middle) assays, respectively. HA-5' forward (5'-TACCCATACGATGTTCCAGATTACGC-3') and Sox4-reverse (Supplementary Table S1) primers were used. Lower: Ishikawa cells were transfected with the p21^{WAF1} reporter constructs, together with Sox4. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means \pm s.d.s. The experiment was performed in duplicate.

and Sox17 was observed in most cases, in contrast to low levels for Sox4, Sox5, and Sox7 (Figure 1), demonstrating significantly positive correlations among these, with the exception of Sox9 status (Table 1).

To examine whether Sox factors have an ability to alter β -catenin/TCF4-driven transcription in Em Ca cells, they were transfected into Ishikawa and Hec251 cells. Transient transfection of Sox4 caused an increase in a β -catenin/TCF4-mediated transcription activity determined by Top reporter constructs, whereas inhibition resulted with Sox3, Sox7, and Sox17 (Figure 2a and Supplementary Figure S1A). The enhancing effects of Sox4 were completely abrogated by co-transfection of Sox7 (Figure 2b), as well as of Sox3 and Sox17 (data not shown). To examine whether Sox4 is directly associated with β -catenin-dependent transcription, we conducted one hybrid assay using a DNA-BD-fused β -catenin fragment (pM- β -cat) in Ishikawa and Hec251 cells. As shown in Figure 2c and Supplementary Figure S1B, the pG5-luc activity was not altered by combination of pM- β -cat with Sox4, in contrast to p300, a known coactivator for β -catenin signaling.¹⁸ In addition, Flag-p300 colocalized with β -catenin, forming aggregates in nuclei as described previously,¹⁸

but this did not occur when Sox4 was combined with β -catenin as well as TCF4 (Figure 2d).

Transcriptional Upregulation of TCF4 by Sox4

Our previous study demonstrated that transcription factor Egr1 participates in upstream regulation of β -catenin signaling through transactivation of the *TCF4* gene in Em Ca cells.¹⁹ To examine whether Sox4 is also involved in control of TCF4 expression, HA-Sox4 or GFP-Sox4 was transfected into Ishikawa and Hec251 cells. As shown in Figure 3a and Supplementary Figure S2A, the overexpression resulted in a significant increase in endogenous TCF4 expression at both mRNA and protein levels. Next, we noted a 1308 bp fragment upstream of the translation start site in the *TCF4* gene (AF522996) to be highly GC rich, containing four potential binding sites for Sox factors (Figure 3b), on the basis of previous data for the consensus binding sequence A/T A/T CAAA T/G.^{6,7} With a series of 5'-truncated promoter constructs (Figure 3c), deletion from -1308 to -590 bp had little effect on induction of promoter activity by Sox4, but lack of -590 to -377 bp resulted in a decrease in responsiveness, indicating that Sox4-responsive elements might be

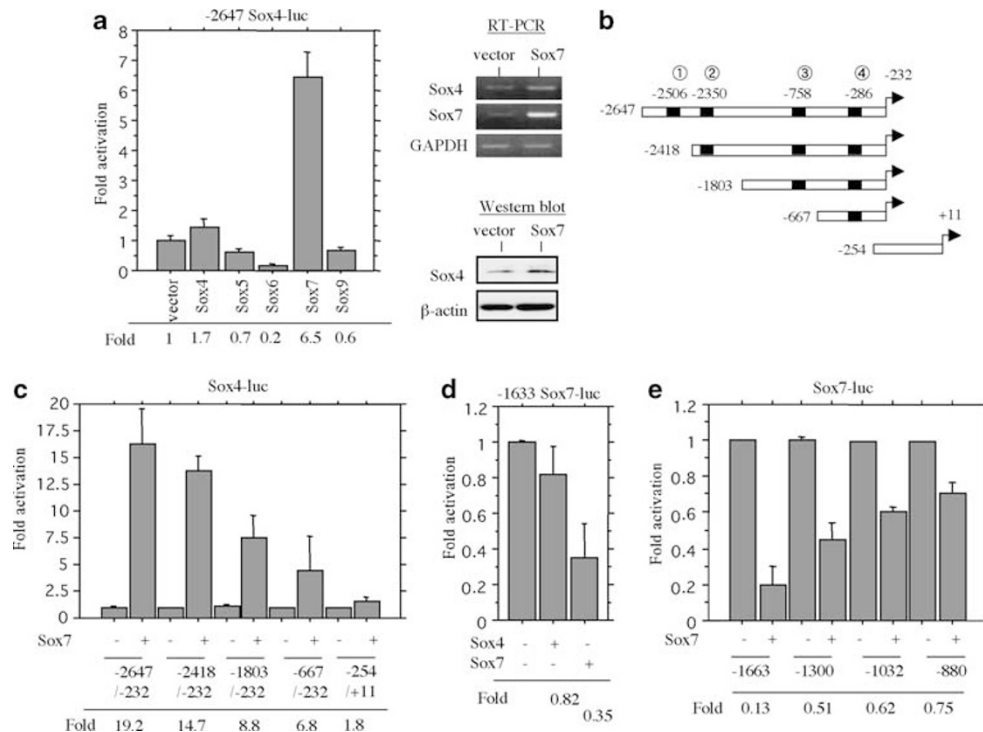


Figure 5 Transcriptional upregulation of Sox4 by Sox7. (a, left) Ishikawa cells were transfected with Sox4 reporter constructs, together with several Sox factors. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means \pm s.d.s. The experiment was performed in duplicate. Right: analysis of mRNA and protein expression levels for the Sox4 gene with total RNA or protein extracted from Sox7-transfected Ishikawa cells using RT-PCR (upper) and western blot (lower) assays, respectively. (b) The Sox4 promoter sequence containing four putative Sox-binding sites. (c) Ishikawa cells were transfected with various 5'-truncated constructs of the Sox4 promoter, along with Sox7 expression plasmids. The experiment was performed in duplicate. (d) Ishikawa cells were transfected with Sox7 reporter constructs, together with expression plasmids for either Sox7 or Sox4. (e) Ishikawa cells were transfected with various 5'-truncated constructs of the Sox7 promoter, along with Sox7 expression plasmids. The experiment was performed in duplicate.

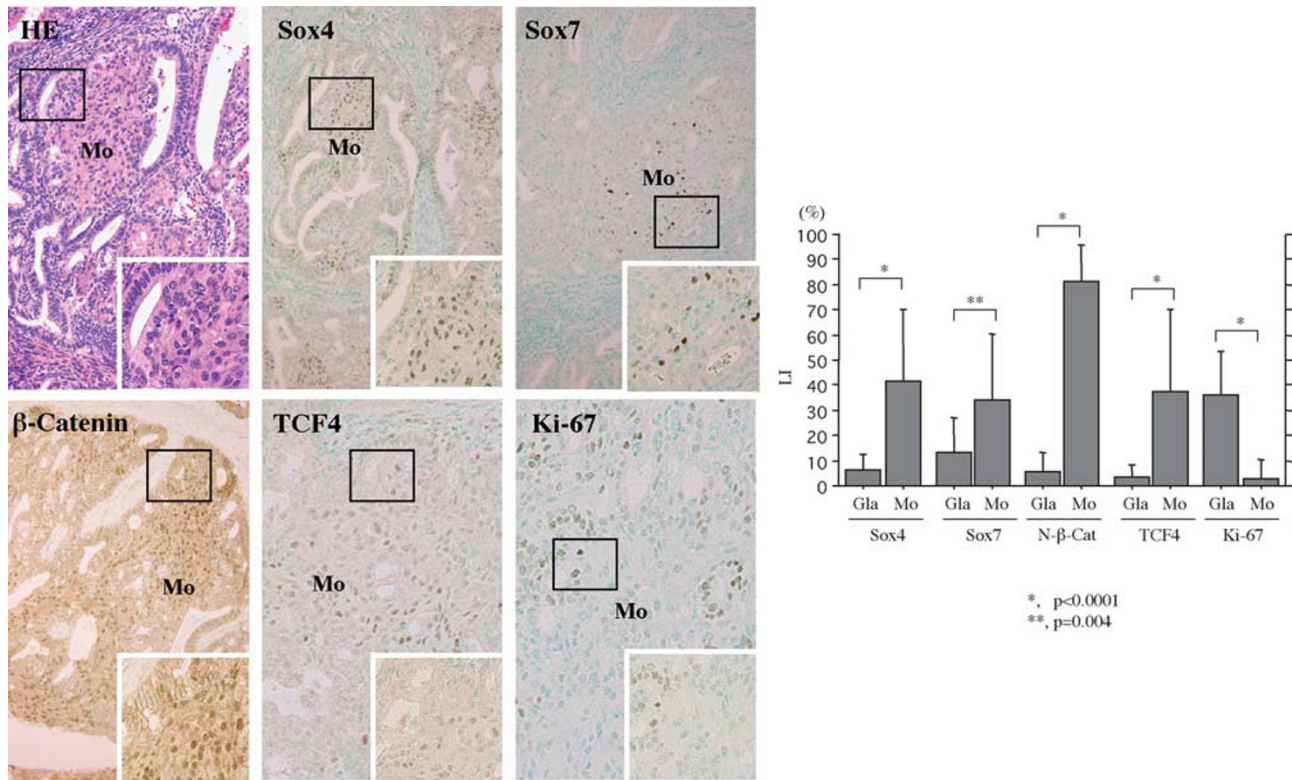


Figure 6 Immunohistochemical findings in serial sections of Em Ca with morules. Left: staining with hematoxylin and eosin (HE), and immunohistochemistry for Sox4, Sox7, β -catenin, TCF4, and Ki-67. Note the predominantly nuclear immunoreactivity for Sox4 and Sox7, as well as β -catenin and TCF4, in morular (Mo) lesions, in contrast to relatively low Ki-67 immunoreactivity. The lesions enclosed by boxes are magnified in the insets. Original magnification: $\times 100$ and $\times 400$ (insets). Right: Lis of Sox4, Sox7, β -catenin, TCF4, and Ki-67 in glandular (Gla) and Mo components in Em Ca tissues. The data shown are means \pm s.d.s.

located in the region from -590 to -377 bp (Figure 3d). Mutations in the putative Sox-responsive element site located between -432 to -426 bp caused significant decrease in responsiveness (Figure 3e), indicating the seven nucleotides CTTTGAA to constitute a functional cis-acting element necessary for *TCF4* promoter stimulation by Sox4. In contrast, such effects were relatively minor with the other Sox factors investigated (Supplementary Figure S2B).

Relationship Between Sox4 and Cell Proliferation in Em Cas

In both Ishikawa and Hec251 cells, Sox4 protein expression was not altered during steady cell growth in the presence of serum, whereas considerable increase of Sox4, as well as TCF4 and p21^{WAF1}, occurred with cell growth inhibition due to serum starvation (Figure 4a). Inhibition of endogenous Sox4 by specific siRNAs caused a relatively increase in Ishikawa cell growth, along with decreased expression of TCF4 and p21^{WAF1} (Figure 4b), whereas cells stably overexpressing HA-Sox4 in nuclei showed a low proliferation rate, particularly in the exponential growth phase, with increased amounts of the two proteins (Figure 4c). In addition, the latter was also upregulated by transfection of Sox4 at both mRNA and

Table 2 Correlations among expression of Sox4, Sox7, nuclear β -catenin, and TCF4 and cell proliferation in Em Cas with morule

	Sox4 LI <i>r</i> (<i>P</i>)	Sox7 LI <i>r</i> (<i>P</i>)	N- β -cat LI <i>r</i> (<i>P</i>)	TCF4 LI <i>r</i> (<i>P</i>)
Sox7 LI	0.4 0.008	*	*	*
N- β -cat LI	0.69 <0.0001	0.44 0.003	*	*
TCF4 LI	0.67 <0.0001	0.46 0.004	0.56 0.0001	*
Ki-67 LI	-0.71 <0.0001	-0.51 0.0008	-0.7 <0.0001	-0.56 0.0002

r: Spearman's correlation coefficient.

*Not examined.

protein levels, in line with the increased promoter activity (Figure 4d).

Transcriptional Upregulation of Sox4 by Sox7

Given the frequent co-expression of Sox factors in most Em Ca cell lines (Figure 1), we examined whether Sox4

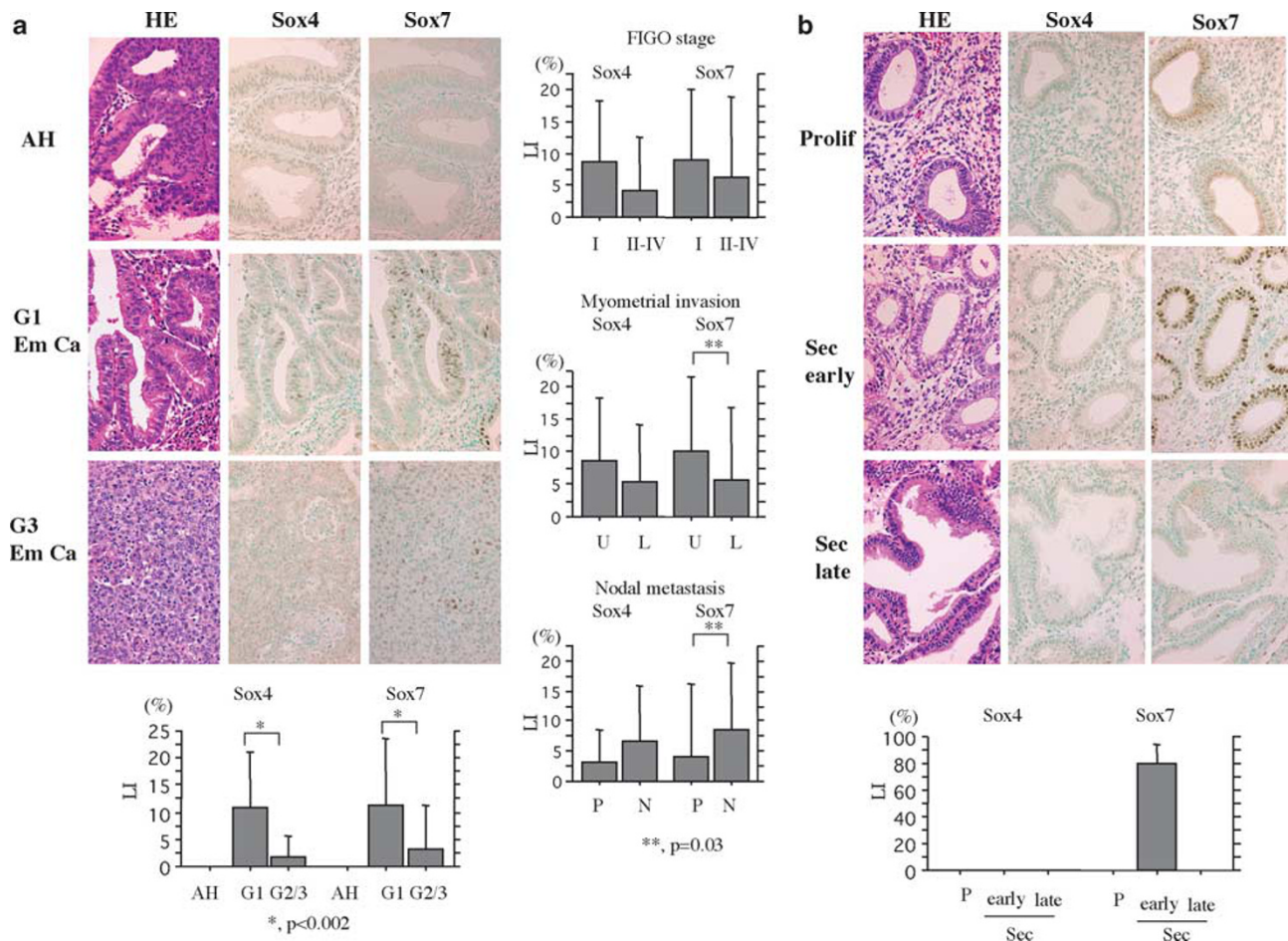


Figure 7 Immunohistochemical findings in serial sections of normal, hyperplastic, and malignant endometrial tissues. **(a, upper left)** Staining is with hematoxylin and eosin (HE), and immunohistochemistry for Sox4 and Sox7 in AH and Em Cas without morules. Note the nuclear immunoreactivity for Sox4 (indicated by arrows), as well as Sox7, in G1 Em Cas, in contrast to the lack or low levels in AH and G3 tumors. Original magnification: $\times 200$. Lower left: nuclear LIs for Sox4 and Sox7 in AH, G1, and G2/G3 Em Ca lesions. The data shown are means \pm s.d.s. Right: relationships of immunoreactivity for Sox4 and Sox7 with several clinicopathological factors, including FIGO stage (upper), myometrial invasion (middle), and nodal metastasis (lower). L, lower MI; N, negative; P, positive for nodal metastasis; U, upper MI. **(b, upper)** Staining with HE, and immunohistochemistry for Sox4 and Sox7 during the menstrual cycle in normal endometrium. Note the nuclear immunoreactivity for Sox7 in glandular cells in an early secretory stage, in contrast to the lack of Sox4 immunoreactivity during the menstrual cycle. Original magnification: $\times 200$. Prolif, proliferative stage; Sec, secretory stage. Lower: LIs of Sox4 and Sox7 in proliferative (P) and early and late secretory (Sec) stages. The data shown are means \pm s.d.s.

expression is affected by other Sox factors. Transfection of Sox7 resulted in activation of the Sox4 promoter about 6.5-fold, leading to the increased expression at both mRNA and protein levels, in contrast to Sox4, Sox5, Sox6, and Sox9 (Figure 5a and Supplementary Figure S3A). On the basis of the evidence that Sox4 promoter (AL136179) had four potential Sox-binding sites in the region between -2647 and -232 bp from the transcription start site, a series of 5'-truncated constructs was generated (Figure 5b). As shown in Figure 5c, the various deletions resulted in stepwise decrease in Sox7 responsiveness, indicating that multiple Sox7-responsive elements might be present in the promoter region. In contrast, transfection of Sox7 showed significant inhibition of its own promoter activity (Figure 5d and Supplementary Figure S3B), but various deleted promoters

resulted in stepwise restoration in the repression (Figure 5e), indicating that the effects might be linked to interaction with the basic machinery at the promoter.

Immunohistochemistry Findings for Normal and Malignant Endometrium

To examine whether such associations also exist in tumor tissues, we compared the expression levels of Sox4, Sox7, β -catenin, TCF4, and Ki-67 in Em Cas with morules. As shown in Figure 6, immunoreactivity for Sox4 and Sox7 was more frequently observed in morular as compared with surrounding glandular carcinoma lesions, the differences in LIs being significant. Similar findings were also observed in cases of nuclear β -catenin and TCF4 staining, as described previously.^{17,18} Average LI values for Sox4 were positively

correlated with LIs for Sox7, nuclear β -catenin, and TCF4, and inversely with Ki-67 LIs. Similar associations of LIs among Sox7, nuclear β -catenin, TCF4, and Ki-67 were also evident (Table 2).

In Em Cas without morules, immunoreactions for Sox4 and Sox7 were significantly greater in G1 than G2/G3 tumors, as well as AH lesions, LIs in the latter being significantly associated with upper myometrial invasion and negative for nodal metastasis (Figure 7a). In addition, Sox4 LIs were correlated positively with Sox7 LIs, and inversely with Ki-67 LIs in Em Cas (Table 3).

In normal endometrium, Sox4 immunoreactivity could not be observed throughout the menstrual cycle, in contrast to the significantly higher LI values of Sox7 in

epithelial but not stromal components in early secretory stage (Figure 7b).

Hypermethylation of Sox7 Promoter in Em Cas

As hypermethylation of the Sox7 promoter has been reported to frequently occur in tumors,^{29,30} we also examined the methylation status in Em Cas for comparison with expression levels. Methylation-specific PCR assays revealed specific signals in seven of eight Em Ca cell lines, with apparent decreases in the mRNA expression levels (Figure 8a). In clinical samples, the frequencies of promoter methylation showed stepwise increase from Em Cas with morules, through G1 to G2/3 tumors lacking such lesions (Table 4), being negatively associated with Sox7 LI values (Figure 8b). In contrast, such findings were not observed in 10 normal and 15 AH lesions investigated.

Table 3 Correlations among Sox4, Sox7, and cell proliferation in Em Cas without morules

	Sox4 LI <i>r</i> (<i>P</i>)	Sox7 LI <i>r</i> (<i>P</i>)
Sox7 LI	0.75 <0.0001	* 0.002
Ki-67 LI	-0.39 0.0005	-0.43 0.002

r: Spearman's correlation coefficient.
*Not examined.

Table 4 Hypermethylation of Sox7 promoter in Em Cas

	<i>n</i>	Unmethylated <i>n</i> (%)	Methylated <i>n</i> (%)
Em Ca with morule	28	26 (92.8)	2 (7.2)
Em Ca w/o morule			<0.02
G1 tumor	32	26 (81.2)	6 (18.8)
G2/3 tumor	17	11 (64.7)	6 (35.3)

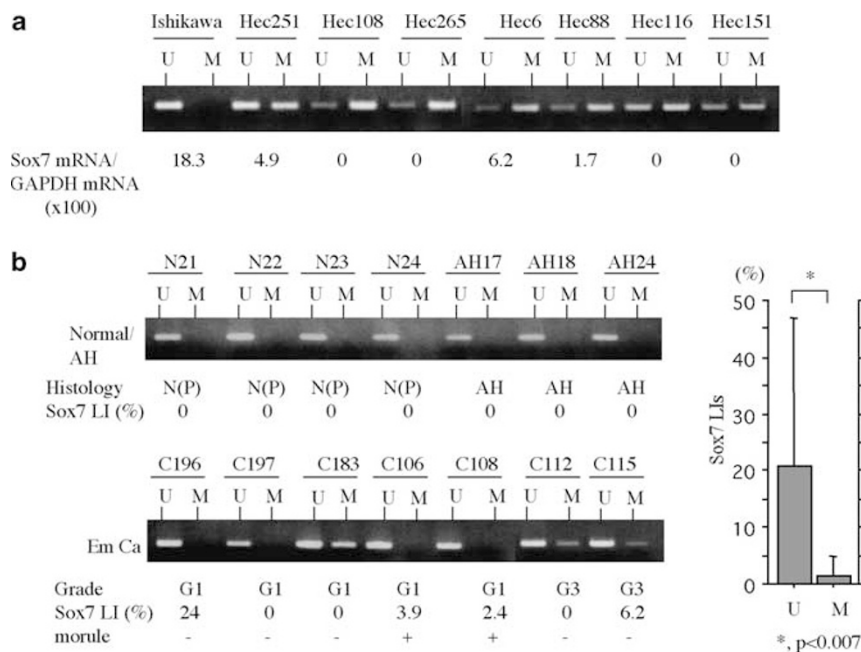


Figure 8 Hypermethylation of Sox7 promoter in normal, hyperplastic, and malignant endometrial tissues. (a) Methylation-specific PCR analysis of Sox7 promoter in eight Em Ca cell lines. Relative expression levels of Sox7 mRNA (demonstrated in Figure 1) are also indicated. (b, left) Methylation-specific PCR analysis of Sox7 promoter in normal proliferative (N(p)), AH, and Em Ca tissues. Average LIs for nuclear Sox7 immunostaining are also demonstrated. Right: Sox7 LIs in unmethylated (U) and methylated (M) Em Ca groups.

DISCUSSION

Overexpression of Sox4 is mediated by many signaling pathways that are commonly activated in malignant cells,³¹ but the significance is not fully understood, as somewhat conflicting data have been reported.^{12,32} In our Em Ca cell lines, inhibition of cell growth due to serum starvation led to an increase in Sox4 expression, whereas cells stably overexpressing Sox4 demonstrated a low proliferation rate, through transactivation of the *p21^{WAF1}* gene. In addition, expression was significantly higher in G1 than G2/3 Em Cas, inversely correlating with cell proliferation. These findings indicate that Sox4 may serve as a negative modulator for cell proliferation in Em Cas, presumably associated with altered cell cycling. Interestingly, it is considered that Sox4 may act as a tumor suppressor and favorable prognostic factor in bladder and hepatocellular carcinomas, respectively.^{12,33}

In line with earlier results for colon carcinoma cells,²¹ overexpression of Sox4 caused enhancement of β -catenin/TCF4-driven transcription, in contrast to inhibitory effects of Sox3, Sox7, and Sox17, in Em Ca cells. Given that Sox4 can directly interact with TCF4 via the respective HMG domains as shown by *in vitro* protein-binding assays,³⁴ we anticipated a formation of active transcriptional complexes containing β -catenin, TCF4, and Sox4, as demonstrated with combination of β -catenin and p300,¹⁸ but such associations proved to be rare events in Em Ca cells. Interestingly, the three proteins are unable to form stable complexes *in vitro*,³² and it was recently reported that transactivation of β -catenin signaling induced by Sox4 was due to stabilization of the β -catenin protein, rather than through induction of β -catenin transcription.³⁵

Several lines of evidence from the present study support the conclusion that TCF4 is under transcriptional control by Sox4. First, Sox4 immunoreactivity significantly overlapped nuclear staining of TCF4, as well as nuclear β -catenin, in morular lesions of Em Ca tissues, with a very significant positive correlation ($\rho = 0.67$, $P < 0.0001$). Second, transient transfection of Sox4 resulted in increased expression of the endogenous *TCF4* gene at both mRNA and protein levels, in line with the results for cells stably overexpressing Sox4. Third, the *TCF4* promoter was activated by transfection of Sox4, through a Sox-binding element located at -432 bp, as evidenced by inhibition of promoter activity by introducing a four-nucleotide alteration in the site, indicating regulation at the transcription level. Taken together with evidence that TCF3 can stabilize β -catenin protein through its competition with axin and APC,³⁶ our results suggest that transactivation of the *TCF4* gene mediated by Sox4 may be the primary mechanism underlying activation of β -catenin/TCF4-driven transcription.

Furthermore, the present data provided the first evidence that Sox7 is a transcriptionally positive regulator of Sox4 expression from the following findings: (1) upregulation of endogenous Sox4 expression at both mRNA and protein levels by exogenous Sox7; (2) increased *Sox4* promoter

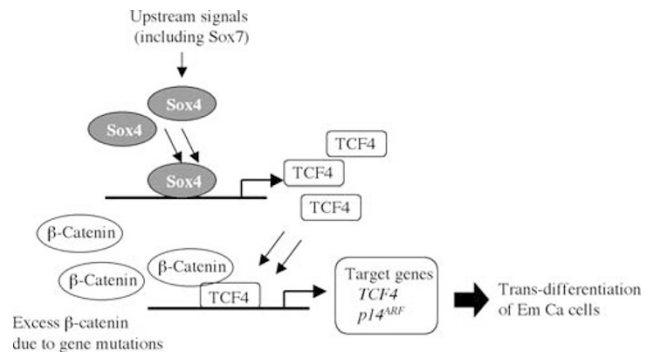


Figure 9 Schematic representation of functional role of Sox4 on β -catenin/TCF4 signaling during transdifferentiation of Em Ca cells.

activity on transfection of Sox7; (3) significant overlap of immunoreactivity between Sox4 and Sox7 in Em Cas, particularly in morular lesions; and (4) occasional *Sox7* promoter hypermethylation in G1 Em Cas with morular lesions, associated with relatively high levels of Sox4 expression as compared with G2/G3 tumors. In contrast to the cooperative function with Sox4, overexpression of Sox7 not only abrogated Sox4-mediated β -catenin/TCF4-driven transcription, but also inhibited its own promoter activity. Given the evidence that Sox7 can degrade active β -catenin protein by direct interaction,²⁸ it is likely that Sox7 may also function as a key factor for modulation of the signal cascade, contributing to a very complex feedback loop system. Further studies of this point are clearly warranted.

As an unexpected result of this study, Sox4 protein expression was not found in any stage of the menstrual cycle in normal endometrium, in contrast to the murine uterus where cyclic changes in mRNA expression occur in response to ovarian steroid hormones.¹⁰ One reason for the discrepancy may be differences in detection using mRNA- and the protein-based methods. In addition, the observed high levels of Sox7 expression in early secretory stage were also surprising, allowing us to speculate that the gene may be important for changes in cell kinetics from proliferative to secretory stages in normal endometrium, as ectopic expression of Sox7 in Sox-null cells has been demonstrated to inhibit cell proliferation.³⁰

Together, our observations suggest a model for functional roles of Sox4 in β -catenin/TCF4 signaling in Em Ca cells (Figure 9), dependent on upregulation of the *TCF4* gene. Newly synthesized TCF4 binds to excess β -catenin, probably present due to gene mutations,^{17,19} and this in turn leads to transactivation of target genes, such as *TCF4* and *p14^{ARF}*, (ref. 17,18) resulting in induction of morular differentiation of Em Ca cells. In addition to many signaling,³¹ Sox7 may also contribute to upregulation of Sox4 expression, exerting antagonistic effects on β -catenin/TCF4-driven transcription.

In conclusion, the present study provided compelling evidence of an association between Sox factors, in particular Sox4, and β -catenin-dependent signaling in establishment

and maintenance of morular differentiation of Em Ca cells through alteration in *TCF4* gene expression.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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