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Tumorigenesis and Neoplastic Progression

Transcriptional Up-Regulation of Sox9 by NF-κB in Endometrial Carcinoma Cells, Modulating Cell Proliferation Through Alteration in the $p14^{ARF}$/p53/$p21^{WAF1}$ Pathway

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The Sox factors are a large family of transcription factors that play important roles in tumor development and progression in a variety of human malignancies and diverse developmental processes, but little is known about their roles in endometrial tumorigenesis. Herein, we focus on the functions of Sox9 in endometrial carcinomas. Cells stably overexpressing Sox9 showed a low proliferation rate, particularity in the exponential growth phase, along with increased amounts of $p21^{WAF1}$. Transient transfection of Sox9 caused transactivation of *$p21^{WAF1}$* and *$p14^{ARF}$* promoters, in cooperation with p53, resulting in activation of the $p14^{ARF}$/p53/$p21^{WAF1}$ pathway. Overexpression of p65, and the constitutively active form myristylated Akt, led to an increase in Sox9 expression through transcriptional and posttranslational mechanisms. In normal endometrium, biphasic up-regulation of Sox9 expression was observed during the menstrual cycle, labeling indices being significantly higher in the proliferative stage than in the secretory stage. Moreover, expression also showed a significant stepwise increase from normal through grade 1 to grade 2/3 tumors, being correlated positively with labeling indices of p53, $p21^{WAF1}$, pp65, and Ki-67, probably due to a feedback system regarding cell proliferation through NF-κB and Akt signaling. These data, therefore, suggest that associations between Sox9 and NF-κB signaling, as well as Akt status, may participate in modulation of the cell kinetics of endometrial carcinomas cells through alteration in the $p14^{ARF}$/p53/$p21^{WAF1}$ pathway. *(Am J Pathol 2012, 181:684–692; http://dx.doi.org/10.1016/j.ajpath.2012.05.008)*

The sex-determining region Y box or Sox factors constitute a large family of transcription factors that share a homologous high-mobility group DNA-binding domain and play important roles in diverse developmental processes in most vertebrates. Based on protein sequence comparisons, the factors are divided into eight groups, A to H, demonstrating highly similar structures within and outside the high-mobility group box in the groups. In addition to the box domain, most factors have one or two other functional domains with transactivation, transrepression, or homodimerization features associated with a control of gene transcription in several signaling pathways.[1–5]

Sox9, known as a member of the SoxE group (Sox8, Sox9, and Sox10), contains three highly conserved domains: the high-mobility group, the C-terminal PQS transactivation, and the PQA domains, the latter two being required for maximum transcription activity.[6,7] The factor is best known for its essential roles in chondrogenesis and in development of the male gonads, neural crest, and spinal cord glial cells.[8–11] Heterozygous mutations have been reported to be the cause of the human disease campomelic dysplasia, a form of dwarfism characterized by extreme cartilage and bone malformation.[8,9,12,13]

Although expression of Sox9 has been reported to be changed in various human malignancies, the possible significance has not been clearly determined. For example, whereas up-regulation of Sox9 contributes to gain of

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Table 1. Primer Sequences Used in this Study

Category	Gene	Sequence	Size (bp)
mRNA	*Sox9*		
	Forward	5′-CAGCAAGAACAAGCCGCACG-3′	222
	Reverse	5′-GTAATCCGGGTGGTCCTTCTT-3′	
	Cyclin A		
	Forward	5′-AGAGCGTGAAGATGCCCT-3′	260
	Reverse	5′-GTGATGTCTGGCTGTTTCT-3′	
	Cyclin D1		
	Forward	5′-AGGAAGAGCCCCAGCCATG-3′	226
	Reverse	5′-GTTCCTCGCAGACCTCCAG-3′	
	p14^{ARF}		
	Forward	5′-GGTTTTCGTGGTTCACATCCCGCG-3′	254
	Reverse	5′-CAGGAAGCCCTCCCGGGCAGC-3′	
Promoter	*Sox9*		
	(−1054)F	5′-AAGCAGCATGACTCCGCCAGAG-3′	1218
	(−229)F	5′-CCCCGCAGCACCCCCCGCCC-3′	392
	(−260)F	5′-TACACACTTGGAAGTCCCGG-3′	423
	(−260)kB2MF	5′-TACACACTTG**TTCA**TCCCGGGTCCCCCGCC-3′	423
	(−260)kB3MF	5′-TACACACTTGGAAGTCCCG**TTGA**CCCCGCCTTCCCCGCAG-3′	423
	(+163)R	5′-AGGCGAATTGGAGAGGAG-3′	
	p21^{WAF1}		
	(−2283)F	5′-AGGAACATGTCCCAACATGT-3′	2305
	(−2253)F	5′-CATAGAAGAGGCTGGTGGCT-3′	2275
	Cyclin A		
	(−592)F	5′-GGCAACCCAAATGATAGTCGCC-3′	509
	(−83)R	5′-CCGGCCAAAGAATAGTCGTAGC-3′	

Bold text indicates inserted mutations.

tumor growth potential and tumorigenicity in lung adenocarcinomas,[14] elevated expression results in a decreased rate of cellular proliferation, cell cycle arrest in grade (G) 0/G1, and increased sensitivity to apoptosis in prostate carcinoma cells.[15] However, little is known about roles in endometrial tumorigenesis.

In this study, we, therefore, extensively investigated Sox9 expression in endometrial carcinoma (Em Ca) cell lines and in normal and malignant endometrial tissues. Thereby, we provided several lines of evidence that upregulation of Sox9 expression in response to NF-κB, as well as active Akt, may serve as a negative regulator of cell proliferation through alteration in the p14^{ARF}/p53/p21^{WAF1} pathway in Em Ca cells.

Materials and Methods

Plasmids, Cell Lines, Antibodies, and Reagents

Full-length cDNAs of human *Sox9* (Open Biosystems, Huntsville, AL) with or without HA tag were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and pEGFP vectors (BD Biosciences Clontech, Worcester, MA). The human *Sox9* promoter sequence between −1054 and + 163 was amplified by PCR using a 4.8-kb *Sox9* promoter fragment (a gift from Dr H. Kawaguchi, Tokyo University, Tokyo, Japan) and a 509-bp fragment of the *cyclin A* promoter (AF518006) and were subcloned into the pGL-3B vector (Promega Corp., Madison, WI). A series of 5′-truncated *Sox9* promoter constructs were generated by PCR-based methods. Site-directed mutagenesis in putative NF-κB–binding sites in the *Sox9* promoter was performed using PCR methods. A series of 5′-truncated *p21^{WAF1}* promoter constructs were also produced in a similar manner. pM-p53 was constructed by inserting full-length wild-type p53 into the pM DNA-BD vector (BD Biosciences Clontech). The dominant-negative type of p53 was also generated by PCR using a pCMV-p53 wild type construct. The identity of these constructs was confirmed by sequencing before use. The sequences of all the PCR primers used in this study are listed in Table 1. The pG5-luc, pGL3B-p21^{WAF1}(−2338)-luc, pGL3B-cyclin D1(−963)-luc, pGL2B-p14^{ARF}(−3704)-luc, pNFκB-luc, pcDNA3.1-mouse p65, and pUSEamp-myrAkt1 constructs were as described previously.[16–18]

Two Em Ca cell lines, Ishikawa and Hec251, were maintained in Eagle's minimal essential medium with 10% bovine calf serum.[16–18] To establish cells stably overexpressing HA-Sox9, the expression plasmid or empty vector were transfected into Hec251 cells, and two independent clones were established, as described previously.[16,19]

Anti-Sox9 (HPA001758) and anti-β-actin were from Sigma-Aldrich (St Louis, MO). Anti-cyclin D1, anti-p21^{WAF1}, anti-p53, and anti-Ki-67 were from Dako (Copenhagen, Denmark). Anti-phospho-p65 at Ser275 (pp65), anti-phospho-Akt at Ser473 (pAkt), anti-total Akt, and anti-p14^{ARF} were from Cell Signaling Technology Inc. (Beverly, MA). Anti-PTEN was from Cascade BioScience (Winchester, MA). Tumor necrosis factor α (TNF-α) and LY294002 were purchased from Sigma-Aldrich. Cycloheximide (Calbiochem, Darmstadt, Germany) was also obtained commercially.

Transfection

Transfection was performed using Lipofectamine PLUS (Invitrogen), in duplicate or triplicate. Luciferase activity was assayed as described previously.[16–19] The small-

interfering RNA (siRNA) for Sox9 was also transfected using the siPORT NeoFX transfection agent (Ambion, Austin, TX), according to the manufacturer's instructions. The siRNA and the negative control are available from Ambion.

RT-PCR

Amplification was performed in the exponential phase (20 to 28 cycles) to allow comparison among cDNAs synthesized from identical reactions using specific primers (Table 1). Primers for *GAPDH* genes were also applied, as described previously.[16–19]

Western Blot and Immunoprecipitation Assays

Total cellular proteins were prepared using radioimmunoprecipitation assay buffer. Western blot assays were performed as described previously.[16–19] The intensity of individual signals was measured using ImageJ software version 1.41 (NIH, Bethesda, MD), as described previously.[17] For immunoprecipitation assays, cells were lysed with TNE buffer. Immunoprecipitation and immunoblotting were conducted using anti-Sox9 and anti-p53 antibodies, as described previously.[19]

Clinical Cases

Histologic findings were reviewed for hysterectomy specimens of endometrioid type Em Cas in the case records of Kitasato University Hospital between January 1, 2000, and December 31, 2008, according to the criteria of the 2003 World Health Organization classification.[20] Each case was also staged according to the 1988 International Federation of Gynecology and Obstetrics staging system.[21] Fifty-five cases of Em Ca, including 24 of G1 and 31 of G2/3, and 25 cases of atypical hyperplasia were investigated. For the carcinoma cases, the mean age of the patients was 54.5 years (range, 30 to 84 years), and 23 were postmenopausal. In addition, 30 of these patients were subcategorized as stage I and 21 as stage II to IV, and 27 demonstrated upper and 24 lower myometrial invasion (MI), 10 being positive and 35 negative for nodal metastasis. Forty biopsy specimens of normal endometrial tissues, including 10 in the proliferative phase and 30 in the secretory phase, were also investigated, with the latter being subclassified into 10 in the early phase and 20 in the middle and late phases according to the criteria of Mutter and Ferenczy.[22] All the tissues were routinely fixed in 10% formalin and were processed for embedding in paraffin wax. Approval for this study was given by the ethics committee of the Kitasato University School of Medicine.

IHC Analysis

Immunohistochemical (IHC) analysis 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.[16–19] As a positive control, cartilage tissue was used since it is well known that Sox9 plays a central role in determining its phenotypic characteristics,[23,24] and rabbit or mouse sera were applied as negative controls. For p53, cases with LIs <30% were selected since high LIs (>30%) have been reported to be typical (90%) of mutated cases.[25] Scoring of immunoreactivity for pAkt, as well as for PTEN, was performed on the basis of percentage of immunopositive cells and the immunointensity with multiplication of values for the two parameters, as described previously.[17] For PTEN, cases were defined as immunopositive and immunonegative when the scores were >1 and 0, respectively.

PIK3CA *Gene Mutation Analyses*

Tumor DNA was extracted from paraffin wax sections of Em Ca cases using proteinase K/phenol-chloroform methods. Exons 9 and 20 of the *PIK3CA* gene were amplified and sequenced as described previously.[26,27]

Statistics

Comparative data were analyzed using the Mann-Whitney *U*-test, Pearson's correlation coefficient, and the χ^2 test. The cutoff value for statistical significance was set at $P < 0.05$.

Results

Relationship between Sox9 and Cell Kinetics in Em Ca Cells

Knowing that Sox9 has potential effects on cell growth,[14,15] we first examined whether its expression is associated with changes in Em Ca cell kinetics using Ishikawa cells with high levels of endogenous Sox9 expression (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). As shown in Figure 1A, Sox9 expression at mRNA and protein levels was increased during steady cell growth in the presence of serum, in contrast to the decrease observed in cells exhibiting growth inhibition due to serum starvation. Similar changes in expression of cyclins A and D1 were observed under both conditions. For $p21^{WAF1}$, the expression was also increased by serum stimulation, but change was relatively minor with serum starvation.

Next, we generated cells stably overexpressing HA-Sox9 using Hec251 cells with low endogenous levels (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Two independent stable clones showed low proliferation rates, particularly in the exponential growth phase, along with increased amounts of $p21^{WAF1}$ but not cyclins A and D1 (Figure 1B), in line with alterations in the promoter activity mediated by transfection of Sox9 (Figure 1C). Because typical siRNA-mediated gene silencing occurred 48 to 72 hours after the transfection,[28] examination of cell proliferation rates with inhibition of endogenous Sox9 by the specific siRNA demonstrated only relatively minor effects during the relatively early growth phase (Figure 1D).

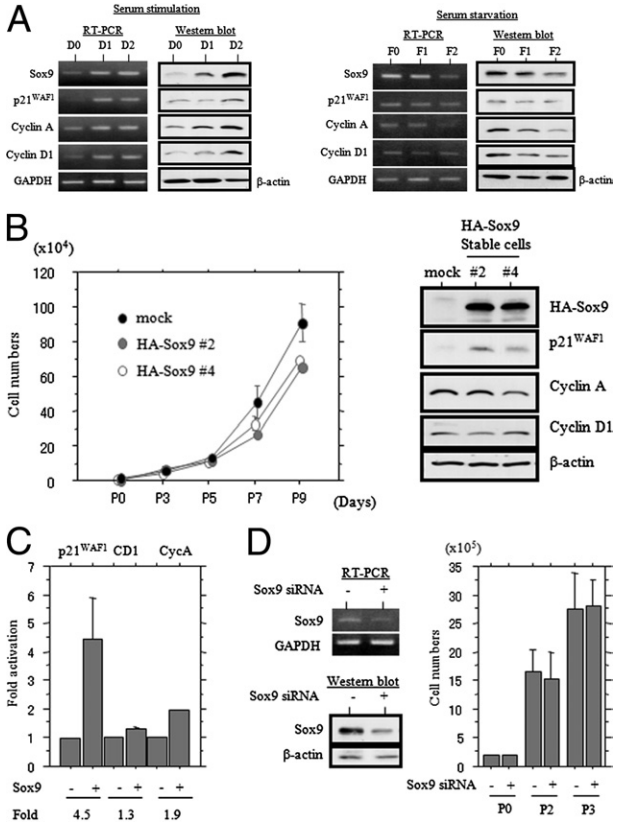

Figure 1. Sox9 expression and Em Ca cell kinetics. **A:** RT-PCR and Western blot analyses of Sox9 expression in Ishikawa cells at different days of cell growth in the presence (**top panel**) or absence (**bottom panel**) of serum. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D0, D1, and D2: 0, 1, and 2 days after serum stimulation; F0, F1, and F2: 0, 1, and 2 days after serum starvation. **B: Left panel:** Two independent Hec251cells stably overexpressing HA-Sox9 (#2 and #4) and mock cells were seeded at low density and monitored for growth. The cell numbers presented are mean ± SD. P0, P3, P5, P7, and P9: 0, 3, 5, 7, and 9 days after cell passage. **Right panel:** Western blot analysis of expression of HA-Sox9, $p21^{WAF1}$, and cyclins A and D1 at different days of cell growth in Sox9-stable cell lines (#2 and #4). **C:** Ishikawa cells were transfected with *p21*^{WAF1} and cyclins D1 (CD1) and A (CycA) reporter constructs, together with Sox9. 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 mean ± SD. The experiment was performed in duplicate. **D: Left panel:** Analysis of mRNA and total protein in Ishikawa cells transfected with 50 nmol/L siRNA for Sox9. An siRNA with no homology of the mammalian genome was used as a negative control. **Right panel:** Ishikawa cells with or without transfection of Sox9 siRNA were seeded at 2×10^5 cells (P0) and monitored for growth. The cell numbers presented are mean ± SD. P0, P2, and P3: 0, 2, and 3 days after transfection of the siRNA.

Sox9 Transcriptionally Up-Regulates p21^{WAF1}*, Cooperating with the p14*^{ARF}*/p53 Pathway*

To examine whether Sox9 can directly regulate $p21^{WAF1}$ expression, it was transfected into Ishikawa cells. As shown in Figure 2A, transient transfection of Sox9 caused an increase in $p21^{WAF1}$ expression at mRNA and protein levels, in line with the reporter assay results (Figure 1C). To determine sequences in the *p21*^{WAF1} promoter involved in Sox9 responsiveness, a series of 5′-truncated promoter constructs were generated (Figure 2B). Deletion from −2337 to −1921 bp, including the p53 responsive element (p53RE), resulted in a significant decrease in responsiveness (Figure 2C), indicating that the region

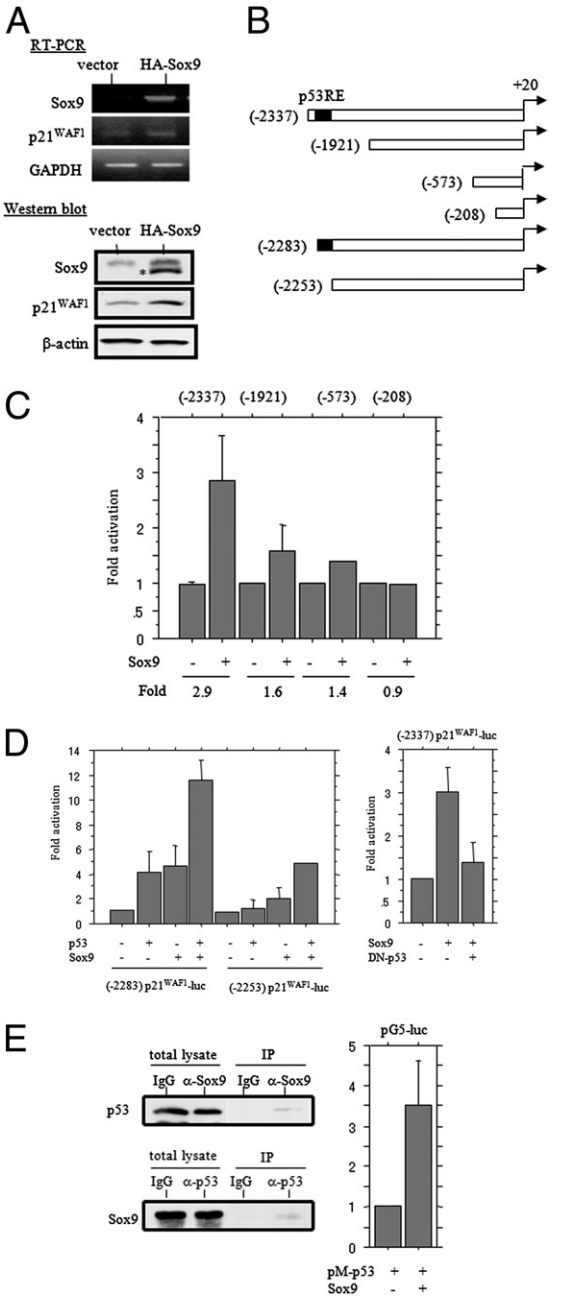

Figure 2. Up-regulation of $p21^{WAF1}$ by Sox9 through cooperation with p53. **A:** Analysis of mRNA and protein expression levels for the *p21*^{WAF1} gene with total RNA or protein extracted from HA-Sox9–transfected Ishikawa cells using RT-PCR (**top panel**) and Western blot (**bottom panel**) assays, respectively. HA-5′ forward (5′-TACCCATACGATGTTCCAGATTACGC-3′) and Sox9 reverse (Table 1) primers were used. In the Sox9 panel in the Western blot assay, the lower band (indicated by an **asterisk**) represents exogenous HA-Sox9, in contrast to the upper bands for endogenous Sox9. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B:** The *p21*^{WAF1} promoter sequence containing the p53RE. **C:** Ishikawa cells were transfected with various 5′-truncated constructs of the *p21*^{WAF1} promoter, along with Sox9 expression plasmids. Data are given as mean ± SD. The experiment was performed in duplicate. **D: Left panel:** Ishikawa cells were transfected with *p21*^{WAF1} reporter constructs with or without a p53RE, together with expression plasmids for p53 and/or Sox9. Data are given as mean ± SD. The experiment was performed in duplicate. **Right panel:** Ishikawa cells were transfected with the *p21*^{WAF1} reporter construct, together with expression plasmids for Sox9 and/or the dominant-negative (DN) form of p53. Data are given as mean ± SD. The experiment was performed in duplicate. **E: Left panel:** Immunoprecipitation (IP) assay data showing Sox9 binding to p53 in Ishikawa cells. **Right panel:** Ishikawa cells were transfected with pG5-luc reporter and pM-p53 constructs, together with the Sox9 expression plasmid. Data are given as mean ± SD. The experiment was performed in duplicate.

of the promoter encompassing p53RE might contain putative regulatory sites that are responsive to Sox9. To test this hypothesis, an additional p53RE deletion construct, targeting −2283 to −2253 bp, was generated (Figure 2B). Enhancement of p53-dependent transactivation of the promoter on co-transfection of Sox9 was significantly reduced by deletion of the p53RE, in line with the effects of a dominant-negative form of p53 (Figure 2D). Although weak activation of the p53RE-deleted reporter was also observed of transfection of sox9 and/or p53, this may be due to the presence of a second p53RE in the proximal region, perhaps at nucleotide −75.[29]

Because Sox function depends on associated interacting proteins,[30] we also examined its interaction with p53 protein. As shown in Figure 2E, distinct interactions between the two were demonstrated by co-immunoprecipitation from lysates of Ishikawa cells. Moreover, one hybrid assay revealed pG5-luc reporter activity to be raised approximately 3.5-fold by co-transfection of DNA-BD–fused wild-type p53 (pM-p53) and Sox9.

Knowing that $p14^{ARF}$ acts as an upstream regulator for the $p53/p21^{WAF1}$ pathway,[31] we examined whether Sox9 has an ability to regulate the *p14ARF* gene. As shown in Figure 3A, elevated amounts of $p14^{ARF}$ and p53 proteins were observed in two stable cell lines compared with in the mock case. Moreover, transient transfection of Sox9 resulted in an increase in endogenous $p14^{ARF}$ expression at mRNA and protein levels, in line with transactivation of the promoter (Figure 3B).

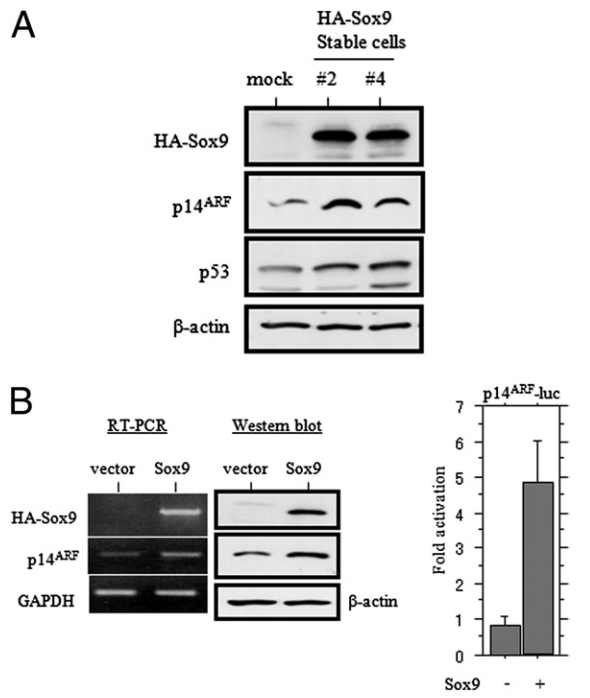

Figure 3. Transactivation of the *p14ARF* gene by Sox9. **A:** Western blot analysis of expression of $p14^{ARF}$ and p53 in Sox9-stable cell lines (#2 and #4). **B: Left panel:** Analysis of mRNA and protein expression levels for the *p14ARF* gene with total RNA or protein extracted from HA-Sox9–transfected Ishikawa cells using RT-PCR (**left panel**) and Western blot (**right panel**) assays, respectively. HA-5′ forward (5′-TACCCATACGATGTTCCAGATTACGC-3′) and Sox4 reverse (Table 1) primers were used. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **Right panel:** Ishikawa cells were transfected with the *p14ARF* promoter construct, along with Sox9. 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 mean ± SD. The experiment was performed in triplicate.

Up-Regulation of Sox9 by NF-κB/p65 and Akt

Because NF-κB has a capacity for transcriptional regulation of the *Sox9* gene,[32,33] we first examined whether Sox9 expression is affected by the NF-κB pathway. Treatment of Ishikawa cells with TNF-α caused induction of Sox9 protein expression and increased p65 stability in nuclei. Transient transfection of p65 also caused an increase in endogenous Sox9 expression at mRNA and protein levels (Figure 4A).

A search of the *Sox9* promoter for a potential NF-κB consensus binding sequence (GGRNNYYCC) revealed the presence of three putative sites (κB1, κB2, and κB3) (Figure 4B), their sequences being well conserved among human, mouse, and chick.[32] To identify p65-responsive elements in the *Sox9* promoter, a series of the 5′-truncated promoter constructs was generated (Figure 4C). Deletion from −1054 to −405 bp had little effect on induction of the promoter activity by p65, but deletion of −405 to −229 bp resulted in decreased responsiveness (Figure 4D).

Mutations in either the κB2 or κB3 site had relatively minor effects on the promoter activity in response of p65, whereas double mutations involving both sites resulted in considerable reduction of p65 responsiveness (Figure 4, C and E), suggesting that p65-dependent transactivation of *Sox9* promoter might require only one or the other site. In contrast, NF-κB–mediated transcription was significantly repressed by transfection of Sox9 (Figure 4F).

Because Akt is known to act as an upstream regulator of several signal pathways, cooperating with the NF-κB pathway,[34–36] we next examined whether Akt is also involved in Sox9 regulation. Increased endogenous Sox9 expression at mRNA and protein levels occurred on transient transfection of a constitutively active form, myristylated Akt (myr-Akt), whereas inhibition was observed in cells on treatment with LY294002, an inhibitor of phosphatidylinositol 3-kinase acting as an upstream effector of Akt (see Supplemental Figure S2A at *http://ajp.amjpathol.org*). Transfection of myr-Akt resulted in activation of the *Sox9* promoter and in NF-κB–driven transcription (see Supplemental Figure S2B at *http://ajp.amjpathol.org*). A cycloheximide charge study also revealed the half-life of Sox9 protein to be significantly increased in myr-Akt–transfected cells compared with in the mock-transfected case (see Supplemental Figure S2C at *http://ajp.amjpathol.org*), suggesting that Akt has the capacity for regulation at the transcriptional and posttranslational levels.

IHC Analysis Findings in Normal and Malignant Endometrium

To examine whether an association also exists in normal and malignant endometrial tissues, we compared the expression levels of Sox9, as well as p53, $p21^{WAF1}$, pp65,

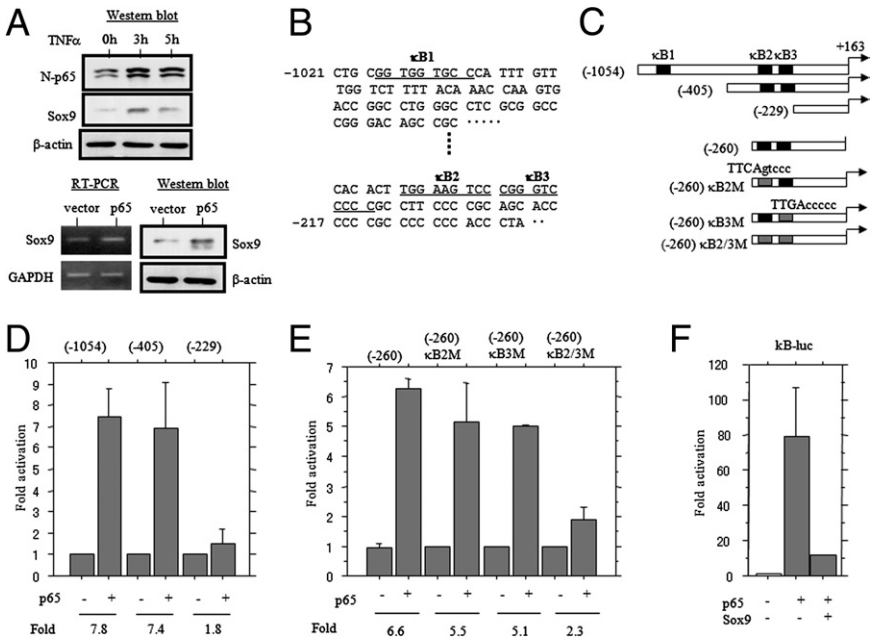

Figure 4. Identification of NF-κB–responsive elements in the *Sox9* promoter. **A: Top panel:** Nuclear or total proteins of Ishikawa cells exposed to TNF-α (20 ng/mL) for the times shown were extracted for analysis of nuclear (N)-p65 and Sox9. **Bottom panel:** Analysis of mRNA and protein expression levels for the *Sox9* gene with total RNA or protein extracted from p65-transfected Ishikawa cells using RT-PCR (**left panel**) and Western blot (**right panel**) assays, respectively. **B:** *Sox9* promoter sequence containing three putative NF-κB–binding sites (underlined). **C:** Various promoter deletion constructs were used to evaluate transcriptional regulation of the *Sox9* promoter by p65. Results of introduction of mutations into pGL3-(−260)-Sox9-luc are also demonstrated. **D:** Ishikawa cells were transfected with various 5′-truncated constructs of the *Sox9* promoter and p65 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 mean ± SD. The experiment was performed in triplicate. **E:** Ishikawa cells were transfected with mutant constructs of the *Sox9* promoter together with p65 expression plasmids. Data are given as mean ± SD. **F:** Ishikawa cells were transfected with κB-luc constructs along with p65 and Sox9. Data are given as mean ± SD.

and pAkt, in addition to cell proliferation determined by Ki-67.

In normal endometrium, a distinct nuclear immunoreactivity for Sox9 was generally observed in epithelial but not stromal components. Average Sox9 LIs were significantly higher in proliferative than in secretory stages. In the latter, the LI values were also significantly higher in the later than in the early secretory stage (Figure 5A).

In atypical hyperplasia and Em Ca tissues, significantly higher values of Sox9 LIs were evident in atypical hyperplasia lesions compared with in normal and Em Ca tissues. In Em Cas, the average LI values showed a significant stepwise increase from G1 through G2 to G3 tumors (Figure 5B), but significant associations with several clinicopathologic factors, including clinical stages, degree of myometrial invasion, and nodal metastasis, were not evident (data not shown). In addition, Sox9 LIs correlated positively with LIs of p53, $p21^{WAF1}$, pp65, and Ki-67 but not with pAkt scores (Figure 6). In addition, there were significant correlations among LIs of p53, $p21^{WAF1}$, and

Figure 5. IHC analysis findings of Sox9 in normal, hyperplastic, and malignant endometrial tissues. **A: Left panel:** H&E staining (**top panels**) and Sox9 IHC staining (**bottom panels**) in the proliferative (P) phase and the early (Sec-E) and late (Sec-L) secretory phases. Note the nuclear staining for Sox9 in epithelial components (indicated by **arrows**). Original magnification, ×200. **Right panel:** Nuclear LIs for Sox9 in the proliferative phase (P) and the early (Sec-E) and late (Sec-L) secretory phases. The data shown are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.02$, and $^{***}P < 0.01$. **B: Left panel:** H&E staining (**top panels**) and Sox9 IHC staining (**bottom panels**) in atypical hyperplasia (AH) and G1 and G3 Em Cas. Original magnification, ×200.**Right panel:** Nuclear LIs for Sox9 in proliferative (P), AH, and G1/2/3 Em Cas. The data shown are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.0005$, and $^{***}P < 0.004$.

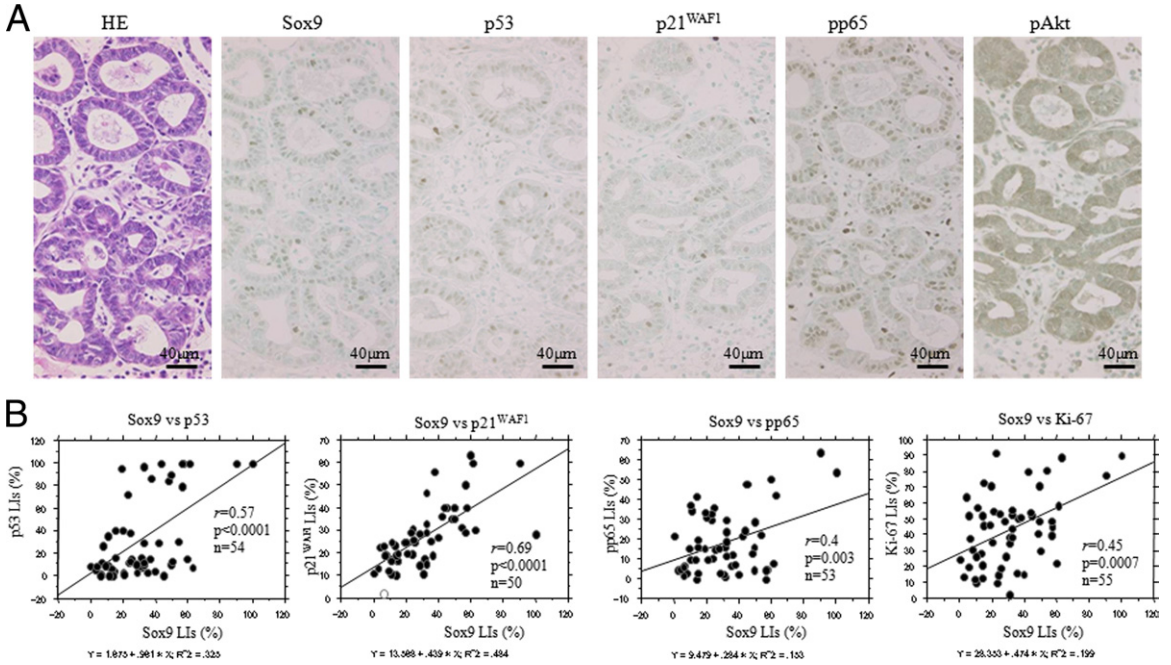

Figure 6. IHC) analysis findings in serial sections of Em Cas. **A:** Staining is for H&E and by IHC analysis for Sox9, p53, $p21^{WAF1}$, pp65, and pAkt in Em Ca. Original magnification, ×200. **B:** Correlations of Sox9 LIs with LIs for p53, $p21^{WAF1}$, pp65, and Ki-67.

pp65 but not of pAkt scores, except for some cases (see Supplemental Figure S3 and Supplemental Table S1 at *http://ajp.amjpathol.org*).

Because the Akt pathway is frequently activated in Em Cas mainly due to *PIK3CA* and *PTEN* alterations,[37,38] we further examined their associations with Sox9 expression. Mutations in the *PIK3CA* gene and loss of PTEN expression (score = 0) were detected in 7 of 47 (14.9%) and 15 of 45 (33.3%) informative carcinomas, respectively, with a significant association between tow (see Supplementaal Figure S4 and Supplemental Tables S2 and S3 at *http://ajp.amjpathol.org*), although the frequencies were relatively lower than those represented by others.[37,38] In addition, they did not show any associations with Sox9 expression (data not shown), indicating that other factors in the Akt pathway may be involved in its regulation.

Discussion

The present study provided evidence of biphasic upregulation of Sox9 expression during menstrual cycles in normal endometrium, with expression being significantly higher in the proliferative and late secretory stages, in contrast to a lack of or extremely low levels in the early secretory stage. In cell lines, endogenous expression levels were apparently altered by the serum stimulation and serum starvation conditions, whereas stable overexpression resulted in a decrease in cell proliferation. Taken together with an earlier report of Sox9 expression in proliferating cells, probably including stem cells, and terminally differentiated Paneth cells in intestinal epithelium,[39] it is, therefore, suggested that the expression may play key roles not only in modulation of cell proliferation but also in the establishment and maintenance of characteristic phenotypes of glandular cells during the menstrual cycle.

Several lines of evidence from the present study support the conclusion that Sox9 is a transcriptionally positive regulator for $p21^{WAF1}$ expression, cooperating with the 14^{ARF}/p53 pathway in Em Ca cells. First, transient transfection of Sox9 and the stable lines resulted in increased expression of the endogenous *p21WAF1* gene at mRNA and protein levels, in line with other studies in melanoma and chondrocytic cells.[40,41] Second, Sox9 could transactivate the *p21WAF1* promoter on binding to the p53RE, probably through formation of active transcription complexes and transactivation of the *p14ARF* gene, resulting in activation of the $p14^{ARF}$/p53/$p21^{WAF1}$

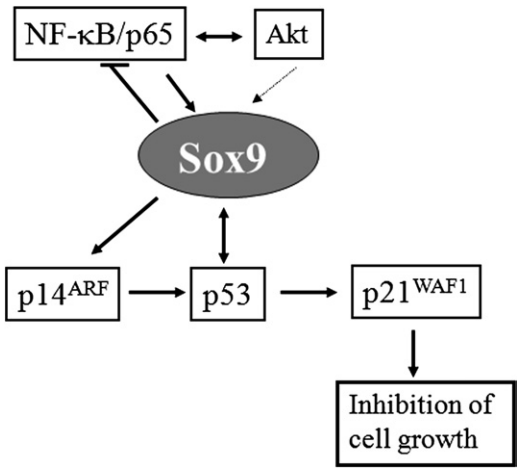

Figure 7. Schematic representation of the functional roles of Sox9 and its associations with NF-κB and Akt, as well as the $p14^{ARF}$/p53/$p21^{WAF1}$ pathway, in Em Ca cells.

pathway. Third, Sox9 immunoreactivity was positively correlated with the status of $p21^{WAF1}$ ($r = 0.69$, $P < 0.0001$) and p53 ($r = 0.47$, $P < 0.0003$) in Em Ca tissues. In contrast, it has been reported that Sox9 expression can cause significant reduction of p53 expression but direct transactivation of the *p21*^{WAF1} gene in chondrocytic cells,[39] whereas knockdown resulted in inhibition of cell growth in lung adenocarcinoma cells, associated with up-regulation and down-regulation of $p21^{WAF1}$ and CDK4, respectively.[14] At the present time, although we are unable to provide an appropriate explanation for the observations, it seems that interactions with other transcription factors may contribute to cell-type specific actions since several specific partners for Sox factors might be involved in the regulation of target genes.[30]

Previous studies have demonstrated that some cytokines, such as TNF-α and IL-1, are potent inhibitors of Sox9 expression through activation of the NF-κB pathway in chondrocytic cells.[42] From the present results, the NF-κB subunit p65 could act as a putative positive regulator of the *Sox9* gene at transcription levels in Em Ca cells, as per the following evidence: i) treatment of cells with TNF-α caused up-regulation of Sox9 expression, along with stabilization of nuclear p65; ii) transient transfection of p65 resulted in increased Sox9 expression at mRNA and protein levels through transactivation of the promoter involving specific NF-κB–binding sites; and iii) Sox9 LI values were positively correlated ($r = 0.4$, $P = 0.0003$) with pp65 LIs in Em Ca tissues. Similar findings have also been reported with HeLa and mouse chondrogenic ATDC5 cells.[31] In addition, the finding of inhibition of NF-κB–mediated transcription by Sox9 allows us to speculate the possible existence of a negative feedback loop between the two.

In addition to NF-κB, overexpression of the active form of Akt (myr-Akt) also caused up-regulation of Sox9 expression through the transcriptional and posttranslational mechanisms, in line with the results of treatment with pharmacologic inhibition of phosphatidylinositol 3- kinase. Given that Akt can phosphorylate many substrates but does not regulate gene transcription,[43,44] it seems that the former is affected in cooperation with other transcription factors, such as NF-κB, since transfection of myr-Akt can enhance NF-κB–mediated transcription, probably through repression of IκBα expression.[45] Due to the lack of such associations in Em Ca tissues, the relatively high levels of pAkt expression in G1 and G2/3 tumors may provide an explanation.

As an unexpected finding in this study, Sox9 expression was significantly associated with high cell proliferative activity and high histologic malignancy in Em Ca cases with functional p53, despite antiproliferative effects of Sox9 in the stable cell lines during the exponential growth phase, whereas knockdown had minor effects on cell proliferation in the relatively early growth phase. In addition, parallel changes in expression of Sox9 and $p21^{WAF1}$ were observed in cells with serum stimulation but not with serum starvation. Taken together with the findings for positive correlations of Sox9 LIs with LIs of Ki-67, as well as p53 and $p21^{WAF1}$, in tumor samples, it seems that a critical threshold may exist for cell proliferation rates in triggering activation of the Sox9-mediated $p14^{ARF}$/p53/$p21^{WAF1}$ pathway. Evidence has recently been presented that increasing proliferation parallels progressive derailment of positive and negative cell cycle regulators in endometrial tumorigenesis.[46,47]

Together, our observations suggest a model for a functional role of Sox9 in Em Ca cells (Figure 7). Overexpression of Sox9 in response to activated NF-κB signaling may cause up-regulation of the *p14*^{ARF} gene at the transcription level, cooperating with p53 function, and this, in turn, leads to activation of the p53/$p21^{WAF1}$ pathway, resulting in inhibition of Em Ca cell proliferation. In addition, the Akt pathway may also serve as an upstream regulator in the process through direct or indirect mechanisms.

In conclusion, the present study provided evidence that associations between Sox9 and NF-κB signaling, as well as Akt status, may participate in modulation of cell kinetics of Em Cas through alterations in the $p14^{ARF}$/p53/$p21^{WAF1}$ pathway.

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