Requirement of the Akt/ β -Catenin Pathway for Uterine Carcinosarcoma Genesis, Modulating E-Cadherin Expression Through the Transactivation of *Slug*

Makoto Saegusa,* Miki Hashimura,*
Takeshi Kuwata,[†] and Isao Okayasu*

From the Department of Pathology,* Kitasato University School of Medicine, Kanagawa; and the Pathology Section,[†] Clinical Laboratory Division, National Cancer Center Hospital East, Chiba, Japan

Uterine carcinosarcomas (UCSs) are considered to represent true examples of the epithelial-mesenchymal transition. Akt plays a key role in the induction of epithelial-mesenchymal transition, but little is known about its involvement in tumorigenesis. Here we examined the functional roles of the Akt/ β -catenin pathway in UCSs. In clinical samples, phospho-Akt (pAkt) expression was found to be significantly increased in mesenchymal compared with epithelial components, exhibiting both positive and negative correlations with nuclear β -catenin and E-cadherin, respectively. Expression levels of the transcription factor *Slug* were also significantly up-regulated in the mesenchymal components and strongly correlated with both pAkt and nuclear β -catenin. In endometrial cancer cell lines, active Akt induced the stabilization of nuclear β -catenin through the phosphorylation of GSK-3 β , and this, in turn, led to the transactivation of *Slug*, which was mediated by nuclear β -catenin. Moreover, *Slug* overexpression itself caused repression of *E-cadherin*, with subtle changes in cell morphology. In addition, knockdown of the retinoblastoma gene product (Rb) up-regulated pAkt and repressed *E-cadherin*, consistent with the *in vivo* finding of significantly decreased Rb expression in mesenchymal components. These findings suggest that changes in the Akt/ β -catenin pathway, as well as alterations in Rb expression, may be essential for both the establishment and maintenance of phenotypic characteristics of UCSs, playing key roles in the regulation of *E-cadherin* through the transactivation of the

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Uterine carcinosarcomas (UCSs) are highly aggressive neoplasms with a biphasic histology featuring both epithelial and mesenchymal elements, the latter being generally further subdivided into two subcategories, homologous with tissues normally found in the uterus and heterologous containing nonnormal elements, most commonly malignant cartilage or skeletal muscles.¹ Recent studies have revealed that the carcinomatous component is the driving force, while the sarcomatous portions are derived from carcinoma or single stem cells that undergo divergent differentiation, indicating that most, if not all, are monoclonal in origin rather than because of collision.^{2,3}

The epithelial-mesenchymal transition (EMT) is a fundamental feature of development that is responsible for initiating pathological alteration in adult organisms. In the process, cells of epithelial origin lose epithelial characteristics and polarity acquiring a mesenchymal phenotype with increased migratory behavior.^{4–7} The molecular mechanisms are characterized by down- and up-regulation of epithelial and mesenchymal markers, respectively, resulting in acquisition of a fibroblast-like morphology with cytoskeleton reorganization and increase in motility, invasiveness, and metastatic capacity.^{4–7} Although observation of full EMT in clinical samples of human and animal tumors is rare,^{8,9} the breast carcinosarcoma is now considered to represent a true example of complete EMT.^{6,10} In contrast, little is known about the association with tumorigenesis of UCSs.

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Address reprint requests to Makoto Saegusa, M.D., Department of Pathology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagami-hara, Kanagawa 228-8555, Japan. E-mail: msaegusa@med.kitasato-u.ac.jp.

A hallmark of EMT is lost expression of E-cadherin, a key component of adhesion junctions that plays an important role in maintenance of epithelial integrity.¹¹ A number of specific transcription factors, including Snail, Slug, SIP-1, and Twist, contribute to induction of EMT, acting as transcriptional repressors of the *E-cadherin* gene.^{12–15} In addition, the oncogenic serine/threonine kinase Akt also promotes the process, modulating several signaling and transcriptional networks linking Wnt/ β -catenin, nuclear factor (NF)- κ B/p65, and the retinoblastoma gene product (Rb).^{16–19} We therefore hypothesized that these factors may serve as mediators for establishment and maintenance of phenotypic characteristics of UCSs. To test this, we here investigated changes in expression of Akt, β -catenin, E-cadherin, and Slug, with reference to the Rb and NF- κ B/p65 status, using *in vivo* and *in vitro* assays.

Materials and Methods

Clinical Cases

We reviewed cases of comprehensively staged high-grade endometrial adenocarcinomas from the patient records of Kitasato University Hospital for the period from 1997 to 2008. According to the criteria of the 2003 World Health Organization classification, tumors were designed as UCS if they had evidence of both malignant epithelial (endometrioid, serous, or clear cell components) and mesenchymal (homologous or heterologous) elements. Endometrioid adenocarcinomas with spindle elements and hyalinized stroma were specifically excluded. Finally, a total of 14 UCSs were investigated (see Supplementary Table S1 at <http://ajp.amjpathol.org>). Thirteen cases of endometrial carcinomas, including six serous, three clear, and four grade 3 endometrioid lesions, were also examined as controls. 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).

Antibodies and Reagents

Antibodies to phospho-Akt at Ser473 (pAkt) (Cell Signaling Technology, Beverly, MA), total AKT (Cell Signaling Technology), β -catenin (clone 14; Transduction Laboratories, Lexington, KY), phospho- β -catenin at Ser33/37/Thr41 (p β -catenin) (Cell Signaling Technology), NF- κ B subunit p65 (p65) (clone 20; Transduction Laboratories), phospho-p65 at Ser276 (pp65) (Cell Signaling Technology), E-cadherin (HECD-1; Takara, Shiga, Japan), p53 (DO7; Novocastra Ltd., Newcastle, UK), cytokeratin (clone AE1/AE3; DAKO, Glostrup, Denmark), vimentin (clone V9, DAKO), GSK-3 β (clone 7, Transduction Laboratories), phospho-GSK-3 β at Ser9 (pGSK-3 β) (Cell Signaling Technology), I κ B α (Cell Signaling Technology), HA (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA), Slug (ab38551; Abcam, Tokyo, Japan), Snail (ab17732, Abcam), Rb (BD Bioscience Pharmingen, San Jose, CA),

phospho-Rb at Ser807/811 (pRb) (Cell Signaling Technology), Ki-67 (DAKO), and β -actin (Sigma Chemical Co., St Louis, MO) were used in this study. Cycloheximide, a specific inhibitor of protein synthesis, was purchased from Calbiochem (Darmstadt, Germany).

Immunohistochemistry

Immunohistochemistry was performed using a combination of the microwave oven heating and polymer immunocomplex (Envision; DAKO, Copenhagen, Denmark) methods. For evaluation of immunostaining, tumor lesions were subdivided into areas containing epithelial and mesenchymal elements. Nuclei immunopositive for β -catenin, pp65, p53, Slug, Snail, Rb, pRb, and Ki-67, were counted in at least 1000 tumor cells in five randomly selected fields each for both components in all cases. Labeling indices (LIs) were then calculated as numbers per 100 cells, as described previously.^{20–22} Cells with nuclear and/or cytoplasmic immunoreactivity for pAkt were also examined in a similar manner. Scoring of membrane and cytoplasmic immunoreactivity for E-cadherin, cytokeratin, and vimentin was performed on the basis of percentages of immunopositive cells and the immunointensity, with multiplication of values for the two parameters, as described previously.^{20–22}

Plasmids and Cell Lines

A full-length cDNA for HA-tagged human *Slug* (GenBank accession number NM003068) was generated by polymerase chain reaction (PCR) and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The promoter sequence between –2125 and –34 bp relative to the translation start site (AF084243) amplified by PCR was subcloned into the pGL3-basic vector (Promega, Madison, WI). A series of 5'-truncated promoter constructs were also generated by PCR, using a combination of specific 5'-forward and common 3' primers (R-235 and R-34). The identity of all constructs was confirmed by sequencing before use. pcDNA3.1-HA- β -cat Δ S45, pcDNA3.1-mouse p65, pCI-Flag-p300, pcDNA3.1-TCF4 Δ N30 (dominant-negative form TCF4), pGL3B (–547/+123) E-cadherin-Luc, pNF κ B-Luc, and Top and Fop reporter constructs were as described previously.^{20–22} The constitutively active myristylated Akt expression plasmid (pUSEamp-myrAkt1) was purchased from Upstate Biotechnology (Lake Placid, NY). pGL3-HBCP containing a β -catenin promoter (–2760/+21 bp) was provided by Dr. Roderick H. Dashwood (Oregon State University, Corvallis, OR). The sequences of PCR primers used in this study are listed in Table 1.

Two endometrial cancer cell lines, Ishikawa and Hec251,^{23,24} were maintained in Eagle's minimal essential medium with 10% bovine calf serum. Ishikawa cells stably overexpressing HA-tagged β -cat Δ S45 (Ish-HA-bcat#25) were also used, as described previously.^{20,21} To establish cells stably overexpressing HA-Slug, the expression plasmid or empty vector were transfected into Hec251 cells. After 2 weeks of culture in the presence of 1 mg/ml of geneticin (Life Technology, Grand Island, NY), three independent clones were established.

Table 1. Primer Sequences Used in This Study

Rb	mRNA	Forward	5'-GCAGTTGACCTAGATGAGATG-3'
		Reverse	5'-CAACATGGGAGGTGAGAGTTT-3'
E-cadherin	mRNA	Forward	5'-CAACATGGGAGGTGAGAGTTT-3'
		Reverse	5'-CGAAGAAACAGCAAGAGCAGCAGAATCAGA-3'
β -Catenin	mRNA	Forward	5'-CCAGCGTGGACAATGGCTAC-3'
		Reverse	5'-CTCTGAGCTCGAGTCATTGC-3'
P65	mRNA	Forward	5'-AATGGCTCGTCTGTAGTGCA-3'
		Reverse	5'-GGTCCTGTGTAGCCATTGAT-3'
Fibronectin	mRNA	Forward	5'-GTGCCTGGGCAACGGA-3'
		Reverse	5'-CCCGACCCTGACCGAAG-3'
Snail	mRNA	Forward	5'-TGCCCTCGACCACTATGCCGC-3'
		Reverse	5'-AGCATTGGCAGCGAGGCGGT-3'
Slug	Cloning	Forward	5'-CAAGATGCCGCGCTCGTTCC-3'
		Reverse	5'-CACCTGAGTTCGCGTCTGGC-3'
	mRNA	Forward	5'-CAAGATGCCGCGCTCGTTCC-3'
		Reverse	5'-TGGCATGGGGTCTGAAAGCT-3'
	Promoter		
	Forward	F-2125	5'-CTGGATTATGCCTCTGTGATCC-3'
		F-1895	5'-GGAGTGAAAAGCAAGGAGGACT-3'
		F-1587	5'-TAGGAAATCTGTGAGTGCCCCA-3'
		F-813	5'-GTACTTAATTTGCACGCGGCC-3'
		F-592	5'-CCACTTCCAAATATAGGCTCTC-3'
		F-444	5'-TCCTGCGCCCTCCTAGCTC-3'
		F-361	5'-ACACTTTTTTTCCTCTCCACTGA-3'
	Reverse	R-235	5'-GAGGTTTCAGATTTCAGCTCCTC-3'
		R-34	5'-GGCGGTCCCTACAGCATCGC-3'

Transfection

Transfection was performed using LipofectAMINE Plus (Invitrogen), in duplicate or triplicate, as described previously.^{20–22} The pRL-TK plasmid (Promega) was used to normalize for transfection efficiency, and luciferase activity was assayed with the dual-luciferase reporter assay system (Promega), as described previously.^{20–22} In addition, siRNAs for Rb, β -catenin, and p65 were transfected using the siPORT NeoFX transfection agent (Ambion, Austin, TX), according to the manufacturer's instructions. The siRNAs, as well as the negative control, are available from Ambion.

Reverse Transcription (RT)-PCR

cDNA was synthesized from 2 μ g of total RNA. 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). The *GAPDH* gene was amplified as an internal control, as described previously.^{20–22}

β -Catenin Gene Mutation Analyses

DNA was extracted from paraffin wax sections of UCS cases, using proteinase K/phenol-chloroform methods. Exon 3 of the β -catenin gene was amplified and sequenced as described previously.²⁰

Western Blot Assays

Total cellular proteins were isolated using RIPA buffer [50 mmol/L Tris-HCl (pH7.2), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate]. Nuclear and

cytoplasmic fractions were also prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). Aliquots of 1 to 10 μ g of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membranes, and probed with primary antibodies, coupled with the ECL detection system (Amersham Pharmacia Biotechnology, Tokyo, Japan).

Immunofluorescence

After transient transfection of pUSEamp-myr-Akt1 into Hec251 cells, they were incubated with primary antibodies. Fluorescein isothiocyanate- or rhodamine-labeled anti-mouse or rabbit IgGs (Molecular Probes, Leiden, The Netherlands) were used as secondary antibodies, as described previously.^{20–22}

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

Relationships Among Expression of Rb, Akt, and E-Cadherin in UCSs

Knowing that Rb plays an important role in maintenance of epithelial phenotype in cells,²⁵ we first examined whether alteration in its expression is associated with genesis of UCSs. As shown in Figure 1A, immunoreactivity for both Rb and its phosphorylated form (pRb) was significantly higher in epithelial than mesenchymal components, independent

Figure 1. Expression of Rb and pRb in UCSs. **A, Top:** H&E staining and immunohistochemistry for Rb, pRb, and Ki-67 are illustrated. The mesenchymal lesion indicated by the arrow is magnified in the inset. **Bottom:** Nuclear LI for Rb, pRb, and Ki-67 in epithelial (Ep) and mesenchymal (Me) components of uterine CS (carcinosarcoma), as well as non-CS tumors. **B, Left and middle:** Comparison of expression levels of several EMT-related molecules for mRNA by RT-PCR (left) and protein by Western blot (middle) between Ishikawa (Ish) and Hec251 cells. E-cad, E-cadherin; FN, fibronectin. **Right:** Ishikawa and Hec251 cells were transfected with Top reporter constructs to determine β -catenin-dependent transcriptional activity. 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 $\pm$ SD. **C:** Analysis of mRNA (at 24 hours after transfection) (left) and total protein (at 48 hours) (right) in Hec251 cells transfected 30 and 50 nmol/L siRNAs for Rb. A siRNA with no homology to the mammalian genome was used as a negative control (con). Original magnifications: $\times 100$ (A, top); $\times 400$ (A, inset).

of cell proliferation determined by Ki-67 immunohistochemistry, suggesting an association between loss of expression and mesenchymal features within UCSs. To verify this hypothesis, we further investigated Hec251 and Ishikawa cells because the former has considerable Rb expression, in contrast to the extremely low levels in the latter.²⁶ As shown in Figure 1B, the Rb status appeared to be closely linked with mRNA and protein expression of several EMT-related molecules, including E-cadherin, fibronectin, Slug, and pAkt, as well as activity of Top (β -catenin/TCF4) and κ B (p65) reporter constructs (see Supplementary Figure S1 at <http://ajp.amjpathol.org>). Furthermore, inhibition of Rb by specific siRNAs caused decreased E-cadherin and increased pAkt expression in Hec251 cells (Figure 1C).

Akt Regulates Expression of E-Cadherin and Nuclear β -Catenin in UCSs

To further show phenotypic characteristics of epithelial and mesenchymal components of UCSs, we conducted

immunohistochemistry for pAkt, E-cadherin, and β -catenin, as well as pp65, p53, cytokeratin, and vimentin. Examples of the findings are illustrated in Figure 2A and Supplementary Figure S2 (see <http://ajp.amjpathol.org>). As shown in Figure 2B, E-cadherin expression was completely lacking in mesenchymal elements, in contrast to moderate levels in epithelial cells, the difference in the scores being significant. Average pAkt and nuclear β -catenin LI also showed significant differences between the two components. The pAkt LI and E-cadherin scores, as well as pp65 LI, did not show any differences between epithelial parts of UCSs and non-UCS tumors, with the exception of nuclear β -catenin LI, despite a low frequency (7.1%) of mutations in exon 3 of the gene (see Supplementary Table S1 and Supplementary Figure S3 at <http://ajp.amjpathol.org>). Significant differences in both cytokeratin and vimentin scores were also observed between the components. A similar tendency was also observed in a case of pp65 LI, but the difference did not reach significance ($P = 0.05$), and similar values were

Figure 2. Akt regulates expression of E-cadherin and β -catenin. **A:** Serial sections through a UCS. H&E and immunohistochemistry for E-cadherin, pAkt, and β -catenin are illustrated. The lesions enclosed by boxes in the **top panels** are magnified in the **bottom panels**. **B:** Immunohistochemical (IHC) scores for E-cadherin (**left**) and LIs for pAkt and nuclear (N) β -catenin (**right**) in epithelial (Ep) and mesenchymal (Me) components of uterine CS (carcinosarcoma), as well as non-CS tumors. **C, Top left:** Western blot analysis of E-cadherin (E-cad) was performed with subcellular protein fractions (Cyt, cytoplasmic; Nu, nuclear) extracted from myr-Akt-transfected Hec251 cells. In the pAkt panel, upper bands (indicated by the asterisk) are exogenous myr-Akt, in contrast to the lower bands demonstrating endogenous pAkt. **Bottom left:** After transfection of myr-Akt into Hec251 cells, staining for pAkt and E-cadherin was performed. Note the lack of E-cadherin expression in cell overexpressing myr-Akt (indicated by arrows). **Top right:** Analysis of E-cadherin (E-cad) mRNA levels by RT-PCR with total RNA extracted from myr-Akt-transfected Hec251 cells. **Bottom right:** Hec251 cells were transfected with reporter constructs containing *E-cadherin* promoter (E-cad Luc), together with expression plasmid for myr-Akt. 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 $\pm$ SD. **D, Top left:** Western blot analysis of HA- β -catenin, total GSK-3 β , and phospho-GSK-3 β (pGSK-3 β) was performed with subcellular protein fractions (Cyt, cytoplasmic; Nu, nuclear) extracted from myr-Akt-transfected Ish-beat#25 cells. **Bottom left:** After transfection of myr-Akt, Ish-beat#25 cells were treated with cycloheximide (25 μ g/ml) for the times shown. Western blotting was performed using anti-HA, anti-pAkt, anti-GSK-3 β , and anti-pGSK-3 β antibodies. In the pAkt panel, upper bands (indicated by the asterisk) are exogenous myr-Akt, in contrast to lower bands demonstrating endogenous pAkt. **Top and bottom right:** Relative amounts of HA- β -catenin were calculated by normalization to the signals for β -actin, using NIH Image. Expression levels of HA- β -catenin observed in myr-Akt-transfected cells in the absence of cycloheximide treatment (0 hours) were set as 100%. The experiment was performed in triplicate. Original magnifications: $\times 100$ (**A, top**); $\times 400$ (**A, bottom**).

found for p53 LIs (see Supplementary Figure S2 at <http://ajp.amjpathol.org>). E-cadherin scores were inversely correlated with LIs of pAkt and nuclear β -catenin, whereas nuclear β -catenin LIs were positively correlated with LIs of pAkt. Moreover, both pAkt and nuclear β -catenin LIs showed negative and positive correlations with cytokeratin

and vimentin scores, respectively, whereas Rb and pRb status were positively correlated with E-cadherin scores only. In addition, LIs of pp65, as well as p53, did not show correlations with any of the markers investigated, with the exception of nuclear β -catenin (Table 2 and see Supplementary Table S2 at <http://ajp.amjpathol.org>).

Table 2. Correlations Among Immunohistochemical Markers Investigated in UCSs

	pAkt ρ^* (P)	N- β -cat ρ (P)	pp65 ρ (P)	E-cad ρ (P)	Snail ρ (P)	Slug ρ (P)	Rb ρ (P)	pRb ρ (P)
N- β -cat	0.85 (<0.01)	*						
pp65	0.2 (0.29)	0.44 (0.02)	*					
E-cad	-0.57 (0.003)	-0.68 (0.0004)	-0.21 (0.28)	*				
Snail	-0.04 (0.82)	0.15 (0.43)	0.26 (0.17)	0.02 (0.9)	*			
Slug	0.52 (0.007)	0.75 (<0.0001)	0.3 (0.08)	-0.61 (0.002)	0.1 (0.56)	*		
Rb	-0.16 (0.41)	-0.1 (0.59)	-0.05 (0.8)	0.45 (0.03)	0.1 (0.57)	-0.3 (0.12)	*	
pRb	0.03 (0.89)	-0.04 (0.83)	-0.19 (0.33)	0.42 (0.03)	0.03 (0.88)	-0.2 (0.26)	0.869 (<.0001)	*
Ki-67	-0.15 (0.44)	-0.24 (0.22)	-0.36 (0.004)	0.3 (0.12)	0.11 (0.98)	-0.19 (0.32)	0.06 (0.76)	0.06 (0.74)

* ρ , Spearman correlation coefficient.

Labeling indices, pAKT, N- β -catenin (nuclear β -catenin), pp65, Snail, Slug, pRb, ppRb, JHC score, E-cad (E-cadherin), Ki-67.

Figure 3. Up-regulation of Slug in UCSs. **A, Left:** Serial sections through a UCS. H&E staining and immunohistochemistry for Slug and Snail are illustrated. Boxes with dotted and solid outlines enclose epithelial and mesenchymal lesions magnified in **a** and **b**, respectively. **Right:** Nuclear LIs for Slug and Snail in epithelial (Ep) and mesenchymal (Me) components of UCSs. **B, Left:** Analysis of mRNA levels for Slug and Snail by RT-PCR with total RNA extracted from Hec251 cells transfected with myr-Akt and β -catenin. **Right:** Hec251 cells were transfected with reporter constructs containing 1.89 kbp of the *Slug* promoter (Slug Luc), together with expression plasmids for myr-Akt and β -catenin. 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 $\pm$ SD. **C, Left:** Analysis of E-cadherin (E-cad) mRNA by RT-PCR (**top**) and protein by Western blot (**bottom**) in HA-Slug-transfected Hec251 cells. Detection of HA-Slug mRNA was performed using a combination of 5'-HA forward (5'-TACCCATACGATGTTCAGATTACGC-3') and Slug mRNA reverse (Table 1) primers. **Right:** Hec251 cells were transfected with reporter constructs containing the *E-cadherin* promoter (E-cad Luc), together with a Slug expression plasmid. **D:** Hec251 cells stably overexpressing HA-Slug. **Left:** Analysis of E-cadherin expression levels for mRNA by RT-PCR (**top**) and protein by Western blot (**bottom**) in three independent stable cell lines (nos. 13, 31, and 50). **Right:** The subtle changes in the morphology in the stable cell lines. Original magnifications: $\times 100$ (**A, top**); $\times 400$ (**A, bottom**).

Based on the above findings and the previous reports that Akt acts as an upstream regulator for several signal pathways,^{16–19} we next examined whether Akt is involved in regulation of E-cadherin and β -catenin, as well as p65, using Hec251 and Ish-HA-bcat#25 cells. Transient transfection of a constitutively active form, myristylated Akt (myr-Akt), resulted in a decrease in endogenous E-cadherin expression at both mRNA and protein levels, in line with repression of promoter activity in Hec251 cells (Figure 2C). In Ish-HA-bcat#25 cells, overexpression also caused an increase in both cytoplasmic and nuclear HA- β -catenin levels, along with an increase in phosphorylated GSK-3 β (Figure 2D), despite no changes in the endogenous mRNA levels and the promoter activity (see Supplementary Figure S4, A and B, at <http://ajp.amjpathol.org>). A cycloheximide chase study revealed the half-life of HA- β -catenin protein to be significantly increased in myr-Akt transfected cells as compared with the mock-transfected case (Figure 2D), in line with the finding of considerable decrease in phosphorylated β -catenin in myr-Akt-transfected Hec251 cells (see Supplementary Figure S4C at <http://ajp.amjpathol.org>). Furthermore, the transfection also caused increase in nuclear p65 levels, as well as inhibition of I κ B α expression, resulting in activation of κ B-dependent transcription in Hec251 cells (see Supplementary Figure S5A at <http://ajp.amjpathol.org>).

Up-Regulation of Slug by Nuclear β -Catenin

Because transcription of *E-cadherin* gene is regulated, to a large extent, by Snail/Slug family members that bind to the E-box in the proximal promoter,^{12,13} we examined whether their expressions are required for development of UCSs. Distinct nuclear Slug staining was predominantly found in mesenchymal rather than epithelial components, the difference in the LIs being significant, in contrast to diffuse Snail immunoreactivity within tumors (Figure 3A). As shown in Table 2, Slug LIs were positively correlated with LIs of pAkt and nuclear β -catenin but not pp65, and inversely with E-cadherin scores. However, such associations were not observed in a case of Snail LIs.

Transient transfection of myr-Akt and β -catenin, as well as p65, resulted in considerable increase in mRNA levels for Slug but not Snail, in line with activation of the promoter in Hec251 cells (Figure 3B and see Supplementary Figure S5B at <http://ajp.amjpathol.org>). However, Akt-mediated up-regulation of Slug expression was not abrogated by inhibition of either β -catenin or p65, respectively, using specific siRNAs (see Supplementary Figure S5C at <http://ajp.amjpathol.org>), again indicating post-translational regulation of β -catenin and p65 by myr-Akt. Moreover, overexpression of HA-Slug caused a considerable decrease in E-cadherin at both mRNA and protein levels, along with repression of promoter activity (Figure

Figure 4. Transactivation of *Slug* promoter by β -catenin. **A:** The various promoter deletion constructs used for evaluating transcriptional regulation of the *Slug* promoter. Numbers for each construct corresponding to the 5'- and 3'-bp locations within the promoter (relative to the translation start site as +1). **B and C:** Hec251 cells were transfected with 5' various deletion constructs of *Slug* promoter, together with the β -catenin expression plasmid. 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 $\pm$ SD. **D:** Hec251 cells were transfected with reporter constructs containing 1.89 kbp of the *Slug* promoter, together with expression plasmids for β -catenin, dominant-negative (DN)-TCF4, and p300.

3C), although Hec251 cells stably overexpressing exogenous *Slug* showed subtle changes in morphology, preserving cell-cell adhesion (Figure 3D).

Searching of an $\sim$ 2-kbp fragment upstream of the translation start site in the *Slug* gene (AF084243) revealed three potential TCF-binding sites (CTTTG T/A T/A), but a lack of putative NF- κ B-binding elements (GGRNNYYCC) (Figure 4A). With the series of 5'-truncated promoter constructs, deletion from -2125 to -361 bp had little effect on induction of promoter activity by β -catenin (Figure 4B). Whereas the -440/-34-bp and -361/-235 fragments responded very similarly to these activators, -257/-34 bp did so to only a very low levels (Figure 4C), indicating that sequences involved in the response for β -catenin are present between -361 to -257 bp. However, β -catenin-mediated transactivation of the *Slug* gene was not affected by co-transfection of either dominant-negative TCF4 or transcriptional co-activator p300 (Figure 4D), in contrast to significant inhibition of the transcription activity by the former as determined with Top and Fop reporter constructs (see Supplementary Figure S5D at <http://ajp.amjpathol.org>). Similar findings were also observed on transfection of p65, and activation of -257/-34-bp fragment was intermediate (see Supplementary Figure S5E at <http://ajp.amjpathol.org>), suggesting that more than one sequence contribute to the stimulation.

Discussion

The present study demonstrated that most UCSs in our series showed not only co-expression of cytokeratin and vimentin, but also identical p53 staining patterns in epithelial and mesenchymal components, supporting the idea that carcinosarcomas are best regarded as meta-

plastic carcinomas.¹ The complete shut-down of E-cadherin expression in mesenchymal elements is in line with evidence that cells undergoing EMT down-regulate E-cadherin,¹¹ suggesting that molecular events responsible for triggering EMT may occur during development of this type of tumor.

Akt is considered to be a key mediator for EMT induction in human epithelial malignancies.^{27,28} In the present study, the expression was up-regulated in mesenchymal components, exhibiting an inverse correlation with E-cadherin. Although transfection of myr-Akt caused transcriptional repression of the *E-cadherin* gene, it is probable that this is effected in cooperation with other transcription factors, because Akt can phosphorylate many substrates but does not directly regulate gene

Figure 5. Schematic representation of possible roles of the Akt/ β -catenin pathway in determination of phenotypic characteristics of UCS.

transcription.^{19,29} It is well accepted that Akt can inactivate GSK-3 β through phosphorylation at Ser9, resulting in stabilization of β -catenin and activation of β -catenin-dependent transcription.^{30,31} Phosphorylation of β -catenin at Ser552 by Akt also facilitates β -catenin dissociation from cell-cell contacts and accumulation in nuclei but does not alter the protein stability.³² In our study, overexpression of myr-Akt could induce stabilization of cytoplasmic and nuclear β -catenin through phosphorylation of GSK-3 β , in line with the *in vivo* finding of a very strong correlation ($\rho = 0.85$, $P < 0.0001$), suggesting that β -catenin is involved in the Akt downstream pathway. In addition, although induction of myr-Akt was also found to activate p65-dependent transcription, probably through repression of I κ B α expression,³³ we failed to confirm such an association in tumor tissues, suggesting that the *in vivo* pp65 status may be affected not only by pAkt but also by other factors, such as transforming growth factor- β and tumor necrosis factor- α .^{34,35}

In our results, expression of Slug, rather than Snail, appeared to be closely associated with the mesenchymal phenotype in UCSs, despite both being capable of repressing E-cadherin expression.^{12,13} A similar finding has also been reported for basal-like breast carcinomas.³⁶ Interestingly, stable overexpression of exogenous Slug inhibited E-cadherin expression at transcriptional levels, although changes in cell morphology were relatively minor. We therefore conclude from the present data that transactivation of the *Slug* gene may be essential in cells that have lost epithelial characteristics in UCSs, acting as an *in vivo* transcriptional repressor of the *E-cadherin* gene. Because cell-cell contacts were still preserved in the stable cell lines, it appears that a critical threshold for E-cadherin must be crossed for complete loss function. Further studies of this point are clearly warranted.

An important finding here is that Slug expression is under transcriptional control of nuclear β -catenin, from the following evidence: i) the very significant correlation ($\rho = 0.75$, $P < 0.0001$) between the two in tumor tissues; ii) up-regulation of Slug expression at both mRNA and protein levels induced by transfection of β -catenin in cell lines; iii) activation of the *Slug* promoter mediated by β -catenin, through the proximal region (–361 to –257 bp). Although most β -catenin targeted genes require TCF/LEF factors for their activation,^{37–39} the observed activation did not require binding sites in the promoter, as evidenced by the failure of deletion or dominant-negative TCF4, in contrast to *Xenopus* and mouse *Slug* promoters activated by β -catenin/TCF complexes through the binding sites.^{40,41} In addition, evidence has been presented that an increase in the cytoplasmic pool of β -catenin occurs after release from interaction with E-cadherin,^{42–44} leading us to speculate on existence of a positive feedback loop of nuclear β -catenin mediated by Slug and E-cadherin. In contrast, p65 might also induce transactivation of the *Slug* promoter, but such an association was not evident in tumor tissues, suggesting that a signal pathway involving β -catenin rather than p65 may be the primary mechanism underlying regulation of *in vivo* *Slug* transcription in UCSs.

There is a rapidly growing body of evidence that Rb plays key roles not only in cell cycle progression but also maintenance of the epithelial phenotype by preventing EMT.^{18,19,25} For example, Rb binds to an E-cadherin promoter sequence in association with the transcription factor activator protein 2 α , suggesting that Rb plays a key role in the EMT/MET (mesenchymal-epithelial transition) switch through regulation of E-cadherin and other EMT-related molecules.²⁵ In agreement with this notion, we demonstrated that expression of Rb, as well as pRb, was decreased in mesenchymal components of UCSs, independent of cell proliferative activity. Further support was provided by the *in vitro* finding that the knockdown was because of changes in expression of several EMT-related molecules, including Akt and E-cadherin.

Together, our observations suggest a model for molecular mechanisms determining phenotypic characteristics of UCSs (Figure 5). In most tumors, constitutive expression of Rb may be required for preserving the epithelial phenotype of the tumor cells, but decreased expression is responsible for activation of the Akt/ β -catenin pathway, and this in turn leads to down-regulation of E-cadherin through transactivation of the *Slug* gene, and consequent changes in morphology toward a mesenchymal phenotype. Cooperation with the NF- κ B pathway may be a minor event during the process.

In conclusion, the present study provided evidence that the Akt/ β -catenin pathway, as well as alterations in Rb expression, may be essential for establishment and maintenance of phenotypic characteristics in UCSs, playing key roles in regulation of E-cadherin through transactivation of the *Slug* gene.

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