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Transforming Growth Factor β Signaling Is Disabled Early in Human Endometrial Carcinogenesis Concomitant with Loss of Growth Inhibition[1]

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ABSTRACT

Transforming growth factor β (TGF-β), a potent ubiquitous endogenous inhibitor of epithelial cell growth, is secreted as a latent molecule (LTGF-β) requiring activation for function. TGF-β signals through the type I (TβRI) and type II (TβRII) receptors, which cooperate to phosphorylate/activate Smad2/3, the transcriptional regulators of genes that induce cell cycle arrest. That carcinomas grow *in vivo* suggests that they are refractory to TGF-β. However, this has been difficult to prove because of an inability to analyze the functional status of TGF-β *in vivo* as well as lack of close physiological paradigms for carcinoma cells *in vitro*. The current studies demonstrate that whereas primary cultures of endometrial epithelial cells derived from normal proliferative endometrium (PE; $n = 10$) were dose-dependently and maximally growth inhibited by 55% ± 5.3% with 10 pM TGF-β1, endometrial epithelial cells derived from endometrial carcinomas (ECAs; $n = 10$) were unresponsive ($P \leq 0.0066$). To determine the mechanism of TGF-β resistance in ECAs, we analyzed the TGF-β signaling pathway *in vivo* by immunohistochemistry using specific antibodies to TβRI and TβRII, Smads, and to the phosphorylated form of Smad2 (Smad2P), an indicator of cells responding to bioactive TGF-β. Smad2P was expressed in all of the normal endometria ($n = 25$), and was localized to the cytoplasm and nucleus in PE, and only nuclear in the secretory endometrium. In marked contrast, Smad2P immunostaining was weak or undetectable in ECA ($n = 22$; $P \leq 0.001$) and reduced in glandular hyperplasia ($n = 25$) compared with normal endometrium. However, total Smad2 and Smad7 (which inhibits Smad2 activation) levels were identical in ECA and normal tissue. Consistent with loss of downstream signaling, both TβRI ($n = 19$) and TβRII ($n = 22$) protein expression were significantly reduced in ECA compared with PE ($n = 11$; $P \leq 0.05$). By *in situ* hybridization, the mRNA levels of TβRI and TβRII were decreased in the carcinoma cells compared with normal PE glands, suggesting that receptor down-regulation occurs at the transcriptional level. Furthermore, a somatic frameshift mutation in the polyadenine tract at the 5′ end of the *TβR-II* gene was detected in two of six cases examined. Finally, the ability of explants of ECA to activate endogenous LTGF-β was deficient compared with normal tissue (23.5% *versus* 7.4%). Therefore, our results suggest that loss of Smad2 signaling in ECA may be because of down-regulation of TβRI and TβRII, and/or decreased activation of LTGF-β. Because disruption of TGF-β signaling occurred independent of grade or degree of invasion and was evident in premalignant hyperplasia, we conclude that inactivation of TGF-β signaling leading to escape from normal growth control occurs at an early stage in endometrial carcinogenesis, thereby defining novel molecular targets for cancer prevention.

INTRODUCTION

ECA[5] is the most common gynecological malignancy with an incidence of ~40,000 cases/year in the United States (reviewed in Refs. 1–3). Whereas the pathogenesis of ECA is poorly understood, adenocarcinomas are generally preceded by premalignant hyperplasia providing an excellent paradigm for identifying molecular events associated with conversion to malignancy. Type I endometrioid ECA, accounting for 85% of all endometrial cancers, is etiologically related to unopposed estrogen (*e.g.*, anovulation, estrogen replacement therapy), which stimulates uterine glandular EEC proliferation resulting in hyperplasia. Similarly, other selective ER modulators (SERMS) can cause endometrial hyperplasia; one such agent is Tamoxifen, which is widely used for the treatment of breast cancer (4). Glandular hyperplasia, especially with cytologic atypia, progresses to ECA at a rate as high as 82% (1–3). Therefore, tissue specimens often include a histopathological spectrum containing normal glands, various degrees of hyperplasia (simple and complex), and grades of adenocarcinoma. Conversely, progestins inhibit EEC growth and, therefore, provide highly effective therapy for endometrial hyperplasia and ECA.

Understanding how different types of cancers escape control by the plethora of growth regulatory proteins is critical for providing insight into mechanisms of carcinogenesis. The TGF-β family of proteins is one of the few known endogenous inhibitors of epithelial cell growth. (reviewed in Refs. 5, 6). Components of the TGF-β signaling pathway are classic tumor suppressors, because their inactivation or absence is often a target for carcinogenesis, and mutations or alterations at any step along this complex growth regulatory pathway can result in uncontrolled proliferation. In the past, the status of TGF-β function *in vivo*, in ostensibly proliferating human cancers has been difficult to assess mainly because TGF-β is produced as a latent molecule, and its abundance in and around cells, even in the presence of TGF-β signaling receptors, does not indicate that it is actively inducing growth arrest. In addition, the responses of human cancer cell lines, including those established from ECAs, to TGF-β *in vitro* are often cell line-specific. TGF-β can be growth inhibitory, have no effect, or even be growth stimulating to ECA cell lines (reviewed in Refs. 3, 7, 8). Thus, it is difficult to know whether human carcinomas are refractory to TGF-β *in vivo*, as tumor growth suggests. Nevertheless, in recent years, studies have centered on unraveling the pathways and mechanisms of TGF-β function in many normal and malignant cell types. These reports have contributed to a body of mechanistic information that can be translated into potential molecular therapeutic targets for cancer (reviewed in Refs. 5, 6, 9–13).

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[5] The abbreviations used are: ECA, endometrial carcinoma; PR, progesterone receptor; ER, estrogen receptor; IHC, immunohistochemistry; EEC, endometrial epithelial cell; RT-PCR, reverse transcription-PCR; HNPCC, human nonpolyposis colorectal cancer; DIG, digoxigenin; TGF, transforming growth factor; cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; PE, proliferative endometrium; SE, secretory phase endometrium; Smad2P, phosphorylated Smad2; ESC, endometrial stromal cell; ISH, *in situ* hybridization; SFBS, charcoal-stripped fetal bovine serum; PAI-1, plasminogen activator inhibitor-1; MSI, microsatellite instability; LTGF-β, latent TGF-β; DAB, 3,3′-diaminobenzidine; NRS, normal rabbit serum.

The three mammalian TGF-β isoforms are secreted as inactive latent precursor molecules, dimers composed of the latent associated protein and mature TGF-β, that require activation to bind to TGF-β receptors and initiate signal transduction (reviewed in Ref. 14). The latent precursor is secreted precleaved as an electrostatically bound complex that can be dissociated *in vitro* by extreme pH or heat to release the M_r 25,000 bioactive dimer. *In vivo*, activation of TGF-β isoforms is not well understood, and different mechanisms that have been described appear to be cell-type specific, including endogenous proteases, integrin binding/activation, and interaction with thrombospondin (15–18). TGF-β isoforms signal through a heteromeric complex usually composed of two TGF-β serine/threonine kinase transmembrane signaling receptors (TβRI and TβRII), that directly activate intracellular transcription factors, designated as R-Smads (receptor-specific substrates), specifically, Smad2 or Smad3 (reviewed in Refs. 19–23). In addition, alternative pathways for TGF-β postreceptor signal transduction, particularly, the mitogen-activated protein kinase or c-Jun-NH_2-terminal kinase pathways, may be involved in cross-talk with the Smad pathway (24–27) or function independently of Smads (27, 28). For classic signal transduction, active TGF-β isoforms bind to TβRII, which subsequently phosphorylates TβRI. Activated TβRI directly phosphorylates R-Smad2 or R-Smad3, which combines with co-Smad4 (common for the TGF-β family of ligand/receptor pairs), and the complex translocates to the nucleus to activate TGF-β-regulated genes. The specificity of the TGF-β-mediated response is determined by cell-type-specific DNA-binding proteins, which direct Smads to specific genes and transcriptional coactivators or corepressors, which are sequestered into the transcription complex (29). In addition, specific inhibitory Smads, Smad6 and Smad7, interact with TβRI without acting as a substrate for their activity thereby blocking TGF-β action by preventing the interaction of R-Smad with TβRI (30–33).

TGF-β functions as an inhibitor of growth by regulating genes that control cell cycle progression in late G_1 phase of the cell cycle (reviewed in Refs. 5, 6, 34). Although complex and probably cell-type-1 specific, basically two classes of gene responses ensue: repression of the proto-oncogene *c-myc* and transcriptional activation of the cdk inhibitors, $p15^{Ink4B}$ and $p21^{cip}$, which cause inhibition of the cdks, that phosphorylate pRb. In addition, TGF-β down-regulates certain cyclins and cdks as well as cdc25A, a phosphatase that removes inhibitory tyrosine phosphorylation from cdks (35). The ultimate effect of cdk inhibition is to maintain pRb in a hypophosphorylated state, thereby preventing transition to S phase. Conversely, hyperphosphosrylated pRb releases the transcription factor E2F, which transactivates genes involved in cell cycle progression, specifically, *c-myc*, which can repress $p15^{ink4B}$ by binding to its promoter (36). The magnitude of the TGF-β response is exemplified by the fact that the levels of 1% of all transcripts are altered 4 h after addition of TGF-β to mammary epithelial cells (6).

Increased expression of TGF-β isoforms in a variety of human cancers (*e.g.*, breast, colon, pancreatic, gastric, brain, prostate) has been correlated with decreased survival, and, therefore, high levels of TGF-β can be considered a biomarker for poor prognosis in these malignancies (5, 37). The elevated levels of TGF-β afford a selective advantage for tumor growth because TGF-β is potently immunosuppressive and angiogenic in the host (5, 16, 38–40). Disabling the TGF-β signaling pathway, by specific mutations or down-regulation of its functional components, is a target for loss of tumor suppressor function in many human cancers (6, 41–45). However, specific human cancers possess a proclivity for inactivating different members of this pathway, even at different stages of carcinogenesis and progression (5).

To better understand the pathogenesis of ECA and, specifically, to determine whether ECAs have lost their response to TGF-β *in vivo*, we produced primary cultures of EECs derived from malignant endometrium and compared growth responses to TGF-β with EECs isolated from both PE and SE. With this approach, we were able to compare the phenotypes and behavior of normal and malignant cells *in vitro*. Moreover, information regarding the TGF-β growth inhibitory pathway was compared by *in vivo* and *in vitro* analyses of the same patient. Our studies show that normal EECs express Smad2P *in vivo*, indicating that the cells are being stimulated by and presumably responding to bioactive TGF-β within their microenvironment. In contrast, Smad2P could not be detected in all grades of ECA, although these cells expressed normal levels of inactivated Smad2. Premalignant hyperplastic glands expressed intermediate levels of Smad2P. The loss of Smad2 signaling in ECA was accompanied by markedly lower levels of both TGF-β receptors and a lack of available activated TGF-β in ECA explant cultures; both could potentially lead to decreased Smad2P signaling. Our results provide the first direct demonstration that malignant progression of human ECA is associated with early loss of TGF-β responsiveness *in vivo* and that the loss of TGF-β growth inhibitory function can be recapitulated *in vitro* with primary cultures of EECs derived directly from ECAs. In addition, we conclude that the signaling pathway for TGF-β-mediated growth inhibition is a primary target for dysregulated growth early on in endometrial carcinogenesis.

MATERIALS AND METHODS

Tissue Procurement, Isolation of EECs, Stromal Cells, and Primary Cell and Explant Cultures

Fresh endometrial tissue from type I endometrioid tumors of women of ages 30–55 was obtained under sterile conditions after hysterectomy at New York University-Tisch and Bellevue Hospitals. Tissue from endometrial curettings was placed in phenol red-free McCoy's 5A (Sigma Chemical Co., St. Louis, MO) medium on ice and cultures initiated within 1 h. Grade and homogeneity of ECA tissue and phase of the menstrual cycle for normal endometrium were confirmed the following day by R. D. and other pathologists of the Surgical Pathology Department of Bellevue Hospital. The ECA samples were graded according to the WHO system (grades I-III, each, for decreased glandular architecture [International Federation of Gynecologists and Obstetricians (upper number)] and for nuclear atypia (lower number; Ref. 46). Tissue considered to be normal was obtained from noncancer-related hysterectomies, and only macroscopically normal tissue was used. EECs and ESCs were isolated from both normal and ECA tissue for cell culture using a modification of a method by Reynolds *et al.* (47). A portion of the tissue was flash frozen in liquid N_2 for RNA isolation, and patient tissue specimens were embedded in paraffin for IHC and ISH. Additional archival paraffin blocks of normal, hyperplastic, and ECA tissue were obtained from the New York University and Bellevue Tumor Registry, and Yale New Haven Surgical Pathology Department. All of the studies using human subjects had full New York University School of Medicine Institutional Review Board approval (Assurance of Compliance number M1177-01).

Separation of Glandular EECs from ESCs. Endometrial tissue was minced, and the tissue was digested for 15 min with type 1 collagenase (1.5 mg/ml; Worthington Biochemical Corp., Freehold, NJ) in McCoy's 5A medium containing ABAM (antibiotic antimycotic solution; Life Technologies, Inc., Grand Island, NY), 1 unit/ml DNase1 (Sigma Chemical Co), and 15 mM HEPES (Life Technologies, Inc.), and the aggregated EECs separated by slow centrifugation (100 × *g*) for 2 min. This was performed three separate times, and supernatants containing the ESCs were pooled. The EEC cell pellets were suspended in phenol-red free McCoy's 5A medium containing 15 mM HEPES, 10% SFBS (48; Hyclone Labs., Logan, UT), ABAM, and $3–4 \times 10^6$ cells were seeded onto Primaria (Falcon/Becton Dickinson, Lincoln Park, NJ) 60-mm tissue culture plates. Primaria polystyrene plates have been stably modified by incorporating a mixture of anionic and cationic functional groups, including a consistent ratio of nitrogen-functional groups. The isolation method yields microaggregates of EECs in which the glandular cells retain their physiological

polarized state. To separate individual cells for accurate counting, a small aliquot was removed and trypsinized for 10 min at 37°C (Trypsin-EDTA; Mediatech Inc., Herndon, VA). Both normal and malignant EECs were isolated and cultured identically in 5% CO_2 humidified air at 37°C. The distribution of normal EECs and ECA cells within the cell cycle was performed over time using propidium iodide (Sigma Chemical Co.) staining followed by fluorescence-activated cell sorter analysis (Ref. 49; Kaplan Comprehensive Cancer Center Core Facility). Briefly, 4×10^6 cells were seeded on 60-mm Primaria plates in McCoy's 5A/10% SFBS and the cells removed by trypsinization at the specified times. Doubling times were determined by seeding the cells at 5×10^4/well/96-well plate in the same medium and cell growth determined every 24 h by the MTS assay (Promega, Madison, WI) or by cell counting using Trypan Blue (Life Technologies, Inc.) after trypsinization. The pooled ESCs from the EEC isolation were centrifuged and the cell pellet resuspended in phenol red-free DMEM/F12 (Life Technologies, Inc.) containing 15 mM HEPES, ABAM, 2.0 nM L-glutamine (Life Technologies, Inc.), and 10% charcoal-stripped calf serum (Hyclone Labs). The purity of the cell cultures, grown on removable chamber slides (LabTek, Naperville, IL) for 6 days, was assessed by IHC using anticytokeratin antisera (to cytokeratins 7, 10, 18, MN 116; Dako, Carpinteria, CA) and by the difference in their morphology. The cells were fixed with 4% paraformaldehyde, immunostained essentially as described below for TGF-β receptors, and assessed by light microscopy (Zeiss Photomicroscope II). ECA cells were fixed with 10% formalin, stained with H&E, and observed using an inverted microscope with phase contrast (Zeiss Axiovert 25). Paraffin-embedded tissue samples and cells adapted to culture, from the same patients, that were plated on chamber slides, were also immunostained for ERs and PRs (antisera from Dako), and TGF-β receptors (TβRI and TβRII; described below) to ensure that steroid receptor and TGF-β receptor levels were similar *in vivo* and *in vitro*. TGF-β receptor levels were determined at 36 h after seeding, the same time TGF-β was added to the cultures for the determination of growth inhibition (described below).

Explant Cultures. Explant cultures were used to test the ability of normal and malignant endometrial tissue to activate LTGF-β. The tissue was dissected into uniform 1–2 mm^3 pieces, and 8–10 explants suspended within Millicell inserts (Millipore, Bedford, MA) near the surface of the air/culture medium interface, as we have described previously (50). The explants were maintained in phenol red-free DMEM-F12 supplemented with 1% insulin, transferrin, and selenium (Collaborative Biomedical Products, Bedford, MA) and 0.1% Excyte (Bayer Corporation, Kankakee, IL), and incubated at 37°C for 48 h in a humidified incubator with 95% air and 5% CO_2. Conditioned medium were collected in prechilled microfuge tubes containing phenylmethylsulfonyl fluoride (1 mM; Sigma Chemical Co.), aprotinin (100 ng/ml; Sigma Chemical Co.), and leupeptin (10 ng/ml; Sigma Chemical Co.), and the samples stored at −80°C until their use in the Pal/L assay described below.

Assay for TGF-β-mediated Growth Inhibition

Primary cultures of EECs from normal PE and SE, and from ECA (5×10^4cells/well on Primaria 96-well plates) were seeded in medium containing 10% SFBS. After 36 h, serum free-medium containing increasing concentrations of recombinant TGF-β1 (0–400 pM; a gift from Dr. M. Palladino, Genentech, South San Francisco, CA) was added to subconfluent cells (80% confluency) and cell proliferation assessed in triplicate 4 days later using an MTS assay kit.

MTS Assay. The cells were incubated for 4 h at 37°C in 5% CO2 with media supplemented with 0.02 ml of MTS/phenazine methosulfate solution, prepared as described by Promega. Absorbance was measured at 490 nm using a 7520 Microplate reader (Cambridge Technology Inc., Cambridge, MA). In certain experiments, the values obtained by the MTS assay were simultaneously confirmed by trypsinization and cell counting using trypan blue exclusion to stain viable cells.

Assay for Activation of LTGF-β by Explants

The percentage activated TGF-β in the supernatants from explant tissue culture was determined using the PAI/L assay as described (51). The quantity of total (potential for activation) LTGF-β was compared with the quantity of endogenously activated TGF-β and the percentage activated quantitated. Supernatants obtained from the explant cultures of normal and malignant endometrium after 48 h in culture were collected, and 50% of the conditioned medium was equilibrated by dialysis with 1.0 M acetic acid to activate all of the LTGF-β present in the sample. The remaining 50% was dialyzed against PBS and used to measure endogenously activated TGF-β. Both the total activated and endogenously activated samples were lyophilized and diluted in PBS and water, respectively. Each sample was diluted from 1:2 to 1:10 to ensure that a dose-response curve could be obtained. Briefly, the PAI/L assay uses mink lung epithelial cells (Mv1Lu) that have been stably transfected with an 800 bp sequence from the human PAI-1 promoter fused to the luciferase reporter gene. Luciferase activity is measured using an enhanced luciferase assay kit (Analytical Luminescence, San Diego, CA) and a luminometer (ML 3000; Dynatech Lab., Chantilly, VA). The values were computed from a standard curve using recombinant TGF-β1 (0–500 pM). The diluted samples were incubated with Mv1Lu cells at 37°C for 72 h at 5% CO_2 and cell growth determined by the MTS assay. To ensure the specificity of the assay, a pan-specific TGF-β neutralizing antibody (Celtrix, Santa Clara, CA) was added to the activated conditioned medium to block promoter reporter activity. Corollary experiments using a modified version of the standard Mv1Lu cell bioassay for TGF-β-mediated growth inhibition to detect TGF-β activity (52) agreed with the results from PAI/L assay. Results from the test samples were compared with control wells receiving medium alone. The amount of active TGF-β in each test sample was extrapolated from the dose-response curve generated with recombinant TGF-β1.

Immunohistochemical Staining for TβRI, TβRII, Activated/Smad2P, Smad2, and Smad7 in Normal Endometrium and ECA

Paraffin-embedded sections (4-μm thick) on Superfrost Plus slides (Fisher Scientific) were immunostained for TβRI and TβRII as described previously (53). The Vectastain Elite kit (Peroxidase) was used for IHC (Vector Labs., Burlingame, CA). Rabbit polyclonal antipeptide antiserum to TβRI (V-22; corresponding to amino acids 158–179 of the internal domain of human TβRI; Santa Cruz Biotechnology, Santa Cruz, CA) and TβRII [C-16; corresponding to amino acids 550–556 of the carboxyl (cytoplasmic) domain of human RII; Santa Cruz Biotechnology] diluted in blocking serum (normal goat serum diluted in Tris-buffered saline containing 0.1% BSA to 1.0 μg/ml) were separately applied to the tissue sections, and the slides were incubated overnight at 4°C followed by biotinylated antirabbit IgG and then by Avidin Biotin Complex. The reaction (brown color) was developed using the substrate DAB (Sigma Chem. Co.) and the slides were counter-stained with Gill's Hematoxylin #2 (Fisher Scientific, Pittsburgh, PA). Normal rabbit serum diluted to the same concentration as the primary antibody was used as a negative control. We have shown previously that TGF-β isoforms are increased in hyperplasia and ECA (54). Because a different cohort of patients was used in this study, all of the tissue samples were likewise immunostained for TGF-β isoforms, as described; the data were confirmatory.

Immunostaining for Smad2, Smad2P, and Smad7. Rabbit antipeptide polyclonal antisera to Smad2P/activated Smad2 was prepared as described (55). Immunostaining required antigen retrieval by microwaving the tissue slides in 10-mM citrate buffer for 5 min (Citra solution; BioGenex, Santa Ramon, CA). The remaining procedures were performed as described for receptor immunostaining (53). The primary antibodies to Smad2/3 (E20, SC#6033; Santa Cruz Biotechnology) and Smad7 (N-19, SC#7004; Santa Cruz Biotechnology) were both used at 10 μg/ml in blocking serum.

Scoring Intensity of Immunostaining. The intensity of immunostaining in the endometrial glands and separately in the stromal cells was assessed by three pathologists and scored 0–3 (immunostaining levels: 0 = none; 1 = weak; 2 = moderate; 3 = intense). Outcome was measured as the product of average staining intensity and the percentage of cells stained at the predominant intensity (usually 100%). As tissue sections often contained areas of normal endometrium, hyperplasia, and carcinoma, these regions were scored separately and also served as internal controls for the immunostaining technique.

ISH for Detection of TβRI and TβII mRNA

Sections of normal and malignant endometrial tissue on Superfrost Plus slides were analyzed for TβRI and TβRII mRNA expression by ISH using DIG-labeled riboprobes prepared from TβRI and TβRII cDNAs (from Murray Korc, University of Southern California, Irvine, CA) produced as described previously (56). The human TβRI (Alk-5) 377-bp fragment and Tβ RII 477-bp

fragment were subcloned into the plasmid vector, pGEM3ZF. Single-stranded antisense and sense riboprobes were labeled with DIG-UTP using the Genius kit, and the riboprobes were detected using the Genius DIG Nucleic Acid Detection kit for immunodetection; both kits were from Boehringer Mannheim, Indianapolis, IN. Both the TβRI and TβRII cDNA-containing plasmids were linearized with BamH1 and HindIII, and transcribed with SP6 polymerase and T7 polymerase for antisense and sense riboprobes, respectively. After overnight incubation at 56°C, the paraffin tissue sections were dewaxed in xylene, rehydrated, and treated with proteinase K [20 μg/ml in 0.1 M Tris-HCl containing 50 mM EDTA (pH 8.0)] for 10 min at 37°C. Subsequently, they were refixed with 4% paraformaldehyde/PBS, acetylated in triethanolamine/acetic anhydride, and then dehydrated through decreasing concentrations of ethanol. The slides were incubated with prehybridization buffer containing 50% formaldehyde (v/v) in 2× SSC for 30 min at 37°C, drained, and incubated with the riboprobes (4 ng/μl) in hybridization buffer containing 50% formaldehyde, 1% SDS, 10% dextran sulfate, and 2× SSC for 16 h at 50°C in a humidified chamber. After hybridization with the probe, the slides were treated with RNase A [20 μg/ml in 10 mM Tris-HCl containing 0.5 M sodium chloride, and 1 mM EDTA (pH 8.0)] for 30 min at 37°C, rinsed twice with 2× SSC, and once with 0.1× SSC at 37°C, each for 30 min. The hybridized probe was detected as described by Boehringer Mannheim. Briefly, the tissue sections were blocked with 0.1 M Tris-HCl containing 0.150 M sodium chloride, 5% BSA, and 0.3% Triton X-100, and then incubated with alkaline phosphotase conjugated anti-DIG IgG (1:500) for 2 h. The color was developed in the dark with the substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in the presence of 2.5 mM Levamisole (Sigma Chemical Co.) for 4 h, and the reaction terminated with 0.02 M Tris-HCl containing 5 mM EDTA (pH 8.0). The slides were counterstained with 1% methyl green in water, dehydrated in Histoclear (National Diagnostics, Mainville, NJ), and mounted with Permount.

Semiquantitative Analysis of TβRI and TβRII mRNA Levels in Primary Cultures of EECs by RT-PCR

After 5 days in culture, total RNA was isolated from EECs on 60-mm plates using RNAzol Tel-Test Inc. (Friendswood, TX). RNA (2.0 μg) was reverse transcribed using SuperScript II Reverse Transcriptase and oligo$(dT)_{12–18}$ primers (Superscript Preamplification System kit; Life Technologies, Inc.). Oligonucleotide primers were synthesized by the Kaplan Comprehensive Cancer Center Core Facility and are as follows: RI: 5′(AATTCCTCGAGATAGGCCGT3′ and 5′ TGCGGTTGTGGCAGATATAG)3′ (244 bp) and RII: (AGGGACCTCAAGAGCTCCAATAT and CACTGCATTACAGCGAGATGACA; 261 bp). As negative controls, SuperScript II RT or the cDNA was omitted from the reaction mixture. After termination of the reaction (50 min), the cDNA was treated with RNase H to enhance polymerase activity. The PCR reactions were optimized using 2–10 ng of corresponding RNA, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM deoxynucleotide triphosphate, 200 nM of each primer, and 0.5 μl Taq DNA polymerase at 28 and 30 cycles for TβRII and TβRI, respectively. The PCR products (10 μl) were analyzed by electrophoresis in 1.0% agarose containing ethidium bromide. Equal loading of RNA was assessed using β-actin mRNA expression with the primers ATCATGTTTGAGACCTTCAA and CATCTCTTGCTCGAAGTCCA (318-bp product).

Mutation Analysis of TβRII by RTPCR

cDNA was prepared exactly as described above. The TβRII cDNA was amplified as two overlapping portions: 5′ half of the coding region of 861 bp (297–1139; sense 5′-CGCTGGGGGCTCGGTCTATG-3′ and antisense 5′-CCACTGTCTCAAACTGCTCT-3′) and 3′ half of 1025 bp (1028–2053; sense 5′-TGCCAACAACATCAACCACA and antisense 5′-CCTCTTTGGACATGCCCAGCC-3′). The PCR products were subcloned into the TA cloning vector (Invitrogen, San Diego, CA) according to the manufacturer's instructions and single isolated clones sequenced. Automated oligonucleotide sequencing of the 5′ and 3′ halves was performed using two antisense primers, of 828–847 bp (5′CTGATGCCTGTCACTTGAAA-3′) and 2009–2028 bp (5′-TGTTTAGGGAGCCGTCTTCA-3′), respectively. The GenBank accession no. M85079 (wild-type) was used as a comparative reference.

Statistical Analysis

The Student *t* test was used to predict statistical significance between the growth inhibitory response to TGF-β obtained from primary cultures of EECs from normal PE and SE compared with primary cultures of ECA cells. The numerical values obtained were used to generate two-tailed *P* values using GraphPad Prism software. The means are considered to be statistically significantly different when $P \leq 0.05$. The intensity of immunostaining for the glandular epithelium and stromal cells was determined for PE, SE, hyperplasia, and ECA. The values for intensity of immunostaining for the respective categories and antibodies used were subjected to the Kruskal-Wallis nonparametric ANOVA test to determine the mean of ranks. The Dunn's Multiple Comparison Test (InStat version 2.01; GraphPad Software) was used to estimate *P* values for statistical significance of mean differences between all of the categories.

RESULTS

Isolation and Characterization of Primary Cell Cultures of ECA and Normal Endometrial Cells. These studies use primary cultures of glandular EECs derived from normal SE and PE tissue and ECAs, as physiological paradigms, to investigate whether loss of TGF-β-mediated growth inhibition is involved in endometrial carcinogenesis. EECs were separated from ESCs derived from the endometria of normal PE and SE, and ECA, as described in "Materials and Methods." Normal tissue and low grade adenocarcinomas (0.5 grams) yielded approximately $20–80 \times 10^6$ ESCs and EECs. As ECA grade increases, glandular architecture is lost, mitotic figures increase, and stromal cell number decreases. Generally, normal endometrium yields a ratio of 1:1 EECs:ESCs. Carcinomas vary with increasing EEC:ESC ratio as a function of histological grade. As much as 60% fewer ESCs are obtained from grade III ECAs. All of the ECAs studied were characterized as (estrogen-induced) endometrioid type I adenocarcinomas. The isolation procedure yields ~90% and 95–99% pure populations of EECs and ESCs, respectively. The purity of EEC and ESC cells is illustrated in Fig. 1 by cytokeratin immunoreactivity of the EECs but not ESCs after 6 days in culture in differential culture medium. Identification of these cells is also aided by their unique morphology. Because the EECs are isolated as microaggregates rather than as single cell suspensions, as shown, they retain a more natural polarized architecture. The normal EECs can be contrasted with identically isolated ECA cells, which are also immunoreactive with the cytokeratin antibody (data not shown). However, the ECA cells displayed a number of typical malignant cell characteristics, most notably, prominent and multiple nucleoli, multinucleated cells (Fig. 1*E*), and increased mitoses and filopodia (not shown). The EECs from ECA tissue required seeding on Primaria tissue culture plates in defined medium. Therefore, both the normal-derived and ECA-derived EECs were grown on this treated plastic. Normal EECs could be subcultured; however, ECA cells usually only survived for up to 10 days and were, therefore, generally used for experiments within the first 7 days. Importantly, EECs derived from PE and SE maintain a phenotype that represents their respective phase of the menstrual cycle (*i.e.,* proliferative *versus* differentiated cell-type behavior). This includes their cell cycle distribution (Table 1), doubling times, and response to TGF-β-mediated growth inhibition *in vitro* (Fig. 2). The *in vivo* differences observed for the levels of TGF-β receptors (Fig. 4 and Fig. 5), subcellular localization of activated Smad2 (Fig. 3), and the cdk inhibitor $p27^{kip1}$ (57) are as one would expect of tissues derived from PE and SE (*i.e.*, less $p27^{kip1}$ in PE and more in SE). Furthermore, tissue sections of normal PE and SE, and lower grades of ECA expressed both ERs and PRs, whereas this varied in higher-grade samples of ECA. Importantly, the presence of these receptors on cells of primary cultures was concordant with the steroid receptor

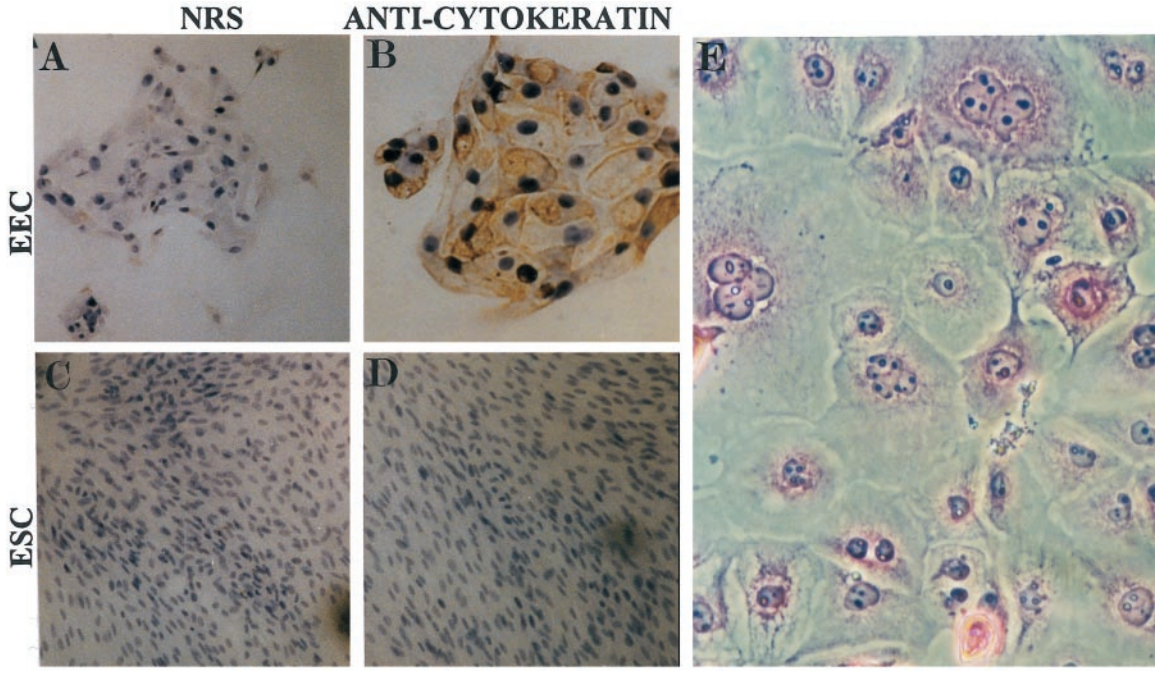

Fig. 1. Characterization of cultures of EEC, ESC, and ECA cells. After 6 days of culture, primary cultures of normal EEC and ESC, plated on removable chamber slides, were immunostained for cytokeratins. *A*, EECs NRS (negative control); *B*, EECs and anticytokeratin; *C*, ESC and NRS; *D*, ESCs and anticytokeratin. The EECs and ESCs are identified by their differential immunostaining and by their unique morphology; only EECs were positive for cytokeratins. The cells were observed by light microscopy. *E*, ECAs (grade III/III) stained with H&E and observed using an inverted microscope with phase contrast. Unlike normal EECs, ECAs are multinucleated and have numerous prominent nucleoli. Original magnification: *B*, ×100; *A*, *C*, and *D*, ×40; *E*, ×200.

Table 1 *Flow cytometric analyses of cell cycle distribution (%) of epithelial cells derived from normal and cancer endometrial primary cultures*[a]

Sample	Time (h)	G_0/G_1	S	G_2/M
PE[b]	0	85.76	1.07	13.17
	48	84.88	11.10	4.02
	144	78.45	10.96	10.59
SE[c]	0	97.72	1.37	0.97
	48	89.59	6.25	4.16
	144	84.93	8.07	6.99
Cancer grade I[c]	0	88.62	2.87	8.50
	48	93.93	2.42	3.65
	144	91.87	7.15	0.98
Cancer grade III[c]	0	77.32	17.33	5.35
	72	74.27	24.01	1.72

[a] Cells (4×10^6) were seeded onto 60-mm Primaria plates under subconfluent conditions. One sample was collected at time 0 and the others were harvested at the times indicated.
[b] Samples for PE are representative of $n = 3$.
[c] One sample tested.

status of the tissues from which they were derived (*e.g.*, PR levels were higher in SE; data not shown).

The cell cycle distribution of EECs from normal PE and SE, grade I/I ECA, and grade III/III ECA was determined before seeding the cells (0 time) and for times thereafter up to 6 days by propidium iodide staining and fluorescence-activated cell sorter analysis. As shown in Table 1, the EECs, in general, displayed a distribution pattern within the cell cycle that one would expect of epithelial cells derived from normal PE and SE, and increasing grades of ECA. Specifically, more cells were in S phase in the higher grade (17%) than the lower grade tumor (2.87%), and the normal cells (1.07% = PE and 1.37% = SE) at seeding time (0 h) and after cell culture (in 10% serum). It is interesting to note that EECs from SE had fewer cells in S phase and more cells in G_0/G_1 than EECs from PE during culture, thereby typifying their differentiated phenotype. We were only able to obtain one sample of grade III ECA with two time points tested for these particular studies. Importantly, the final time point of 72 h is within the time frame that the TGF-β growth inhibition assay was performed. At this time in cell culture, the grade III ECAs displayed 24% of the cells in S phase whereas the grade I ECAs and EECs from PE and SE generally showed >50% less cells in this phase of the cell cycle over time. The low number of EECs in S phase in the grade I ECA may be specific to the particular sample used. As expected, the cell doubling time for normal EECs from PE and SE were different, and from SE was also age-dependent, such that PE showed the fastest doubling time of 38 h, and SE samples ranged from 48 to 72 h ($n = 3$ SE and 1 PE).

Primary Cultures of ECA Cells Lack Response to TGF-β-mediated Growth Inhibition, and EECs from PE and SE Respond Differently. In a previous study, we showed that TGF-β isoforms mRNA and protein were increased in hyperplasia and ECA compared with normal endometrium (54) suggesting that tumor growth *in vivo*

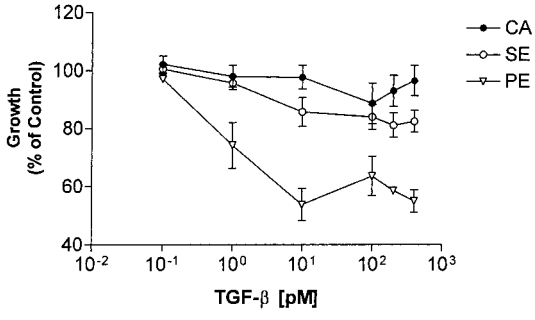

Fig. 2. Assay for TGF-β-mediated growth inhibition in primary cultures of normal EECs and ECA. Primary cultures of EECs (5×10^4cells/well/Primaria 96-well plates) were seeded in medium containing 10% SFBS. After 36 h, serum free-medium containing increasing concentrations of recombinant TGF-β1 (0–400 pM) were added to subconfluent cells and cell proliferation assessed (in triplicate) 4 days later using the MTS assay. EECs from normal PE exhibited a dose-dependent inhibition of growth by TGF-β that reached 55% ± 5.3% (at 10 pM TGF-β; $n = 10$), whereas ECA cells were not growth inhibited by TGF-β at 2.37% ± 4.0%. Values are presented as mean of percentage growth of untreated cells; *bars*, ±SD.

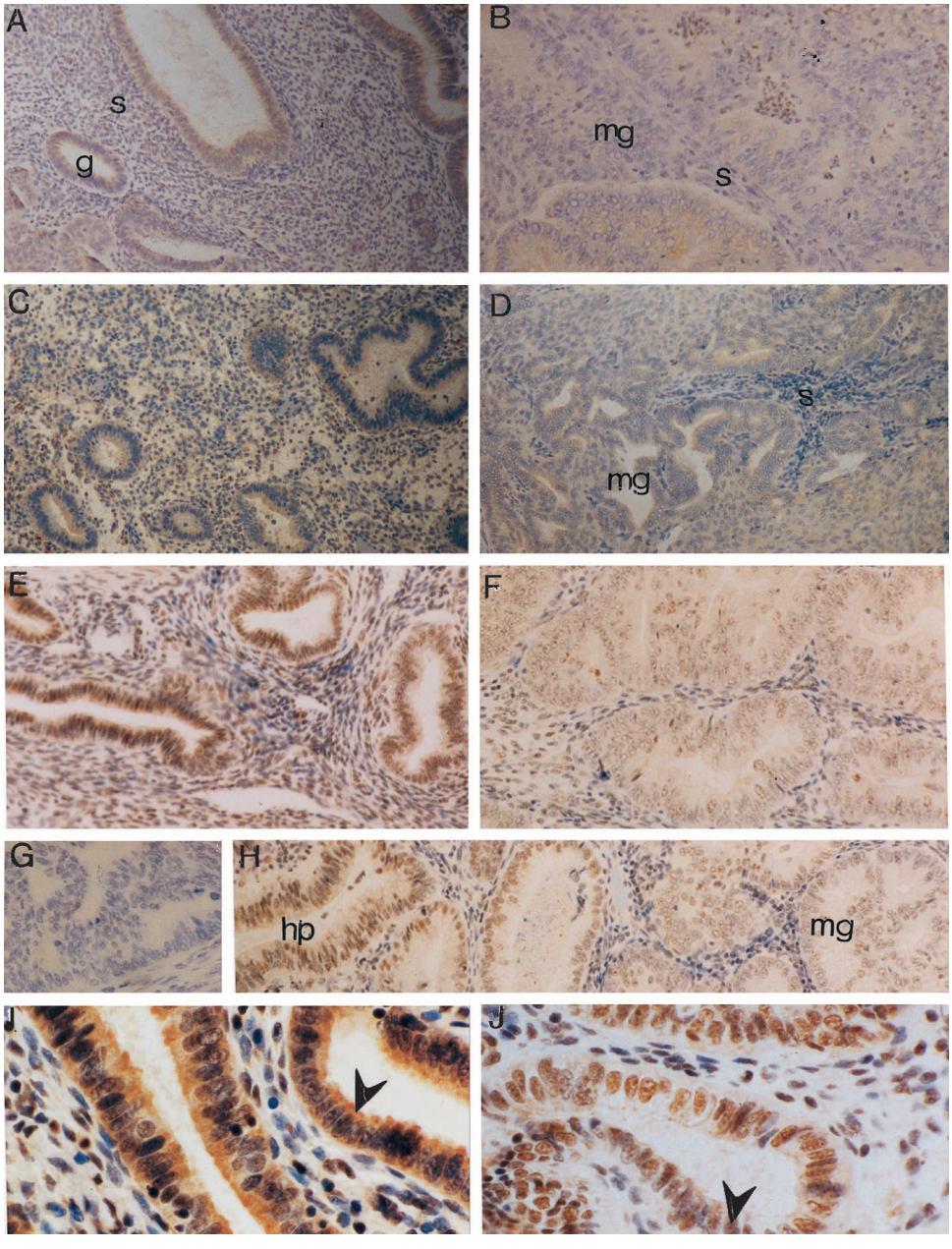

Fig. 3. Immunohistochemical staining for Smads in tissues derived from normal endometrium and ECA. Slides from paraffin-embedded tissue sections were microwaved in citrate buffer for antigen retrieval and incubated with an antipeptide antibodies to Smad2P at 10 μg/ml, Smad2 and Smad7 at 1.0 μg/ml overnight at 4°C in blocking buffer. The slides were immunostained using the Vectastain immunoperoxidase kit and DAB as the substrate (shown as a *brown* positive stain), as described in "Materials and Methods." *A,* Smad2, PE; *B,* Smad2, ECA grade I/I; *C,* Smad7, PE; *D,* Smad7, ECA grade II/I (patient with BATRII mutation); both the normal glands (*g*) and malignant glands (*mg*) show similar weak immunostaining for Smad2 and Smad7. *E,* Smad2P, SE; *F,* Smad2P, ECA grade I/I; whereas normal glands (*g*) are intensely immunoreactive for the phosphorylated/activated form of Smad2P, the malignant glands (*mg*) show negligible to complete absence of immunoreactivity; stromal cells (*s*) show little immunostaining. *G,* NRS; the negative control shows complete absence of immunoreactivity in ECA grade I/I; *H,* Smad2P in a tissue sample of ECA grade I/I (same sample as *F*) showing a spectrum of malignant progression from hyperplasia (*hp*) and malignant glands (*mg*); it is notable that intensity of immunostaining ranges from moderate and mainly nuclear in hyperplasia to absent in malignant glands. *I,* Smad2P, PE showing cytoplasmic and nuclear immunostaining in the glandular epithelial cells (*arrowhead*); *J,* Smad2P, SE showing nuclear immunoreactivity in the glands (*arrowhead*). Many stromal cells are immunoreactive in both PE (*I*) and SE (*J*). Original magnification: *C,* ×25; *A, B, D–H,* ×40; *I* and *J,* ×100. *Box plots* of the immunostaining data are shown in Fig. 5.

is not from lack of expression of this normally potent endogenous growth inhibitor. To determine whether TGF-β could inhibit the growth of primary cultures of normal PE and SE, and of ECA, increasing concentrations of recombinant TGF-β1 (0–400 pM) in serum-free medium were added to the isolated EECs from normal and malignant endometrium 36 h after seeding. Because PE proliferate and SE do not proliferate but perform differentiating functions, we anticipated that their growth response to TGF-β might be different and, thus, analyzed them separately. As shown in Fig. 2, whereas EECs from normal PE ($n = 10$) exhibited a dose-dependent inhibition of growth by TGF-β (55% ± 5.3% of the growth of untreated cells at 10 pM TGF-β), the carcinoma cells derived from ECA patients, representing grades I/I to III/III ($n = 10$), were not growth inhibited by TGF-β (2.3% ± 4.0%; $P \leq 0.0066$). Importantly, no grade-related differences were observed. EECs derived from SE ($n = 5$) were maximally inhibited by 15% ± 4.9% with 10 pM TGF-β. To determine whether any factors present in serum could affect the response to TGF-β, the growth assay was also tested in the presence of 2% and 5% SFCS. No significant differences were observed with either the normal EECs or ECA cells (data not shown). ESCs isolated from the tissue of the normal or malignant endometrium were not growth inhibited by TGF-β, and at low seeding density (500 cells/well compared with 1000 or 1500 cells/well/96-well plates) they were stimulated by TGF-β1 (data not shown).

Decreased Expression of the Smad2P/Activated Smad2 (TGF-β Transcription Factor) in ECA Cells Resistant to TGF-β Growth Inhibition. Because primary cultures of ECA cells were not growth inhibited by TGF-β, we determined whether the TGF-β signaling pathway was disrupted. As an optimal way to determine which cell types are responding to TGF-β *in vivo*, IHC using an antiserum directed against the phosphorylated (activated) form of Smad2 (55), was used. Both antisera to total Smad2 (does not react with the phosphorylated form) and to Smad2P were used on tissue samples of normal PE ($n = 17$) and SE ($n = 8$), glandular hyperplasia (simple, complex, and complex with nuclear atypia were grouped together; $n = 25$), and ECA patients ($n = 22$). Fig. 3 shows that both the glands

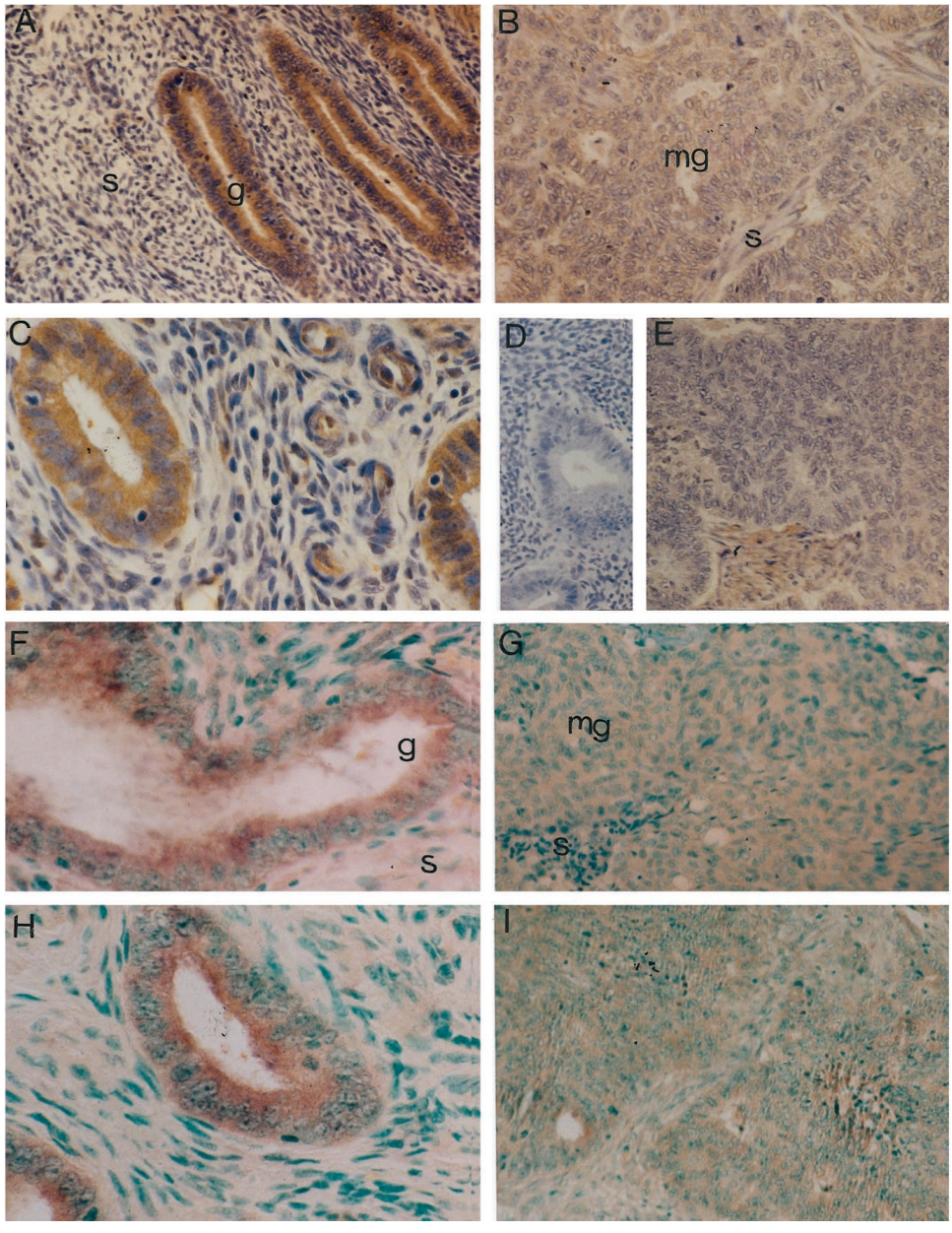

Fig. 4. IHC staining and ISH for TGF-β receptors (TβRI and TβRII) protein and mRNA, respectively, in tissues derived from normal endometrium and ECA. IHC, *A–E*. Slides from paraffin-embedded tissues were immunostained for TβRI and TβRII using polyclonal antisynthetic peptide antibodies with the Vectastain immunoperoxidase kit and DAB as the substrate, as described in "Materials and Methods." *A*, TβRI, PE; *B*, TβRI, ECA grade II/I (patient with BATRII mutation patient and same tissue sample as shown above in Fig. 3*D*); *C*, TβRII, PE (same case as *A*); *D*, NRS control shows negative immunostaining of PE; *E*, TβRII, ECA, grade II/II. In the ECA tissue samples, TβRI and TβRII immunoreactivity was weak to absent (*B* and *E*) in the malignant glands (*mg*). In contrast, tissue from normal endometrium showed strong immunoreactivity in the proliferative glands (*g*; *A* and *C*). Some stromal (*s*) cells (subpopulation) are immunoreactive for TβRI and TβRII in both the normal and ECA tissues. Endothelial cells are immunoreactive. *Box plots* of the immunostaining data are shown in Fig. 5. ISH, slides were hybridized with single-stranded DIG labeled antisense and sense riboprobes prepared from TβRI and TβRII cDNAs. *F*, TβRI mRNA, PE; *G*, TβRI mRNA, ECA, grade II/I (case with BATRII mutation); *H*, TβRII mRNA, PE; *I*, TβRII mRNA, ECA grade II/I (same case as *B* and *G*). Whereas hybridization of the riboprobe was strong in the glands of normal PE for both TβRI and TβRII (*F* and *H*), only a weak signal was observed in the glands of the ECA tissue. The sense probe for both TβRI and TβRII did not yield a signal in any of the samples (data not shown). Original magnification: *A*, *B*, *D*, *E*, *G*, and *I*, ×40; *C*, *F*, and *H*, ×100.

of normal PE (Fig. 3*A*) and ECA tissue (grade I/I; Fig. 3*B*) have similar mild to moderate immunoreactivity for the unphosphorylated inactive form of Smad2. In marked contrast, whereas the normal glands in SE (Fig. 3*E*) showed intense Smad2P immunostaining, the carcinoma cells from a grade I/I ECA (Fig. 3*F*) lacked immunostaining. Notably, all of the grades of ECA demonstrated negligible Smad2P immunostaining (see Fig. 5; $P \leq 0.001$). A few malignant cells contained speckles of apparently cytoplasmic immunostaining; the nature of these structures is not known. It is also important to note that cells derived from the tissue samples of the same patient used for Smad2P immunostaining agreed with the status of their growth response to TGF-β *in vitro* (Fig. 2). That is, Smad2 activity/TGF-β signaling, assessed by positive Smad2P immunostaining was intact in normal PE glands consistent with their ability to be growth inhibited by TGF-β *in vitro* ($n = 6$ PE patients). Similarly, the carcinoma cells of the same ECA patient that did not respond to TGF-β *in vitro* did not have intact TGF-β signaling ($n = 6$ ECA patients). In Fig. 3*H*, the same tumor as shown in Fig. 3*F* (grade I/I ECA) illustrates hyperplasia on the left and malignant tissue on the right. This spectrum of pathology is often present in tissue sections of ECA affording an optimal comparison for immunostaining including an internal control. As shown here, Smad2P immunoreactivity and TGF-β signaling, therefore, is progressively decreased from moderate in hyperplasia to absent in ECA. This is contrasted with the strong immunoreactivity shown in normal SE, Fig. 3, *E* and *J*, and in PE in Fig. 3*I*. Also notable was a statistically significant decrease in Smad2P immunoreactivity in the stromal cells surrounding the carcinomatous glands compared with those surrounding the glands in PE and SE (compare left of Fig. 3, *E* and *F*; $P \leq 0.03$), thus, suggesting possible paracrine effects between the glands and stromal cells. Smad7 inhibits Smad2 signaling, because it competes with Smad2 for binding to TβRI but is not a substrate for its activation. Therefore, we also examined whether loss of TGF-β-mediated growth inhibition might be associated with (and because of) increased levels of Smad7 in ECA glands. We found weak levels of Smad7 immunoreactivity in normal PE, hyperplasia, and ECA, shown in Fig. 3*C*, and ECA, shown in Fig. 3*D* (grade II/I;

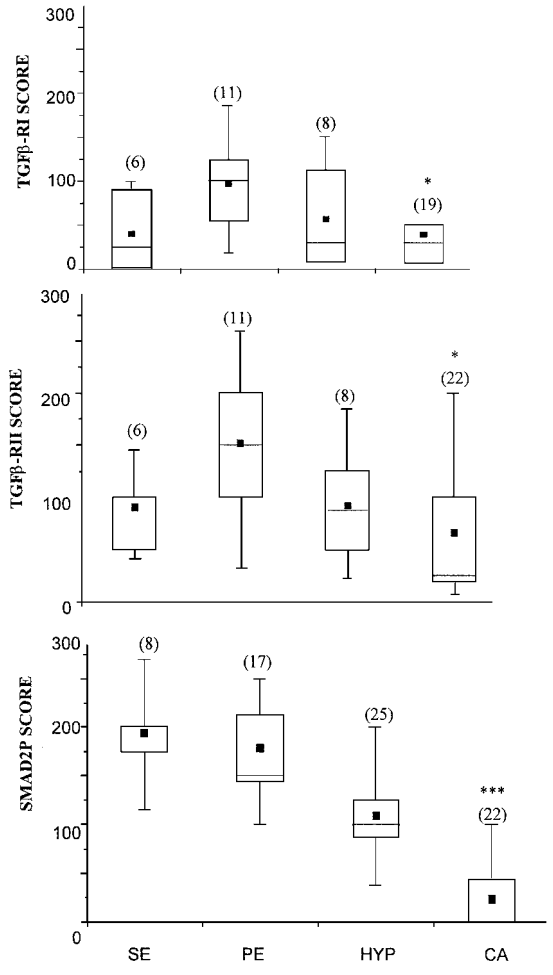

Fig. 5. Box plot analyses comparing intensity of Smad2P RI, RII and immunostaining in tissue samples of normal proliferative (*PE*) and secretory endometrium (SE), endometrial hyperplasia, (*HYP*), and ECA. The intensity of immunoreactivity (*Y* axis; scale 0–300) was assessed for all cases and categories (*X* axis) shown below. The samples were ranked, and the mean of the ranks computed using the Kruskal-Wallis Nonparametric ANOVA Test. Statistical significance among the samples was assessed using the Dunn's Multiple Comparison Test. The mean for each category is depicted by the internal box (■), the median is shown by a *horizontal line*, and the upper and lower range marks the 90th and 10th percentiles; the number of cases in each category is shown in *parentheses* above each *plotted box*. The scores were significantly decreased in ECA glands for RI and RII compared with PE $*(P < 0.05)$ and for activated/phosphorylated Smad2P compared with SE, PE, and HYP $***(P < 0.001)$; *bars*, ±SD.

$n = 12$ PE, $n = 7$ SE, $n = 7$ hyperplasia, and $n = 11$ ECA). Fig. 5 presents box plots of the intensity of immunostaining for Smad2P for all categories of endometrial tissue examined. Interestingly, we found a significant difference in the intracellular localization of immunoreactive Smad2P between normal PE and SE glands. Fig. 3*I* shows that intense Smad2P immunostaining localized predominantly to the cytoplasm and nucleus in normal PE ($n = 17$). However, as shown in the glandular epithelial cells in Fig. 3*J*, Smad2P immunostaining appeared to be only nuclear in SE ($n = 8$); this finding was consistent in all of the cases.

Decreased Expression of TGF-β Receptors, TβRI and TβRII Protein, and mRNA in ECA Compared with Normal Endometrium. To determine whether loss of Smad2 activation was associated with attenuated TGF-β receptor expression in ECA, the same patients tested for the presence of activated Smad2 were analyzed for TβRI and TβRII protein expression by IHC using antipeptide-specific antibodies to TβRI and TβRII. As shown in Fig. 4, a decrease in the expression of both receptors in the glands of ECA tissues compared with normal PE ($P \leq 0.05$ for both receptors) was observed. Fig. 4*A* (TβRI) and Fig. 4*C* (TβRII) of normal PE tissue illustrate intense homogenous cytoplasmic immunoreactivity in the glands and sporadic staining in the surrounding stromal cells. In contrast, as illustrated in Fig. 4*B* (ECA Grade II/I) and Fig. 4*E* (ECA grade II/II), little (TβRI) to no (TβRII) immunostaining was observed in the carcinoma cells. A mutation in TβRII (BATRII) was found in both tissues of the ECA patients depicted here (described below). Although the mutation may be responsible for the lack of TβRII expression in this ECA patient (Fig. 4*E*), the down-regulation of both TGF-β receptors is a significant finding associated with the group of ECA patients examined (Fig. 5). Consistent with the *in vitro* response of EECs derived from PE to TGF-β, the glands show higher receptor levels compared with SE (Fig. 5). All of the ECA samples analyzed for TGF-β receptor expression (10 of 22) were included in the growth inhibition assay above (Fig. 2). Complete loss of TGF-β receptors was generally consistent with the loss of the growth-inhibitory response to TGF-β, except that 2 of 10 showed low to moderate immunoreactivity for TβRI and 1 of 10 for TβRII. Consistent with the status of TGF-β receptors shown by IHC of the tissues, we found that whereas primary cultures of ECA cells did not express TGF-β receptors at 36 h after seeding, these receptors were present on endometrial cells from PE (data not shown). This data are significant in terms of correlating the *in vitro* TGF-β growth inhibitory response with the status of TGF-β receptors *in vivo* and *in vitro*. To gain insight into whether the observed down-regulation of TGF-β receptors was at the transcriptional level, ISH was performed on normal and malignant endometrial tissue. The labeled riboprobes showed that both TβRI (Fig. 4*F*) and TβRII (Fig. 4*H*) mRNA were expressed in the glands of PE and also of SE (not shown). The expression of both receptors by stromal cells was sporadic in both the normal and ECA tissue. Conversely, only a slight hybridization signal for TβRI (Fig. 4*G*) and TβRII (Fig. 4*I*) was evident in the carcinoma cells of ECA tissue (same case, grade II/I). The sense probes did not hybridize in both cases (not shown). The tissue sample of the same patient is depicted in Fig. 4, *B* (protein), *G*, and *I*, and thus, both the message and protein were absent in this patient as well as loss of TGF-β responsiveness. These data suggest that the decrease in TGF-β receptors observed in ECA may be because of decreased gene transcription. Fig. 4, *F–I* exemplifies the results with all of the carcinomas ($n = 7$) and normal tissue ($n = 6$) examined. By semiquantitative RT-PCR, we also showed low levels of expression of both TβRI and TβRII mRNAs derived from primary cultures of ECA cells after 6 days in culture compared with EECs

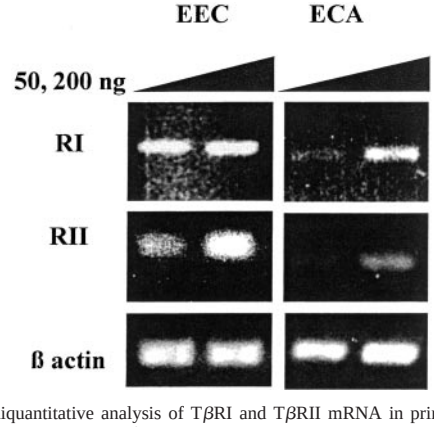

Fig. 6. Semiquantitative analysis of TβRI and TβRII mRNA in primary cultures of normal EECs and ECA cells. After 5 days in culture, RNA was isolated from the cells using RNAzol and subjected to RT-PCR as described in "Materials and Methods." The PCR products (50 and 200 ng) were analyzed by electrophoresis in 1% agarose containing ethidium bromide. The PCR product of actin mRNA was used to ensure equal loading. The gel shows that TβRI mRNA is decreased and TβRII nearly absent in ECA compared with normal PE. The results represent $n = 3$.

from normal PE (Fig. 6). These data corroborate the results obtained by ISH and show that TβRII mRNA receptor level is nearly absent, and TβRI markedly decreased compared with normal PE. Thus, TβRI and TβRII mRNA and protein are down-regulated in ECA both *in vivo* and *in vitro* ($n = 3$), the latter probably accounting for the loss of response of ECA cells to TGF-β *in vitro*.

A Somatic Frameshift Mutation Was Found in TβRII in ECA. Inactivation of TβRII because of a specific mutation in a 5′ poly(A) regions of the mRNA (extracellular domain), termed the BATRII mutation, was shown to be present in HNPCC and a few ECAs (58, 59). This mutation was reported to cause a frameshift and truncated message (reviewed in Ref. 45). To determine whether this mutation in TβRII might be related to inactivation and decrease in TGF-β receptor expression, RNA derived from six primary cultures of ECAs that did not respond to TGF-β *in vitro* and two cultures of normal EECs that were shown to be responsive was amplified by RT-PCR using TβRII-specific primers that span the 5′ half of TβRII mRNA (extracellular domain). Comparing to the wild-type TβRII amplified and cloned from normal EECs, Fig. 7 shows that an adenine peak was absent (Fig. 7, *B* and *C* of ECA compared with normal endometrium shown in Fig. 7*A*) within the poly adenine sequence (between 709–718) of the mRNA, indicating that a point mutation occurred 129-bp upstream from the 5′ sequencing primer (828–847 bp). This inactivating mutation was somatic because it was found in 50% of the clones analyzed (three of six) in two of the patients examined. Consistent with the loss of TβRII (by mutation), these same patients showed loss of TβRII protein and mRNA expression, loss of TGF-β function/Smad2 signaling *in vivo*, and loss of TGF-β-mediated growth inhibition *in vitro*. This same mutation was found in the PCR product of another patient, but it was not confirmed by cloning.

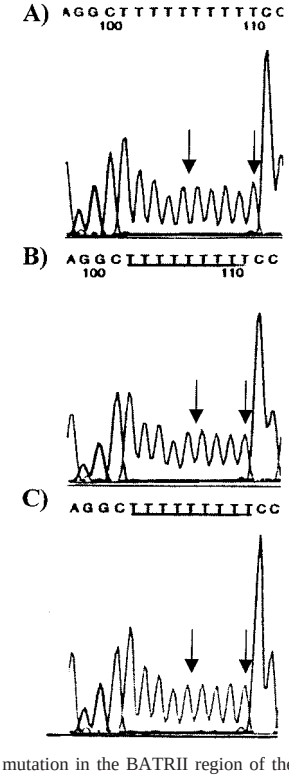

Fig. 7. Analysis for mutation in the BATRII region of the TβRII gene by RT-PCR. Total RNA (2.0 μg), isolated from primary cultures, using RNAzol, was reverse transcribed using Superscript RT and oligo$(dT)_{12-18}$ primers, and the PCR products subcloned into the TA cloning vector and single isolated clones were used for sequencing, as described in "Materials and Methods." Automated sequencing of the 5′ and 3′ halves was performed using two antisense primers, of 828–847 bp and 2009–2028 bp, respectively. The graph shows one adenine deletion in the poly adenine sequence of TβRII (BATRII) located at the 5′ half (between 709–718) of the mRNA in two (*B* and *C; n* = 6 examined) cases of ECA compared with one normal patient (*A*), showing all 10 adenine peaks, expected for the wild-type (GenBank accession no. M85079). This was a somatic mutation found in 50% of the clones (three of six) examined.

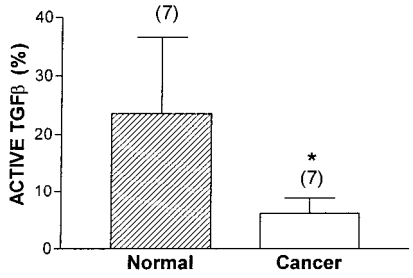

Fig. 8. Quantitation of activated LTGF-β by explants of normal and ECA tissue using the PAI/L assay. The percentage of activated LTGF-β was determined in the supernatants of explants tissue grown for 48 h, by the PAI/L assay (52), as described in "Materials and Methods." The samples were divided into those that were dialyzed against 1.0 M acetic acid, to obtain the total amount of activatable LTGF-β and those that were dialyzed against PBS, to obtain the portion of activated LTGF-β in the cultures. Both total and endogenously activated samples were lyophilized, diluted, and the percentage activated TGF-β in each sample was determined using the PAI/L assay, which uses MvLu1 transfected with the PAI-1 promoter fused to a luciferase reporter gene to calculate TGF-β activity. The values were computed using a standard curve of recombinant TGF-β (0–500 pM) and TGF-β specificity confirmed using a TGF-β neutralizing antisera. ECA explants (n = 7) showed a statistically significant decreased capacity to activate LTGF-β compared with normal endometrial explants ($n = 7$) at 7.4% compared with 23.5%, respectively *($P \leq 0.05$). Values represent the means; *bars*, ±SD.

Loss of the Ability to Activate LTGF-β in ECA. The activation of LTGF-β is a critical step in the physiological regulation of TGF-β growth inhibitory activity and, therefore, it is important that tumor cells have the ability to activate LTGF-β or have access to the activated form of TGF-β for receptor-bound induction of signal transduction. Whereas the results shown above indicate that TGF-β unresponsiveness in ECA is most likely because of a disrupted signaling pathway, lack of the active form of TGF-β would also obviate TGF-β signaling (receptor-binding). Therefore, we tested the capacity of normal and malignant endometrium to activate LTGF-β by using supernatants from cultured endometrial explant tissues. The ratio of activated mature TGF-β to secreted LTGF-β from the cultured explants was quantitated using an assay that uses the highly TGF-β-sensitive PAI-1 promoter conjugated to a luciferase reporter in the mink lung cell line, Mv1Lu (51). Fig. 8 shows that explants from ECA tissue ($n = 7$) did not produce activated LTGF-β to the same extent as normal tissue explants ($n = 7$). The fraction (%) of total LTGF-β activated by the normal explants was 23.5%, whereas ECA tissue explants activated only 7.4% ($P \leq 0.011$). An average of 484 pg/ml compared with 171.3 pg/ml of active TGF-β was produced by the normal compared with ECA tissue explants, respectively ($P \leq 0.05$). Although the ECA tissue explants showed little activation of LTGF-β in the samples, they secreted an average of 2572 pg/ml compared with 2383 pg/ml from normal explants of similar size. There was no difference between the quantity of activated LTGF-β produced by normal PE ($n = 3$) and SE ($n = 4$). This finding is inconsistent with previous results using IHC, which showed that ECA tissue displayed higher levels of TGF-β isoforms than normal endometrium (54). As we have shown here that TGF-β receptors are down-regulated in ECA, the nature of the receptor that binds TGF-β isoforms *in vivo* is unclear.

DISCUSSION

Clues that TGF-β may not be functioning as a growth inhibitor in human cancers *in vivo* were derived from observations of high levels of expression of TGF-β isoforms in many human malignancies, including ECA (reviewed in Refs. 5, 11). Therefore, our main objec-

tive was to gain insight into mechanisms of loss of TGF-β growth inhibition in ECA *in vivo*. Although immortalized cell lines derived from various malignancies including ECAs have provided important mechanistic information concerning oncogenesis, results are often cell line-dependent and vary within a certain cancer type. The fact that all of the primary carcinoma cells derived directly from ECA tissue were not growth inhibited by TGF-β in our studies suggests that certain established ECA cell lines, such as HEC-1A, that are inhibited by TGF-β (7), may not be representative of their *in vivo* response to TGFβ. That these primary cultures of normal EECs and malignant ECAs are close corollaries of physiological function and, therefore, excellent paradigms for study is supported by the fact that the cells retain many of their respective phenotypes after adaptation to cell culture. For example, we were able to differentiate between EECs that had been derived from PE and SE endometrium. This is well exemplified by their different sensitivity to TGF-β-mediated growth inhibition *in vitro*. Whereas EECs from PE were growth inhibited by 55% at 10 pM TGF-β, EECs from SE were inhibited by only 15%. We reason that EECs from SE display less sensitivity to TGF-β because they are nonproliferating, differentiated cells (probably in G_0 of the cell cycle *in vivo* as demonstrated in Table 1 at zero time). Thus, the differential response of these normal cells is related to their respective growth states, which continue to be displayed in culture (Table 1). Consistent with this idea and the results of cell cycle distribution shown in Table 1, we have found that 48 h after seeding EECs derived from tissues, cultures of SE have 40–50% fewer cells than those from PE and, furthermore, that the doubling times and cell cycle distribution for both of these normal cells reflect their specific growth-related phenotypes. Therefore, we reason that on seeding, proliferative phase EECs are already in the cell cycle and are, thus, responsive to TGF-β. We also noted that the levels of TβRI and TβRII were lower in SE (Fig. 5), which may contribute to their decreased responsiveness to TGF-β. In addition, the presence of Smad2P in the nucleus in SE (Fig. 3*J*) suggests that TGF-β may already be functioning in these cells causing them to be refractory to additional inhibition by exogenous TGF-β. In this regard, TGF-β may act to maintain the differentiated state of SE by continued activation of genes (through activated Smad2) that block cell proliferation. In the case of PE, the fact that Smad2P is localized to the cytoplasm is consistent with its position to be phosphorylated/activated by the TβRI kinase for subsequent translocation to the nucleus (as shown by the nuclear staining) for gene activation. Therefore, it does not appear that Smad2P is activated by the TβRI kinase in SE, because it is not present in the cytoplasm. In a separate study, we have shown that PE glands express lower levels of the cdk inhibitor, $p27^{kip1}$, than secretory glands and that, *in vitro*, EECs from PE but not from SE can degrade exogenous recombinant $p27^{kip1}$ through the ubiquitin-proteasome pathway in culture (57). This is a result one would expect of cycling cells, which should have lower levels of $p27^{kip1}$ to enable proliferation. In addition, similar to their respective tissue samples, we have found that both normal and carcinoma cells in culture continue to express both ERs and PRs (data not shown).

Because we showed that the primary cultures of ECA cells had lost their ability to be growth inhibited by TGF-β *in vitro*, using an antibody to Smad2P, ostensibly as an indicator of TGF-β signaling and function *in vivo*, we demonstrated a statistically significant marked decrease or negligible amount of Smad2P immunostaining in both the glands and stromal cells in all grades of ECA (Figs. 3 and 5) compared with PE. These results suggest that defective TGF-β signaling may be responsible for the escape of ECA cells from negative growth regulation by TGF-β *in vivo*. In studies using various cancer cell lines, intact receptors were shown to be required for Smad2 activation/phosphorylation in response to TGF-β (55, 60). Although elevation of (inhibitory) Smad7 levels is a possible mechanism for blocking TGF-β-mediated growth inhibition, we detected equally low levels of Smad7 in both normal and malignant endometrium. Conversely, it was shown recently that Smad7 mRNA and protein expression was increased in pancreatic cancer and furthermore, that transfection of Smad7 into a pancreatic cancer cell line obviated the TGF-β-mediated growth inhibitory response (reviewed in Refs. 61, 62). Interestingly, it has been shown that TGF-β induces Smad7, both to elicit certain functions (63) and as a negative feedback control (reviewed in Refs. 31, 64, 65). Loss of TGF-β-mediated growth arrest as well as loss of tumor suppressor activity (increased tumorigenesis) in a variety of human cancers have been associated with mutations in Smad2 and Smad4; both are located on chromosome 18q21, and mutations in both their MH1 and MH2 functional regions disrupt their interaction with DNA and TβRI, respectively (reviewed in Ref. 6). Specifically, biallelic loss of Smad4 is observed in 50% of pancreatic cancers (66) and 30% of metastatic colon cancers (67). Homozygous deletion of both Smad2 and Smad4 in mice are embryonically lethal. However, Smad3-deficient mice develop aggressive metastatic colorectal cancer (68), and Smad4-deficient heterozygotes have gastric polyps that develop into adenocarcinomas (69). TGF-β-induced growth inhibition was shown recently to be mediated by Smad2 or Smad3/Smad4 bound to the Sp1 transcription factor, which rapidly induced transcription of the $p15^{ink4B}$ promoter (70). These data support our observations that loss of Smad2P signaling in ECA is associated with the inability of cells derived from these same tumors to be sensitive to TGF-β-mediated growth inhibition. Moreover, we also detected a marked decrease in $p15^{ink4B}$ immunoreactivity in tissue samples from ECA patients ($n = 11$) compared with normal SE ($n = 7$; data not shown). Finally, it appears loss of Smad2 activation may be specific to endometrial carcinogenesis, because we show that 100% and 87% of ECA patients had decreased or negligible levels of Smad2P immunoreactivity, respectively, whereas in similar studies of cohorts of both breast and colon cancer, loss of Smad2P immunostaining *in vivo* as a marker for TGF-β signaling was not observed (only 6.6% to 6.1% had decreased Smad2P; Refs. 60, 71).

As a possible explanation for lack of Smad2P activation/signaling in ECA, we examined TGF-β receptor expression in ECA compared with normal endometrium and found a statistically significant nongrade-dependent decrease in both TβRI and TβRII proteins in ECA compared with normal PE (Figs. 4 and 5). Furthermore, our studies suggest that down-regulation of both TβRI and TβRII may be at the level of mRNA transcription, because there was a clear loss of receptor expression by ISH (Fig. 4) in the glands of ECA tissue compared with normal PE. Transcriptional repression has been designated as a principal mechanism of down-regulation of TβRII in several human cancers (13, 43, 45, 72). Consistent with the *in vivo* status of TGF-β receptors and lack of TGF-β responsiveness *in vitro*, cultured ECA cells had significantly decreased TβRI and TβRII mRNA (Fig. 7), and protein expression compared with normal EECs from PE. Reconstitution of normal TGF-β receptor levels is an important cancer therapeutic target and, therefore, understanding mechanisms for down-regulation of TGF-β receptors is critical to this goal. Methylation of 5′CpG islands in the promoter of the *TβRI* gene, particularly at the transcriptional start site, has been suggested as a mechanism for down-regulation of TβRI (45), and high-density methylation of *TβRII* and *TβRI* promoter sequences with loss of gene expression was found in esophageal carcinoma (73) and gastric cancer (72), respectively. Both TβRII transcription and TGF-β sensitivity was attained by treatment of breast and colon carcinoma cell lines, shown to have decreased activity of the Sp1 transcription factor on the *TβRII* promoter, with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (13, 74). In addition, vitamin D3 and analogues in-

creased expression of TβRII in MCF-7 breast cancer cells (75), and in this same cell line, transcriptional repression by the Sp3 transcription factor reduced expression of both *TβRI* and *TβRII* (43). It is also possible that TGF-β receptors may undergo intracellular protein degradation in cancer, particularly via the ubiquitin-proteasome pathway, as has been shown for Smad2 and Smad3 (76–78).

The BAT-RII mutation (10-bp polyadenine repeat in the 5′ end of the TβRII gene) was discovered in HNPCC concomitant with loss of TGF-β-mediated growth inhibition (reviewed in Refs. 45, 58). Similarly, we found this identical deletion mutation in two of six ECA cases examined; cells from these same ECA tissue specimens were also not inhibited by exogenous TGF-β *in vitro*. As reported for HNPCC, the BAT-RII mutation was only present in 50% of the cloned RNA indicating that a somatic frameshift mutation had occurred. Although it is not known why mutations in TβRII are associated with mRNA instability, this mutation has been shown to cause a truncated TβRII. The lack of both TβRII mRNA and protein expression, and complete absence of Smad2 activation in the two ECAs with the BATRII mutation is ostensibly related to this mutation. The BATRII mutation has been shown to be associated with MSI in HNPCC, other colorectal carcinomas, gastric carcinomas, and gliomas (59, 79, 80). It has been suggested that the specific genetic defects in DNA mismatch repair proteins may be responsible for the lack of repair of the TβRII gene (45). It is interesting to note that an inherited syndrome of Type III ECA occurs in 20–40% of females in HNPCC affected families (2). However, in our cohort of patients, the BATRII mutation was found to be at a high rate in the estrogen-induced differentiated type I endometrioid ECAs, and although MSI is notably common in this ECA tumor type, it has not been correlated with mutations in mismatch repair genes (81) or in TβRII in other studies (59, 82). In a study that examined the entire coding region of TβRI and TβRII in sporadic ECAs, several mutations in the kinase domain of TβRI (1 of 39 cases) and TβRII (7 of 42 cases) were found (83). Taken together, it appears that the coding sequence of TβRII is uniquely vulnerable to replication errors (MSI) during normal mitosis, thus providing a selective advantage for cancer cells. In regard to TβRI, reports show an inactivating mutation in this receptor in 30% of ovarian cancers with no concomitant mutations in TβRII (84), in metastatic breast cancers (85), and at a low rate in other cancers (5, 6).

In endometrial hyperplasia and carcinoma, we show a disrupted TGF-β signaling pathway related to loss of expression of TGF-β cell surface receptors and lack of activation of the downstream signaling transcription factor Smad2 most likely explaining the loss of TGF-β-mediated growth inhibition that we observed *in vitro*. Furthermore, ECA tumors have a reduced capacity to activate LTGF-β, and lack of available activated TGF-β may lead to decreased signaling and/or even down-regulation of TGF-β receptors. Whether lack of TGF-β activation and/or down-regulation of TGF-β receptors (particularly TβRI) is the initial event obviating Smad2 phosphorylation/activation or, inactivation of Smad2 is an independent event is a significant problem to solve for a better understanding of the abrogation of TGF-β signaling in the pathogenesis of ECA. Because mutations in Smad2P have not been analyzed in ECA, it is possible that a disruptive mutation in the Smad2 gene may cause receptor down-regulation at the mRNA level through a negative feedback mechanism. In future studies, if rescue of Smad2 signaling and, thus, growth inhibition after up-regulation of TGF-β receptors can be shown (*e.g.*, by transfection of receptor cDNAs or promoter demethylating agents), we should be able to determine whether inactivation of the components of the TGF-β signaling cascade are independent or interrelated events. Concomitant loss of both TGF-β-signaling receptors and Smads has been observed in other human cancers (reviewed in Refs. 5, 6, 11, 45, 60). Because obviating only one component of this signaling pathway may be sufficient to render cancer cells unresponsive to TGF-β, it is likely that there is multiplicity and overlap of TGF-β signaling components with other pathways related to tumor suppressive activity or cell growth. Although the TGF-β signaling pathway is disrupted in many human cancers, the components of the pathway that are affected are cancer-type specific. Examples are the specific inactivation of Smad4 in pancreatic cancer (DPC4-deleted in pancreatic cancer; Ref. 66), loss of TβRI in prostate cancer (86), an increase in TGF-β receptor levels but decrease in Smads mRNAs in glioblastoma multiforme (87), and loss of downstream signaling of TβRI in ovarian cancer (88). In addition, whereas loss of TGF-β receptors and Smad2 inactivation does not occur at a high rate in human breast cancer (60) and breast cancer cell lines (89, 90), recently, loss of TGF-β responsiveness in breast cancer cell lines was attributed to loss of *c-myc* down-regulation through failure of Smad complex formation (91). Thus, as the lesions resulting in escape from (TGF-β-mediated) growth control are unique for different cancers, our studies provide support for the notion that primary cultures of different carcinomas may provide optimal paradigms to define specific molecular targets for possible therapeutic protocols tailored for different human cancers. Finally, because we did not show a grade-related correlation with the magnitude of loss expression of TGF-β signaling components and also, decreases were noted in endometrial hyperplasia, our studies suggest that inactivation of TGF-β signaling is an early event in endometrial carcinogenesis. Therefore, the study of endometrial hyperplasia and ECA should provide a unique opportunity to define molecular targets for intervention of the escape of cancer cells from growth regulation by TGF-β. We herein define an overwhelming need to address how TGF-β receptor levels can be up-regulated, and signaling restored in endometrial hyperplasia and carcinoma, because this would have excellent translational value for the development of both cancer-preventative and therapeutic modalities.

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