Transforming Growth Factor-β3 Increases the Invasiveness of Endometrial Carcinoma Cells through Phosphatidylinositol 3-Kinase-dependent Up-regulation of X-linked Inhibitor of Apoptosis and Protein Kinase C-dependent Induction of Matrix Metalloproteinase-9*\(^\text{S}\)

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Tumor cells often acquire intrinsic resistance to the growth inhibitory and pro-apoptotic effects of transforming growth factor-β (TGF-β); moreover, TGF-β can confer invasive properties to established tumor cells. In the present study, we show that TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) trigger proper Smad signaling in human endometrial carcinoma cell lines and efficiently inhibit cellular proliferation. These cells, however, exhibit a high degree of resistance to TGF-β pro-apoptotic effects; we found that this resistant phenotype would be acquired through up-regulation of X-linked inhibitor of apoptosis protein (XIAP) levels. In addition, using RNA interference and pharmacological inhibitors, we show that TGF-β increases cellular invasiveness via two distinct signaling pathways in endometrial carcinoma cells: phosphatidylinositol 3-kinase/AKT-dependent up-regulation of XIAP and protein kinase C-dependent induction of matrix-metalloproteinase-9 (MMP-9) expression. Additionally, these findings were correlated with clinical observations showing abundant TGF-β immunoreactivity in human endometrial carcinoma tumors in vivo, extending from the epithelial compartment to the stroma upon acquisition of an invasive phenotype (gradually from grades I to III). Collectively, our results describe for the first time a role for TGF-β3 in tumor invasiveness.

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Endometrial carcinoma is the leading type of gynecological cancer in the Western world. Dysregulation of apoptosis is an early event in the pathogenesis of endometrial carcinomas \(^1\); this inherent resistance to various pro-apoptotic signals may contribute to endometrial tumor resistance to current chemotherapeutic agents.

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TGF-β3 are pleiotropic cytokines that can exert tumor suppressing effects, such as growth inhibition and induction of apoptosis, as well as tumor promoting effects, such as stimulation of invasiveness, depending on cell type and cancer stage. The three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) can activate TGF-β receptors (TBR1, TBR2, and TBR3) with unequal affinity \(^2\). Activated TBR2 serine/threonine kinase receptor recruits, phosphorylates, and activates TBR1; Smad2 and Smad3 directly interact with and become phosphorylated by activated TBR1 and subsequently associate with Smad4 to form heterodimeric complexes that translocate to the nucleus, where they can trigger downstream transcriptional responses \(^4\). TGF-β can also trigger ERK activation \(^5\), again leading to modulation of cellular transcriptional activity.

In numerous cell types, TGF-β receptor engagement can also trigger apoptosis; notably, TGF-β signaling leads to the activation of caspases \(^6\), a family of cysteine proteases that are considered as the main effectors of apoptosis. TGF-β can also promote cell death by down-regulating X-linked inhibitor of apoptosis protein (XIAP) \(^7\), an endogenous inhibitor of effector caspases, and AKT \(^8\), a central element in the PI3-K/AKT survival pathway.

Although the three TGF-β isoforms are detectable at high levels in endometrial tumors \(^9\), intracellular signaling cascades triggered by individual isoforms in these cells remain largely unknown. Moreover, although TGF-β can modulate the invasive properties of established tumor cells \(^10\), little information regarding the effect of specific TGF-β isoforms on the invasiveness of resistant endometrial carcinoma cells is available. We have thus assessed the ability of each TGF-β isoform to activate signaling pathways in human endometrial carcinoma cells and compared their effect on cellular proliferation, apoptosis, and invasiveness. We have also correlated in vitro results with clinical data from human endometrial carcinoma tissue samples.

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2 The abbreviations used are: TGF, transforming growth factor; HEC, human endometrial carcinoma; XIAP, X-linked inhibitor of apoptosis protein; MMP, matrix metalloproteinase; PI3-K, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKC, protein kinase C; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase.
**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—Human endometrial carcinoma KLE and Hec-1A cell lines were purchased from ATCC. KLE cells were maintained in Dulbecco’s modified Eagle’s medium-Ham’s F-12 medium without HEPES supplemented with 10% fetal bovine serum and 50 mg/ml gentamycin; Hec-1A cells were maintained in McCoy’s medium supplemented with 5% BGS (bovine growth serum) and 50 mg/ml gentamycin. All of the antibodies were from Cell Signaling Technology (Beverly, MA) except for anti-rabbit secondary antibody (Bio-Rad). Recombinant TGF-β3 were purchased from Calbiochem (San Diego, CA). LY294002 was purchased from Cell Signaling Technology.

**RNA Isolation and Analysis**—Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was synthesized from 0.4 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Primers for PCR amplification are listed in Table 1. PCRs were conducted in a MJ Research Thermal cycler (model PTC-100), using the following parameters: 30 s at 94 °C, 30 s at Tm (Table 1), and 1 min at 72 °C for 36 cycles, except for GAPDH (25 cycles). The reaction mixture was size-separated on an agarose gel and visualized using SYBR-Safe™ (Invitrogen) staining upon ultraviolet transillumination.

**Western Blot Analysis**—The cells were washed and submitted to lysis in cold radioimmune precipitation assay buffer containing protease inhibitors (Complete™ from Roche Applied Science) followed by three freeze-thaw cycles. Equal amounts of cell lysates (as determined using Bio-Rad DC protein assay) were separated onto 15% polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% milk in PBS and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Detection was performed using SuperSignal West Femto™ substrate (Pierce), as described by the manufacturer.

**MTT Proliferation Assay**—The cells were plated in 96-well plates at a density of 9 × 10^3 cells/well in 200 μl of culture medium and incubated overnight at 37 °C in a 5% CO2 atmosphere, after which they reached 80% confluence. Recombinant TGF-β3 (Cell Signaling Technology) were added to selected wells at the indicated concentrations in 100-μl culture medium, and plates were incubated for the indicated times at 37 °C. MTT reagent (tetrazolium salt; Sigma) was added to the wells (10 μl of a 0.5% solution in PBS) 3.5 h before the end of incubation period, after which conversion of yellow tetrazolium salt to blue thiazol crystals by metabolically active cells was stopped by adding 100 μl of a 10% SDS, 0.1% HCl in PBS solution to each well. The plates were incubated overnight at 37 °C to allow complete solubilization of thiazol crystals, and the intensity of blue emission in each well was measured using a Fluostar multiwell plate reader (BMG Laboratories, Durham, NC). The percentage of proliferating cells was calculated as the ratio of optical densities of treated to nontreated (control) cells.

**Hoechst Nuclear Staining**—The treated cells were collected, washed twice in PBS, resuspended at an approximate density of 2 × 10^5 cells/ml in PBS containing Hoechst 33258 (Sigma), and incubated for 24 h at 4 °C before fluorescence microscopy analysis of apoptotic cells. At least 200 cells were counted for each sample, and a percentage of apoptotic cells was calculated as the ratio of apoptotic cells (with characteristic apoptotic morphology such as nuclear shrinkage and condensation) to total cell count.

**Invasion Assay**—The invasive properties of treated KLE and Hec-1A cells were measured using Matrigel-coated Transwell inserts (Costar, Corning, NY). Briefly, the cells were treated for a 24-h period with TGF-β3, following a 1-h pretreatment in the presence or absence of 2 μM P13-K inhibitor LY294002 (Cell Signaling Technology), 50 nM MPP-9 Inhibitor I (VWR, Montreal, Canada) or 1 μM PKC inhibitor chelerythrine chloride (Sigma), or after down-regulating intracellular XIAP levels using siRNA technology. Transwell inserts with a 8-μm pore size were coated with 2 mg/ml Matrigel, and the cells were collected, washed, and resuspended in respective basal medium without serum. The lower chambers were filled with 600 μl of respective culture medium, and 2 × 10^5 cells were added to the upper chamber inserts. The plates were incubated for 72 h at 37 °C. After that incubation period, invasive cells had reached the porous insert, where they adhered, but a proportion of these invasive cells had crossed the porous barrier and reached the lower compartment. Medium filling the lower chamber was collected, and floating invasive cells were recovered by centrifugation (1 min at 6,000 × g) before they were resuspended in 30 μl of PBS and counted using an hemocytometer. A percentage of cell invasion was calculated from the ratio of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment.

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**Table 1**

| Gene   | Forward primer 1 | Reverse primer 1 | Tm (°C) | Product size (bp) |
|--------|------------------|------------------|---------|------------------|
| TGF-β3 | gattaccatcctccacccga | ggtgctctcattgtgagt | 66      | 756              |
| TGF-β3 | gcttctccctccacccga | ctcattctcattgtgagt | 58      | 218              |
| TGF-β3 | gactctcctccacccga | ctcattctcattgtgagt | 58      | 556              |
| TGF-β3 | gactctcctccacccga | ctcattctcattgtgagt | 60      | 180              |
| TGF-β3 | gactctcctccacccga | ctcattctcattgtgagt | 58      | 121              |
| TGF-β3 | gactctcctccacccga | ctcattctcattgtgagt | 60      | 151              |
| XIAP   | gaaaccacacacctttacaccagc | ggaaccacacacctttacaccagc | 60      | 395              |
| GAPDH  | gcctgctctcattgtgagt | gcctgctctcattgtgagt | 58      | 1485             |
| GAPDH  | gcctgctctcattgtgagt | gcctgctctcattgtgagt | 58      | 212              |
Gelatin Zymography—Serum-free conditioned medium from KLE cells treated with recombinant TGF-β3 for the indicated times was assessed by zymography for the presence of gelatinolytic enzymes. The samples were applied without heating or reduction to a 7.5% polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After size separation upon electrophoresis, the gels were washed twice for 60 min with 2.5% Triton X-100 to remove SDS and incubated for 24 h in 50 mM Tris, 5 mM CaCl₂, 1% Triton X-100 at 37 °C. The gels were stained with Coomassie Brilliant Blue and destained in 30% (v/v) methanol, 10% acetic acid. The proteolytic activity was identified as a clear band on a blue background.

Targeting of XIAP or Smad4 by siRNA—The cells were seeded in 6-well plates at a density of 2.5 × 10⁵ cells/well (required to reach ~75% confluency in 24 h) and allowed to adhere overnight. On the day of the experiment, XIAP (5’-cacuguaagcucuugga-3’ and 5’-augcuaaacccaacaccu-3’), control (5’-acucuaacucgcgcgcua-3’ and 5’-aucgcgcugcagauaggu-3’), or Smad4 (5’-ggucuuugauuugcguca-3’ and 5’-cugaccaaccaacgcctttc-3’) siRNAs were mixed with Superfect™ transfection reagent following the supplier’s instructions (Qiagen) and added to the cells (working concentration, 100 nM). The plates were incubated for 24 h at 37 °C, and medium was replaced with fresh medium containing TGF-β or other agents as indicated in the figure legends. The plates were incubated for 24 h at 37 °C before the cells were collected.

Immunofluorescence-based Detection of TGF-β3 in Clinical Samples—Human endometrial carcinoma tissue section slides (Cybrdi, Frederick, MD), containing 17 grade I tumor specimens, 33 grade II tumor specimens, 5 grade III tumor specimens, and 3 normal endometrial specimens, were used. The tissues, obtained from biopsies, were already formalin-fixed, paraffin-embedded. The slides were deparaffinized by heating at 60 °C for 30 min followed by two washes in Neoclear solvent (VWR Canlab, Mississauga, Canada) and progressively hydrated with successive washes at room temperature in 100% ethanol, 95% ethanol in PBS, 70% ethanol in PBS, and finally PBS. After permeabilization for 6 min in boiling citrate solution (0.1% sodium citrate, 0.1% Triton X-100 in water), the tissue slides were washed twice in PBS at room temperature. Nonspecific binding sites were blocked by 1-h incubation with goat serum at room temperature in a humidified chamber, and the tissues were probed with rabbit anti-human TGF-β3 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. The slides were washed twice in PBS, and the tissues were probed with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) for 1 h at room temperature in a humidified chamber protected from light. The slides were washed once in PBS and counterstained with Hoechst nuclear dye followed by two rinses in water. The tissues were covered with prolong anti-fade mounting medium and observed under a fluorescence microscope.

Statistical Analysis—The data were subjected to one-way analysis of variance (PRISM software version 3.03; GraphPad, San Diego, CA). Differences between experimental groups were determined by the Tukey’s test. Statistical significance was accepted when p < 0.05.

RESULTS TGF-βs and Their Receptors Are Produced by KLE Endometrial Carcinoma Cell Line—Endometrial tumors have been reported to contain the three TGF-β isomers in vivo (9). We examined whether KLE and Hec-1A human endometrial carcinoma cell lines produced TGF-βs and their receptors. In KLE cells TGF-β1 and TGF-β3 transcripts were abundantly expressed, whereas we could only detect a minimal level of TGF-β2 (Fig. 1A), and high levels of the three TGF-β receptors transcripts were present (Fig. 1B), suggesting that TGF-β can exert autocrine action in this cell line. In Hec-1A cell line all three TGF-β receptors, and only TGF-β3, are produced (data not shown).

TGF-β Isoforms Inhibit the Growth of Endometrial Carcinoma Cells—We have compared the growth inhibitory effects of TGF-β isomers on endometrial carcinoma cells using MTT proliferation assays. We found that KLE cells are equally sensitive to the growth inhibitory effects of the different TGF-βs, except after a prolonged exposure of 72 h when TGF-β1 and TGF-β3 were more potent than TGF-β2 to suppress KLE cell proliferation (Fig. 1, C–F). Similar results were also obtained with the Hec-1A cell line (data not shown).
TGF-β Isoforms Are Unable to Trigger Apoptosis in Endometrial Carcinoma Cells—We have determined the effect of TGF-β isoforms on apoptosis of endometrial carcinoma cells using Hoechst nuclear staining. The results showed no significant increase in apoptotic cell numbers in TGF-β-treated KLE cells when compared with control cells (Fig. 1F); the Hec-1A cell line was also resistant to pro-apoptotic effects of TGF-β isoforms (data not shown). These results indicate that TGF-β-activated pathway(s) involved in apoptosis are disrupted in endometrial carcinoma cells.

TGF-β Isoforms Similarly Activate Smad Signaling in Endometrial Carcinoma Cells—We have examined Smad signaling triggered by TGF-β isoforms in endometrial carcinoma cells. Although Smad2, Smad3, and Smad4 levels were not modulated by TGF-β isoforms, we observed a similar increase in the phosphorylation/activation of Smad2 and Smad3 in KLE cells treated with each TGF-β isoform (Fig. 2). Each TGF-β isoform is thus able to activate proper Smad signaling in endometrial carcinoma cells.

TGF-β Isoforms Induce the Activation of Effector Caspases in Carcinoma Cells—We have examined whether deficient caspase activation was responsible for the lack of apoptotic effect of TGF-β isoforms. TGF-β isoforms similarly increased the levels of cleaved caspases-3 and -6 in KLE cells (Fig. 3), suggesting that TGF-β signaling disruption takes place downstream from the effector caspases activation in KLE endometrial carcinoma cells.

XIAP Expression Is Up-regulated by TGF-β Isoforms—XIAP can block pro-apoptotic caspase signaling upon binding and inhibition of effector caspases activity (11). We have characterized the regulation of expression of XIAP in carcinoma cells by TGF-β isoforms. XIAP is constitutively expressed and dramatically up-regulated upon treatment of both KLE (Fig. 4) and Hec-1A (supplemental Fig. S1) cells with either TGF-β isoform. These results thus identify XIAP as a candidate molecule for the late inhibition of TGF-β pro-apoptotic signaling in resistant carcinoma cells.

TGF-β3 Increases Cellular Invasiveness by Activating PI3-K—We have evaluated the effect of TGF-β isoforms on the invasiveness of resistant endometrial carcinoma cells, using Matrigel invasion assay. KLE cells only exhibited moderate intrinsic invasive properties, but invasiveness was selectively increased by TGF-β3 (Fig. 5A). TGF-β3 also selectively up-regulated the.
TGF-β3 Increases Cell Invasion

XIAP Is Involved in TGF-β3-increased Cellular Invasiveness via PI3-K—We investigated whether PI3-K activity was involved in the up-regulation of XIAP by TGF-β3 in these cells. Indeed, PI3-K inhibitor LY294002 blocked the TGF-β3-induced up-regulation of XIAP (Fig. 5, C and D) but not the increase of XIAP induced by TGF-β1 and TGF-β2 (Fig. 5, E and F). Because activation of PI3-K by TGF-β3 leads to up-regulation of XIAP levels, as well as increased cellular invasiveness, we have examined whether XIAP could be involved in the invasiveness of KLE cells. When KLE cells were transfected with XIAP siRNA, endogenous XIAP levels were reduced more than 50%, and TGF-β3 could only minimally increase XIAP expression to reach a level similar to cells that had been transfected with control siRNA (Fig. 6, A and B). In these conditions, intrinsic invasive properties of KLE cells were not modulated, but reducing XIAP levels partially impeded the ability of TGF-β3 to increase the invasiveness of KLE cells (Fig. 6C). We also observed that reducing XIAP levels partially blocked the ability of TGF-β3 to increase the invasiveness of Hec-1A cells (supplemental Fig. S1). These results indicate that although endogenous XIAP levels do not dictate intrinsic invasive properties of endometrial carcinoma cells, the increase of cellular invasiveness by TGF-β3 would be mediated, at least in part, by up-regulation of XIAP levels.

XIAP Up-regulation Confers Resistance to Pro-apoptotic Effects of TGF-β3—We have determined whether XIAP up-regulation was involved in resistance to pro-apoptotic effects of TGF-β3. After exposure to TGF-β3, the apoptotic index was significantly higher in KLE cells that had been transfected with XIAP siRNA compared with cells treated with control siRNA (Fig. 6D), indicating that the presence of XIAP blocks TGF-β3-induced apoptosis in KLE cells. In addition, we found that knockdown of XIAP also caused increased apoptotic index in KLE cells upon exposure to TGF-β1 and TGF-β2 (supplemental Fig. S2), indicating that increased XIAP content in these cells protects from apoptosis induced by each TGF-β isoform.

MMP-9 Is Involved in TGF-β3-increased KLE Invasiveness via PKC Activation—Because MMP-9 actively participates in tumor cell invasion (12), we have examined whether TGF-β3 increased the invasiveness of KLE cells through up-regulation of MMP-9 expression in KLE cells. We found that resting KLE cells do not reproducibly express detectable levels of MMP-9, but exposure to TGF-β3 induced a transient expression of MMP-9 transcript (Fig. 7A) and protein (Fig. 7B). TGF-β1 and TGF-β2 were unable to increase the expression of MMP-9 in...
KLE cells (supplemental Fig. S3). Treatment with LY294002 did not impede the induction of MMP-9 expression in KLE cells by TGF-β3 (Fig. 7C), nor did treatment with PD98059 and SB203580 (supplemental Fig. S3), but selective PKC inhibitor chelerythrine chloride partly blocked this effect (Fig. 7D), showing a role for PKCs in MMP-9 induction by TGF-β3. Noteworthy, this up-regulation of MMP-9 via PKC activation is involved in the increase in KLE invasiveness by TGF-β3, because MMP-9 Inhibitor I, a selective inhibitor of MMP-9 activity, completely abolished TGF-β3-triggered invasiveness (Fig. 7E), and given that chelerythrine chloride also inhibited TGF-β3-triggered invasiveness (Fig. 7E).

**TGF-β3-activated Pathways Mediating XIAP and MMP-9 Up-regulation Are Independent**—We have examined whether in addition to MMP-9 induction, PKC activation was involved in XIAP up-regulation by TGF-β3 in these cells and whether XIAP, which can act as a transcriptional activator (13), was involved in MMP-9 expression in KLE cells. PKC inhibitor chelerythrine chloride did not block the up-regulation of XIAP by TGF-β3 (Fig. 7D), indicating that PKCs were not involved in XIAP up-regulation. Moreover, reduction of XIAP levels by siRNA did not interfere with MMP-9 induction by TGF-β3 (Fig. 7F), indicating that XIAP is not involved in MMP-9 induction by TGF-β3. Because PI3-K activation, which partly mediates XIAP up-regulation by TGF-β3, was not involved in MMP-9 up-regulation (Fig. 7C), we conclude that PKC-dependent up-regulation of MMP-9, and PI3-K-dependent up-regulation of XIAP, occur independently in KLE cells upon exposure to TGF-β3.

**TGF-β3-mediated Cell Invasion Is Smad-dependent**—To determine whether Smad signaling, which is properly activated by TGF-β3 in KLE cells (Fig. 2), was involved in TGF-β3-induced cellular invasiveness, we silenced Smad4, a necessary component of Smad pathway, in both KLE and Hec-1A cell lines using RNA interference, before treating the cells with TGF-β3. Our results showed that down-regulation of Smad4 protein (Fig. 8, A and C) impaired the ability of TGF-β3 to increase cellular invasiveness (Fig. 8, B and D); in addition, we found that knockdown of Smad4 impaired the ability of TGF-β3 to up-regulate XIAP (Fig. 8, A and C), but not to induce MMP-9 in KLE cells (Fig. 8, E and F). These results indicate a role for Smad signaling in TGF-β3-induced XIAP up-regulation and increased cellular invasiveness.

**TGF-β3 Is Present in Endometrial Carcinoma Tumors in Vivo**—To correlate our observations of a role for TGF-β3 in HEC cell survival and invasiveness with clinical data, we have conducted immunofluorescence analysis on a large number of endometrial carcinoma tissue samples representing various
stages of the disease (Fig. 9). In normal endometrial tissue samples, TGF-β3 expression was found to be almost exclusive to epithelial cells, although sparse TGF-β3-expressing cells were present in the stromal compartment (Fig. 9D). In stage I endometrial carcinoma samples, TGF-β3 localized to the basal surface of epithelial lining and, to a similar extent, to adjacent stromal cells (Fig. 9A); in stage II endometrial carcinoma tissues, weak TGF-β3 immunoreactivity was found at the basal surface of epithelial lining, and a few cells in the stromal compartment showed intense TGF-β3 signal, whereas most of the stromal cells showed a constant but weak signal (Fig. 9B); in stage III endometrial carcinoma tissue samples, TGF-β3 immunoreactivity was observed at the basal surface of epithelial compartment and in the stroma where TGF-β3 immunoreactivity was strongly observed (Fig. 9C). Collectively, these results confirm that TGF-β3 is produced in endometrial tumors in vivo, by epithelial as well as stromal cells, consistent with a role for this TGF-β isoform in HEC cells survival and invasiveness.

**DISCUSSION**

In the early events of epithelial tumorigenesis, TGF-β generally exerts growth inhibitory and pro-apoptotic effects on cancer cells, thereby suppressing tumor growth. However, tumor cells become increasingly resistant to growth inhibition by TGF-β, and in the late stages of carcinogenesis, TGF-β would rather exert tumor promoting effects on tumor cells, notably by stimulating invasion and metastasis.

Different types of malignant cells have acquired the ability to override the growth inhibitory effects of TGF-β through various mechanisms that include down-regulation or mutation of TGF-β receptors (14, 15), down-regulation of Smads (16), and increased AKT activity (17). The three mammalian TGF-β isoforms are present in endometrial tumors (9), and several lines of evidence suggest that endometrial carcinoma cells are resistant to TGF-β growth inhibitory effects (reviewed in Ref. 18); underlying mechanisms, however, are poorly understood.

We have examined the signaling pathways activated by each TGF-β isoform in endometrial carcinoma cells and compared their effect on cellular proliferation, apoptosis, and invasiveness, using the KLE and Hec-1A endometrial carcinoma cell
lines. High levels of TGF-β1 transcripts are present in KLE cells, in agreement with previous reports of TGF-β1 production by endometrial carcinoma cells in vitro and in vivo (19, 20). In addition, the KLE cell line is tumorigenic when injected subcutaneously in nude mice and invasive (21), which indicates a late stage phenotype and legitimates the evaluation of the invasion promoting effects of TGF-β3. Hec-1A cell line is also widely used as a model of endometrial carcinoma cells (22).

We have shown that endometrial carcinoma cell lines KLE and Hec-1A are sensitive to the growth inhibitory effects of TGF-β, although to a varying level depending on the isoform. Smad signaling activated by TGF-β reportedly interferes with cell cycle regulation and cellular growth properties, and this growth inhibitory effect of TGF-β3 was indeed accompanied by proper activation of Smad components. We also showed that endometrial carcinoma cells were resistant to the pro-apoptotic effect of TGF-β3. Effector caspase-3 was cleaved/activated in KLE carcinoma cells upon exposure to each TGF-β isoform, suggesting that the apoptotic program was indeed initiated in KLE cells upon exposure to TGF-β3 but that other cellular mechanisms interfered with late apoptotic events. Indeed, XIAP, which directly inhibits the activity of effector caspases (11), is up-regulated in KLE cells after exposure to each TGF-β isoform and efficiently blocks TGF-β-induced apoptosis in KLE cells. To our knowledge, this is the first report of a positive regulation of XIAP by TGF-β3. Others had reported that in hepatoma cells, TGF-β1 down-regulates XIAP levels, which would facilitate initiation of apoptosis by caspase-3 (23); this discrepancy may result from cell type specificities.

It is known that TGF-β1 can directly confer invasive properties to established cancer cells (10, 24). We found that TGF-β isoforms differ in their ability to modulate the invasive behavior of endometrial carcinoma cells, because only TGF-β3 could increase the invasiveness of KLE and Hec-1A cells. In this regard, only TGF-β3 triggered PI3-K activation and increased AKT phosphorylation/activation; moreover, we showed that TGF-β3 increased the invasiveness of endometrial carcinoma cells through activation of PI3-K.

We also found that only TGF-β3 induces the expression of MMP-9 in KLE cells, and MMP-9 activity was necessary for TGF-β3-increased invasiveness of KLE cells; in the clinic levels of MMP-9 were shown to correlate with an advanced stage (involving tissue invasion) of endometrial tumors (25). TGF-β1 and TGF-β2, which were unable to trigger MMP-9 expression by these cells, did not stimulate the invasiveness of KLE cells. Others had reported that TGF-β1 increased invasiveness in KLE cells (20), but in the latter study TGF-β1 increased MMP-9 expression by KLE cells, which nonetheless supports our findings that increased MMP-9 expression confers invasive properties to endometrial carcinoma cells. The fact that TGF-β3 induces the expression of MMP-9 by KLE cells suggests the presence of a positive loop for regulation of autocrine TGF-β3 tumor promoting effect, because MMP-9 can activate latent TGF-β isoforms into their active forms (26). We also demonstrated that PKC activity was involved in TGF-β3-triggered MMP-9 induction and invasiveness of KLE cells, in accordance with several other studies describing a positive role for PKCs in MMP-9 expression (27) and in cellular invasiveness (28).

Our results showed for the first time that XIAP was involved in the regulation of invasiveness of endometrial carcinoma cells that had been exposed to TGF-β3. Given the previous indication that XIAP can function as a cofactor for TGF-β1 in the regulation of gene expression (13), it is possible that endometrial carcinoma cells, up-regulation of XIAP by TGF-β3 leads to the modulation of transcriptional activity of particular genes involved in cellular motility/invasiveness. Our results showed, however, that XIAP alone was not sufficient to induce the expression of MMP-9 in KLE cells, which probably explains why TGF-β1 and TGF-β2 are unable to induce MMP-9 expression, and KLE invasiveness even though XIAP is up-regulated to a level similar to that resulting from TGF-β3 exposure. Alternatively XIAP could, in combination with one or more cellular components selectively mobilized by TGF-β3, activate the expression of components involved in MMP-9 expression. Such a functional link between XIAP and MMP-9 is highly improbable, however, considering that inhibition of PI3-K, which completely abolished XIAP up-regulation by TGF-β3, did not impede MMP-9 induction. Because PKCs are not involved in XIAP up-regulation by TGF-β3 in KLE cells, we conclude that TGF-β3 independently triggers PKC-dependent MMP-9 up-regulation and PI3-K-dependent XIAP up-regulation. Interestingly, TGF-β3 did not induce MMP-9 expression in Hec-1A cells but did increase their invasive properties, indicating that induction of MMP-9 expression is not obligatory for TGF-β3-induced invasion in these cells. Similarly, in KLE cells, blockade of PKC-mediated MMP-9 induction only partially reduced TGF-β3-induced invasion. Importantly, this further suggests that although increased MMP-9 activity promotes HEC cells invasiveness, increased MMP-9 expression is not necessary for TGF-β3-induced invasion in endometrial carcinoma cells in general. In addition, our finding that in Hec-1A cells TGF-β3 promoted XIAP-dependent invasiveness, as observed with KLE cells, but was not able to induce MMP-9 expression in Hec-1A cells strengthens our conclusion that TGF-β3 induces HEC invasiveness through independent up-regulation of both MMP-9 and XIAP. This suggests that XIAP is the critical and key factor in TGF-β3-induced invasion in endometrial carcinoma cells.

Finally, we have highlighted the importance of Smad pathway in TGF-β3-induced cellular invasiveness using RNA interference. Others had already reported the involvement of Smad signaling in regulating cancer cell invasiveness in vitro (29, 30), notably upon regulation of the expression of particular MMPs (30). However, in the case of endometrial carcinoma cells, TGF-β3-activated Smad signaling is probably not involved in the regulation of MMP-9, which we have showed to play a role in TGF-β3-induced invasiveness. Smad4 is, however, involved in the increase of XIAP protein content by TGF-β3. Noteworthy, our finding that knockdown of Smad4 impairs the up-regulation of XIAP as well as the increase in cellular invasiveness triggered by TGF-β3 strengthens our conclusion of a role for XIAP in cellular invasion.

The selective ability of TGF-β3 to increase invasiveness in a XIAP-dependent manner and the fact that each isoform up-regulates XIAP can be explained as follows: 1) only TGF-β3 increases XIAP in a PI3-K-dependent manner in both KLE and
Hec-1A cells, suggesting that other factors are also regulated in this TGF-β3-specific pathway and are necessary for XIAP to exert its invasive promoting role, and 2) in KLE cells only TGF-β3 induces MMP-9 expression, which we have shown to partially mediate TGF-β3-induced invasiveness.

In conclusion, we showed for the first time that TGF-β3 confers invasive properties to endometrial cancer cells that are resistant to its pro-apoptotic effects through PI3-K-dependent up-regulation of XIAP and through PKC-dependent induction of MMP-9 expression and in a Smad-dependent manner (Fig. 10). Such a role for TGF-β3 in HEC cells survival and invasiveness correlates with clinical data, as we highlighted the presence of TGF-β3 in endometrial carcinoma tumors in vivo. The results demonstrated that upon progression toward a more invasive phenotype (from stage I to stage III endometrial adenocarcinoma), TGF-β3 immunoreactivity gradually extends from the epithelial compartment (in normal tissues) to the stroma (in adenocarcinoma), consistent with a role for TGF-β3 in HEC invasiveness. Currently an experimental metastasis model is under development that will allow us to investigate more precisely the involvement of TGF-β3 in endometrial carcinoma metastasis in vivo.

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FIGURE 10. Proposed model for tumor promoting effects of TGF-β3 in endometrial carcinoma cells. In endometrial carcinoma cells, TGF-β3 would trigger PI3-K-dependent and Smad-dependent XIAP up-regulation, which would increase survival and invasiveness. In a subset of endometrial carcinoma cells, MMP-9 would also independently activate PKC-dependent MMP-9 expression, conferring increased invasive properties.