Regulation of PDGF production and ERK activation by estrogen is associated with TSC2 gene expression

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Mechanisms that regulate the growth response to estrogen (17β-estradiol, E2) are poorly understood. Recently, loss of function of the tuberous sclerosis complex 2 (TSC2) gene has been associated with E2-related conditions that are characterized by benign cellular proliferation. We examined the growth response to E2 in vascular smooth muscle cells (VSMCs) that possess wild-type TSC2 and compared them with ELT-3 smooth muscle cells that do not express TSC2. In TSC2-expressing VSMCs, growth inhibition in response to E2 was associated with downregulation of platelet-derived growth factor (PDGF), PDGF receptor (PDGFR), and limited activation of extracellular signal-regulated kinase (ERK). In contrast, the growth-promoting effect of E2 in TSC2-null ELT-3 cells was associated with induction of PDGF, robust phosphorylation of PDGFR, and sustained activation of ERK. Furthermore, in ELT-3 cells, cellular growth and ERK activation by E2 were inhibited by the PDGFR inhibitor tyrphostin AG 17 and by PDGFR-neutralizing antibody. These results demonstrate that autocrine production of PDGF and augmentation of the ERK pathway leads to estrogen-induced cellular proliferation in TSC2-null cells, a pathway that was downregulated in cells that express TSC2. Understanding the mechanisms that regulate the diverse responses to the steroid hormone estrogen could lead to novel approaches to the treatment of estrogen-related diseases that are characterized by aberrant cell proliferation.

tuberous sclerosis complex 2 gene; tuberin; cell growth; mitogen-activated protein kinase

ESTROGEN HAS BEEN SHOWN to inhibit vascular smooth muscle cell (VSMC) growth in vitro and contributes to the atheroprotective effect observed in some animal models (35, 49). In contrast, estrogen has also been implicated as a cellular mitogen in the growth of reproductive tract malignancies as well as in the cellular growth observed in benign conditions such as primary pulmonary hypertension and lymphangioleiomyomatosis (LAM) (15, 51). Thus estrogen can mediate two diverse growth responses. The mechanism that underlies this diverse response is poorly understood.

The genomic and nongenomic effects of estrogen are mediated by estrogen receptors (ER) (19). Unlike other growth factors, estrogen diffuses through the cell membrane, binds to ER, and localizes to the nucleus to activate gene transcription (19). However, not all the effects of ER are mediated genomically. Accumulating evidence indicates that some of the effects of ER are mediated through the early nongenomic activation of signaling intermediates within the cytosol including members of the ras-raf-MEK-1-mitogen-activated protein kinase (MAPK) pathway (34). The ras-raf-MEK-1 pathway is frequently activated during cell growth (7, 8). This results in the downstream activation of extracellular signal-regulated kinase-1 and -2 (ERK-1/p42mapk and ERK-2/p44mapk), members of the family of MAPKs (7, 8). ERK-1 and ERK-2 play a vital role in the downstream activation of several nuclear transcription factors that regulate genes that are involved in governing the cell cycle. It has been demonstrated that cross talk exists between the MAPK pathway and steroid hormone signaling (31, 36). MAPK is frequently activated by ER and, conversely, activated MAPK mediates translocation of ER to the nucleus (23, 31, 36). Understanding the genomic and nongenomic effects of ER, including the regulation of the MAPK pathway, is key to understanding the mechanism that underlies the diverse effects of estrogen.

The factors that predict mitogenesis in response to estrogen are likely very complex. For example, it has been demonstrated during development that specific gene expression profiles in VSMCs are associated with a growth response (55). In response to estrogen, the exact nature of such genes and the cellular processes that they alter are unknown. It has been shown that expression of the tumor suppressor gene tuberous sclerosis complex 2 (TSC2), which encodes the protein tuberin, can regulate ER-mediated gene transcription (30). Loss of function of the TSC2 gene results in a well-defined clinical syndrome, tuberous sclerosis complex (TSC), that is associated with the development of...
multiple benign tumors (1, 17), some of which express increased amounts of ER (3, 26, 29, 39). Loss of function of TSC2 has also been implicated in the pathogenesis of pulmonary LAM, a disease with a female preponderance that is characterized by smooth muscle cell proliferation and is also associated with increased ER expression (2, 6, 48, 50). The Eker rat, which is heterozygous for TSC2, spontaneously develops tumors of mesenchymal cell origin including tumors of the reproductive tract (24, 58). ELT-3 cells are Eker rat uterine leiomyoma-derived smooth muscle cells that are null for the TSC2 gene and grow in response to estrogen (20). In contrast, VSMCs highly express TSC2 and are growth inhibited by estrogen (10, 11, 14, 33, 53). In this study we used TSC2-expressing vascular cells and TSC2-null ELT-3 cells to compare and contrast the mechanisms that regulate cellular growth in response to estrogen. We describe the coordinate regulation by estrogen of platelet-derived growth factor (PDGF) and MAPK to regulate cell growth. The growth-inhibiting effect of estrogen is associated with the downregulation of PDGF and MAPK in TSC2-expressing cells. In contrast, upregulation of PDGF and MAPK was essential for estrogen-mediated growth of TSC2-null cells. These data suggest that TSC2 gene expression may be associated with the cellular growth response to estrogen.

EXPERIMENTAL PROCEDURES

Cell culture and growth factor stimulation. Vascular endothelial cells (VECs) and VSMCs from mouse aorta (kindly provided by Dr. Richard Karas, Tupper Institute, New England Medical Center) were grown in culture by the explant technique and maintained in DMEM supplemented with 10% serum as previously described (22). Experiments were performed on VSMCs between passages 5 and 12. ELT-3 cells were obtained from and characterized by Dr. Cheryl Walker (M. D. Anderson Cancer Center). ELT-3 cells were grown and maintained in DF8 medium as previously described (20). Experiments were performed on ELT-3 cells between passages 26 and 30. For cell growth experiments, cells were seeded at a density of 0.5 × 10^4 cells/ml on 35-mm petri dishes. When cultures achieved 10–20% confluence, serum was withdrawn from the culture medium for 24 h. Cells were treated with 17β-estradiol (E2; Sigma, St. Louis, MO), PDGF-BB, basic FGF (bFGF), acidic FGF (aFGF), or transforming growth factor (TGF)-β1 (R&D Systems, Minneapolis, MN) in the absence of serum and counted with a Coulter counter (Technical Communications, Coulter Electronics) at the intervals indicated in RESULTS. All cell culture experiments were performed in quadruplicate and repeated at least twice to ensure reproducibility. For all other experiments, cells were seeded at a density of 0.5 × 10^5 cells/ml on 100-mm petri dishes. When cultures achieved 90–100% confluence, serum was withdrawn and cells were treated with E2 (under serum-free conditions). After the specific intervals indicated in RESULTS, cells were harvested and extracts were prepared. All experiments were performed at least in triplicate with appropriate vehicle controls.

Inhibitors. Inhibitors used in these studies included the MEK-1 inhibitor PD-98059 (Calbiochem, La Jolla, CA); suramin, which inhibits growth factor-receptor interactions (Sigma); the specific estrogen receptor inhibitor ICI-182780 (Tocris, Ellisville, MO); and PDGF receptor (PDGFR) inhibitors 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile (tyrophostin AG 17) and 6,7-dimethoxy-2-phenylquinoxaline (AG 1296), which block PDGFR kinase and signaling from the activated PDGFR (25, 28) (Calbiochem). Length of treatment and doses are indicated in RESULTS.

PDGF neutralizing antibody. For neutralizing antibody experiments, cells were treated with E2. Polyclonal antihuman PDGFB-BB neutralizing antibody (R&D Systems) was added to culture medium at 8 h of E2 treatment. Cell extracts were prepared 12 h after the addition of neutralizing antibody. Nonspecific immunoglobulin was used as a control and added in the same manner to culture medium. The neutralizing antibody dose (ND) used was 20 times the ND50 recommended by the manufacturer.

Preparation of cell extracts. Preparation of whole cell extracts was described previously (13). Membrane extracts for PDGFR and epidermal growth factor receptor (EGFR) immunoblotting experiments were prepared in a similar manner with EB buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.1% BSA) plus inhibitors, 50 mM NaF, 1 mM PMSF, 2 mM Na3VO4, and 20 μg/ml aprotonin.

Immunoprecipitation and kinase assays. MAPK activity was measured as previously described (13). Immunoprecipitation experiments were performed by binding 5 μg of PDGFR-β polyclonal antibody (R&D Systems), EGFR antibody (Cell Signaling Technology), or phosphotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA) with 1 mg of protein extracts prepared as described (13). Samples were rocked at 4°C overnight. Protein A Sepharose beads (Repligen, Cambridge, MA) were then added and rocked at 4°C for a further 2 h. The precipitated immune complexes were washed, and the reaction was terminated by the addition of 2× Laemmli sample buffer. Samples were boiled and resolved by 7% SDS-PAGE. In all experiments, membranes were subsequently stripped and rebotted with appropriate antibody to ensure actual immunoprecipitation of the protein of interest.

Immunoblot analysis. Cell extracts were electrophoresed on SDS-PAGE gels. Gel proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (wet transfer). The blot was blocked with 5% nonfat dry milk or 1% milk at room temperature for 1 h. Blots were incubated with primary antibodies to ERK-1, phosphotyrosine, ER-α, tuberin (C-20), actin (Santa Cruz Biotechnology) PDGF, PDGFB-BB (R&D Systems), phospho-ERK, phospho-EGFR, and EGFR (Cell Signaling Technology) at 4°C overnight, washed, and incubated for 1 h with appropriate secondary horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling Technology). The chemiluminescent signal was detected with enhanced chemiluminescence (ECL) (Cell Signaling Technology). Immunoblots for PDGFB-BB included a positive control for accurate identification of the BB isomer of PDGFB. All experiments were performed in triplicate. Densitometric semiquantitative analysis was performed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data were expressed as fold activation relative to control. Results are expressed as means ± SD. Statistical analysis by a nonparametric test (Mann-Whitney U-test) was performed with SPSS software. Significance was assumed at a P value of <0.05.

RESULTS

E2 differentially regulates cell growth in TSC2-expressing cells compared with TSC2-null cells. Mutations of the TSC2 gene have been associated with increased ER-α expression in conditions such as LAM, angiomyolipoma (AML), and hepatic angioma (3, 26, 29, 39). The expression of tuberin, the protein product
of the TSC2 gene, and ER-α was compared in three E2-responsive cell lines by subjecting equal amounts of protein extract from each cell line to Western immunoblot for α-tuberin and α-ER-α. Figure 1A demonstrates that VSMCs highly expressed TSC2 compared with VECs and with ELT-3 cells, which did not express any TSC2. Immunoblot for ER-α demonstrated that ER-α was expressed in low amounts in VECs and VSMCs and highly expressed in ELT-3 cells (Fig. 1A). Immunoblot for actin demonstrated equal protein loading for the representative samples loaded.

The growth response to E2 (0–1,000 nM) was next examined. Growth of VECs was unaffected by E2 (Fig. 1B), and, as previously shown by others, VSMCs were growth inhibited by E2 (Refs. 10, 14, 53; Fig. 1C). In contrast, a significant dose-dependent growth response to E2 was observed in ELT-3 cells (Fig. 1D). The dose of E2 used for the remaining experiments was 10 nM, a dose that induced significant and consistent inhibition of VSMC growth (mean ± SD: 21 ± 12.2%) and promotion of ELT-3 cell growth (2.05 ± 0.53%). In ELT-3 cells, cell proliferation or apoptosis was not directly measured. However, cell morphology and trypan blue exclusion dye testing were similar between control and treatment groups (results not shown), suggesting that the growth effects of estrogen were not mediated through decreased apoptosis.

**PDGF participates in E2-mediated growth of TSC2-null cells.** The induction of autocrine factors by E2 has been implicated in hormonally regulated cell growth (27, 61). To investigate potential growth factors that might be involved in E2-mediated cell growth, TSC2-null ELT-3 cells were treated with the peptide growth factors PDGF-BB (0.1–10 ng/ml), aFGF and bFGF (5–10 ng/ml), and TGF-β1 (2–5 ng/ml) and growth was examined with Coulter counting. Figure 2A demonstrates significant ELT-3 cell growth in response to PDGF-BB and both forms of FGF. Stimulation of cell growth was not observed in response to TGF-β1. To examine the possibility of synergy between E2 and the mitogens identified from this experiment, ELT-3 cell growth was assessed after treatment with E2 together with either PDGF-BB or bFGF. E2 combined with PDGF-BB resulted in cell counts that were moderately synergistic, an effect not observed with bFGF (Fig. 2B). To further explore this synergy, lower doses of bFGF and PDGF-BB were used together with E2 treatment. Again, moderate synergy was observed for PDGF-BB plus E2 but no additional stimulation was noted for bFGF plus E2 (results not shown). These results suggested that E2 and PDGF-BB are synergistic in promoting TSC2-null ELT-3 cell growth.

To investigate the hypothesis that PDGF participates in E2-induced ELT-3 cell growth, the proliferative effect of E2 was assessed in the presence and absence of the PDGFR inhibitor AG 17 (tyrphostin), which inhibits the phosphorylation of the PDGFR (28). Complete inhibition of E2-stimulated ELT-3 cell growth was achieved with 10 μM AG 17 without significantly affecting baseline control cell growth (Fig. 3A). A similar significant inhibition of E2-induced growth was observed with a second PDGFR inhibitor, AG 1296 (20 μM) (Ref. 25; results not shown). Successful inhibition of PDGF-BB-induced ELT-3 cell growth, which served as a positive control, was also seen in response to AG 17 (Fig. 3B). Thus these results suggest that PDGF plays an integral part in mediating TSC2-null ELT-3 cell growth in response to E2.
E2 differentially regulates an autocrine pathway involving PDGF-BB in TSC2-expressing cells compared with TSC2-null cells. The preceding experiments indicated that PDGF was involved in mediating a positive growth response to E2 in TSC2-null ELT-3 cells. To investigate the participation of PDGF in E2-regulated cell growth, VECs, VSMCs, and ELT-3 cells were treated with E2 (0–48 h) and cell lysates were subjected to immunoblot analysis for PDGFRβ and PDGF-BB protein (Fig. 4). In VECs, PDGFRβ and PDGF-BB protein remained unaltered by E2. In VSMCs, downregulation of PDGFRβ and PDGF-BB protein was observed. In contrast, time-dependent induction of PDGFRβ protein was observed in TSC2-null ELT-3 cells beginning at 2 h, with maximum sustained induction by 24–48 h (1.89 ± 0.31-fold by densitometry). A more pronounced induction of PDGF-BB protein was also observed in ELT-3 cells (2.56 ± 0.89-fold by densitometry), which temporally paralleled PDGFRβ induction by E2. To investigate whether this pathway was activated by E2 in an autocrine fashion, PDGFRβ was immunoprecipitated from E2-treated lysates and subjected to immunoblotting for phosphotyrosine (Fig. 5A). Again, TSC2-expressing VECs and VSMCs were compared with TSC2-null ELT-3 smooth muscle cells. No phosphorylation of PDGFRβ was observed in TSC2-expressing VECs or VSMCs despite adequate amounts of PDGFRβ in immunoprecipitated samples (Fig. 5A). In contrast, substantial phosphorylation of PDGFRβ by E2 was observed in TSC2-null ELT-3 cells (Fig. 5A). Furthermore, inhibition by the ER antagonist ICI-182780 (100 nM) of E2-induced PDGFRβ phosphorylation in TSC2-null ELT-3 cells was also observed, confirming that this response was ER mediated (Fig. 5A).

Previous authors have shown that the response to estrogen can be mediated through EGFR (9, 12, 27, 46). Furthermore, it has also been shown that AG 17 can also inhibit the phosphorylation of EGFR (42) and can inhibit growth by disrupting mitochondria (4). Thus AG 17 is not truly specific as an inhibitor of PDGFR activation. The effect of estrogen on the activation of EGFR and the effect of AG 17 on phosphorylation of both PDGFR and EGFR were next examined. ELT-3 cells were treated with estrogen in the presence and absence of AG 17. Cell lysates were subjected to immunoprecipitation with a polyclonal antibody for phosphotyrosine followed by immunoblotting for PDGFRβ and EGFR (Fig. 5B). Both estrogen- and PDGF-BB-induced
phosphorylation of PDGFR-β were significantly inhibited by AG 17. In contrast, estrogen did not activate EGFR and AG 17 did not significantly inhibit EGF-induced phosphorylation of EGFR. To ensure that phosphorylation of EGFR was not occurring at an earlier time point, estrogen-treated lysates were subjected to Western immunoblotting with an antibody for phospho-EGFR (Fig. 5C). No significant phosphorylation of EGFR was observed over the time period examined.

Fig. 4. Estrogen differentially induces the protein expression of PDGF receptor (PDGFR)-β and PDGF-BB. Protein expression of PDGFR-β and PDGF-BB was determined with Western immunoblot for both proteins. Immunoblot for ß-actin was used as a control for protein loading for each experiment. VECs, VSMCs, and ELT-3 smooth muscle cells (SMC) were treated with estrogen (10 nM) for the times indicated.

Fig. 5. Estrogen differentially activates PDGFR-β. A: PDGFR-β was immunoprecipitated (IP) with polyclonal PDGFR-β antibody from estrogen-treated cell extracts of VECs, VSMCs, and ELT-3 cells. Western immunoblot with phosphotyrosine antibody (p-tyr) was then performed. Cells were treated with estrogen (10 nM) for 24 h. Phosphorylation of PDGFR-β by estrogen was compared with vehicle-treated cells (negative control) and PDGF-BB-treated cells (positive control). Protein expression of PDGFR-β in immunoprecipitated samples was determined with Western immunoblot. Control (C), E2, ICI-182768o (ICI), and PDGF-BB treatments were as indicated. B: phosphotyrosine was immunoprecipitated with polyclonal phosphotyrosine antibody from E2-treated cell extracts of ELT-3 cells. Western immunoblot with phosphotyrosine and epidermal growth factor (EGF) receptor (EGFR) antibody was then performed. Cells were treated with E2 (10 nM) for 24 h. Phosphorylation of PDGFR-β and EGFR by E2 was compared with vehicle-treated cells (negative control) and PDGF-BB- or EGF-treated cells (PDGF-BB-positive control; EGF-positive control) in the presence and absence of AG 17 (10 μM). Control (C), E2, PDGF-BB, EGF, and AG 17 treatments were as indicated. C: phosphorylation of EGFR (p-EGFR) by E2 (10 nM) and total EGFR protein (EGFR) were also determined with Western immunoblot in ELT-3 cells. Cells were treated with E2 for the times indicated. Phosphorylation of EGFR by EGF (10 ng/ml) was used as a positive control (EGF-positive control). Immunoblot for total EGFR also served as a control for protein loading.
E2-induced cell growth and ERK activation in TSC2-null cells are MEK-1 dependent. The ras-raf-MEK-1-ERK pathway is frequently activated during cell growth (7, 8), and E2 has been shown to result in the immediate activation of the ERK pathway (36, 54). To investigate the potential mechanisms used by E2 to regulate cell growth, TSC2-expressing VECs and VSMCs and TSC2-null ELT-3 smooth muscle cells were compared for ERK activation in response to E2. Cell extracts were subjected to Western immunoblot for phospho-ERK (0–48 h). In TSC2-expressing VSMCs and VECs the activation of ERK-1 and ERK-2 by E2 was immediate, occurring at 15 min and slowly declining to baseline levels by 24 h (Fig. 6A). Densitometry of phospho-ERK-2 revealed a fourfold activation of ERK in VECs and a twofold activation of ERK in VSMCs at 15 min, returning to baseline levels by 24 h (Fig. 6A). In contrast, the pattern of E2-induced ERK-1 and ERK-2 activation in TSC2-null ELT-3 cells was biphasic (Fig. 6B). Immunoblotting did not reveal any increase in the amount of ERK protein in E2-treated samples, and immunoprecipitation kinase assay also confirmed this pattern of ERK activation in response to E2 in ELT-3 smooth muscle cells. This suggests that ERK activation occurred via phosphorylation rather than induction of ERK protein. Densitometry, performed on Western immunoblots of phospho-ERK, revealed a twofold activation of ERK-2 at 15 min, returning to baseline levels by 1–2 h (Fig. 6B). A second wave of ERK activation began at 4–8 h, with maximum activation by 24–48 h. The biphasic pattern of ERK activation by E2 in ELT-3 cells was not observed in TSC2-expressing VSMCs or VECs.

To investigate the role of E2-induced ERK activation in TSC2-null ELT-3 cell growth, cells were treated with E2 in the presence and absence of PD-98059, a MEK-1 inhibitor, and cell growth was assessed. Figure 7A demonstrates that E2-stimulated growth was sig-
EI significantly inhibited by PD-98059, indicating that E₂-induced growth of ELT-3 cells is MEK-1 dependent.

E₂-induced late activation of ERK in TSC2-null cells is dependent on MEK-1 and autocrine induction of PDGF-BB. In ELT-3 cells, the delayed second wave of ERK-1 and -2 activation and the known induction of PDGF by E₂, which temporally paralleled E₂-induced

activation of ERK, suggested that an autocrine factor, possibly PDGF, mediated this later phase of ERK phosphorylation. To test this hypothesis, cultured ELT-3 cells were treated with E₂ in the presence and absence of PD-98059 (10 μM), a MEK-1 inhibitor, suramin (300 μM), an inhibitor of growth factor-receptor interactions, and AG17 (10 μM), a PDGFR inhibitor, and PDGF-neutralizing antibody (20 μg/ml). The inhibitors and neutralizing antibody were added 8 h after E₂ treatment, at a time when PDGF induction by E₂ was previously observed, and lysates were prepared 12 h later. Samples were subjected to immunoblot analysis with phospho-ERK-1/2 antibody. Significant inhibition of E₂-induced ERK-1/2 activation was observed for PD-98059, suramin, and AG17 (Fig. 7B). More specifically, significant inhibition of E₂-induced ERK-1/2 activation was also observed for PDGF-neutralizing antibody (Fig. 7C). Early activation (15 min) of ERK by E₂ was also inhibited by pretreating ELT-3 cells with PD-98059 1 h before E₂ treatment (results not shown). These results demonstrated that late-phase ERK-1/2 activation by E₂ in ELT-3 smooth muscle cells is MEK-1 dependent and mediated by PDGF-BB.

**DISCUSSION**

In the present study we demonstrate that growth inhibition by estrogen of TSC2-expressing cells occurs in association with the downregulation of PDGF and limited activation of ERK. In contrast, the growth-promoting effect by estrogen of TSC2-null cells involves augmentation of PDGF production and ERK activation. Collectively, these data suggest that the growth response to estrogen is determined by the autocrine production of PDGF-BB and ERK activation. This pathway may be regulated by TSC2 expression and is depicted in Fig. 8.

This study shows that the growth response to estrogen is mediated, not by the immediate activation of the ERK pathway or by direct genomic transactivation of
cell cycle genes, but rather by the induction of a PDGF autocrine loop that results in a more sustained pattern ERK activity. Conversely, the growth inhibitory response to estrogen is associated with the downregulation of these proteins. Studies have shown that PDGF is induced by estrogen (18, 47). This study now demonstrates that the autocrine production of PDGF can be upregulated and downregulated by estrogen to switch on or switch off cellular growth. Furthermore, Lange et al. (27) showed that potentiation of EGF-induced cell growth by the steroid hormone progesterone involves the enhanced activation of all members of the MAPK families, ERK, JNK, and p38, as well as the cell cycle proteins, cyclin D1, cyclin E, and p21WAF1. This suggests that the autocrine production of polypeptide growth factors by steroid hormones is designed to augment or potentiate specific signaling pathways within the cell to promote cell growth.

In the present study we demonstrate that the divergent growth response to estrogen is associated with the altered expression of ER-α and TSC2. TSC2-expressing cells contained comparatively low levels of ER-α expression and were growth inhibited by estrogen. TSC2-null cells highly expressed ER-α and exhibited a significant growth response to estrogen. Thus loss of TSC2 expression may be associated with enhanced expression of ER-α as a mechanism to promote a growth response to estrogen. In support of this theory, increased ER expression has been observed in conditions associated with functional loss of TSC2 such as LAM, AML, and hepatic hemangioma (3, 26, 29, 37, 39). Furthermore, estrogen has been implicated as a growth-promoting hormone in the pathogenesis of both LAM and AML, which are associated with aberrant expression of TSC2 and characterized smooth muscle cell proliferation (51).

The regulation of cellular growth by estrogen is likely determined by the expression of a gene or set of genes that are cell or tissue specific. One weakness of this study is that the effects of estrogen were not tested in comparable TSC2-expressing and TSC2-null cell lines. Furthermore, the cells tested were not derived from the same tissue source. However, our data and those of others do suggest that TSC2 may be one of the genes that promote or inhibit the growth response to estrogen. Other groups have observed that TSC2-null ELT-3 cells, derived from uterine leiomyomas in the Eker rat, grow in response to estrogen (21). In contrast, mature myometrium from this model, which possesses one wild-type allele for TSC2, does not grow in response to estrogen (5). Furthermore, in Hep G2 cells, Lou et al. (30) demonstrated reduced ER-mediated gene transcription by the expression of TSC2. Interestingly, neither the PDGF-B chain promoter nor PDGFR promoter contain a classic estrogen response element (ERE) site (National Center for Biotechnology Information GenBank accession no. NM0026009). However, the promoters for these genes do contain consensus binding sites for transcription factors, AP-1 and SP-1, which are known to directly activate transcription in response to the estrogen-ER complex (52, 56). It could be speculated that expression of TSC2 regulates ER-mediated transcription of PDGF and PDGFR via transcription factors such as AP-1 and SP-1. Collectively, these data support the hypothesis that TSC2 gene expression regulates the growth response to estrogen.

We observe that the altered production of PDGF and its receptor was associated with opposing functions for estrogen. TSC2 expression has the potential to regulate the described autocrine pathway at multiple levels. In this study we showed that a major component of estrogen-regulated cellular growth of TSC2-null cells involved the MAPK pathway. It is widely believed that the rapid activation of MAPK after estrogen treatment is a signaling effect of membrane-associated ER (mER) (12, 44, 45). The function of the early-immediate activation of ERK was not fully examined in this study. Although early activation of ERK was not different in TSC2-expressing and TSC2-null smooth muscle cells, the second phase of estrogen-induced ERK activation was very apparent in TSC2-expressing ELT-3 smooth muscle cells. This second wave was mediated by the autocrine induction of PDGF-BB. It has been demonstrated that tuberin, the protein product of the TSC2 gene, possesses Rap1GAP activity (57). Rap1GAP GTPases share homology with the ras family of signal molecules, and B-raf is activated by Rap1 (40, 41). When microinjected into cells, Rap1 can induce DNA synthesis and dysregulation of the ERK pathway, which has been implicated as a mechanism through which Rap1 can alter cell growth (59, 60). Furthermore, a prostate computer search (ExPaSy; http://www.expasy.ch/) of the tuberin sequence reveals a MAPK phosphorylation site. Thus TSC2 has the potential to play a vital role in modulating at least the pattern of MAPK/ERK activation to alter estrogen-regulated cell growth. Recently, much attention has been paid to the trans-activation of membrane receptors such as EGFR by mER (12, 45). Trans-activation usually occurs as an early membrane-associated event that affects downstream signaling events including the activation of MAPK. TSC2 localizes to the cell membrane, and it could be speculated that loss of function of TSC2 potentially regulates receptor function at the cell membrane to alter downstream signaling events (38). Finally, TSC2 has been shown to antagonize insulin and growth factor-mediated cell growth by antagonizing the phosphatidylinositol 3-kinase pathway distal of Akt (16, 32, 43). Thus it is reasonable to speculate the converse, i.e., that loss of function of TSC2 is associated with augmented growth factor-regulated pathways to promote cell growth, a hypothesis that is supported by the observations in this study.

In conclusion, we have described an ER-regulated autocrine loop involving PDGF in TSC2-null smooth muscle cells that is not present in cells that express TSC2. Estrogen initiates a coordinated cellular response to ensure continued cell growth via amplification of the MAPK pathway. Understanding of the complex integration of signaling pathways between steroid and peptide growth factors may bear relevance to es-
trogen-related diseases that are characterized by aberrant cell proliferation.

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**DISCLOSURES**

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