Peroxisome Proliferator-activated Receptor γ and Transforming Growth Factor-β Pathways Inhibit Intestinal Epithelial Cell Growth by Regulating Levels of TSC-22*

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Peroxisome proliferator-activated receptor γ (PPARγ) and transforming growth factor-β (TGF-β) are key regulators of epithelial cell biology. However, the molecular mechanisms by which either pathway induces growth inhibition and differentiation are incompletely understood. We have identified transforming growth factor-simulated clone-22 (TSC-22) as a target gene of both pathways in intestinal epithelial cells. TSC-22 is member of a family of leucine zipper containing transcription factors with repressor activity. Although little is known regarding its function in mammals, the Drosophila homolog of TSC-22, bunched, plays an essential role in fly development. The ability of PPARγ to induce TSC-22 was not dependent on an intact TGF-β1 signaling pathway and was specific for the γ isoform. Localization studies revealed that TSC-22 mRNA is enriched in the postmitotic epithelial compartment of the normal human colon. Cells transfected with wild-type TSC-22 exhibited reduced growth rates and increased levels of p21 compared with vector-transfected cells. Furthermore, transfection with a dominant negative TSC-22 in which both repressor domains were deleted was able to reverse the p21 induction and growth inhibition caused by activation of either the PPARγ or TGF-β pathways. These results place TSC-22 as an important downstream component of PPARγ and TGF-β signaling during intestinal epithelial cell differentiation.

The pathways that induce intestinal epithelial differentiation are complex and multigenic (1, 2). Two biologically important regulators of intestinal epithelial cell growth are the peroxisomal proliferator-activated receptor-γ (PPARγ) and transforming growth factor-β (TGF-β) pathways. PPAR isoforms α, δ, and γ constitute a family of orphan nuclear hormone receptors that serve to integrate dietary fat intake with changes in the expression of genes involved in both fatty acid oxidation and storage (3). PPARγ was originally defined as an essential component of the adipocyte differentiation program (4). However, the range of biological functions that PPARγ regulates is much wider than originally thought and the receptor has been shown to modulate the growth of a wide variety of cell types (5–7). This is perhaps most evident in the colon, where activation of the receptor inhibits the growth of a broad range of colon cancer cell lines and induces markers of differentiation (8, 9). The effects of PPARγ on colorectal carcinogenesis may be dependent on the model system used because activators of the receptor slightly increase the number of colonic adenomas in the min mouse (a murine model of familial adenomatous polyposis) (10, 11). However, ~8% of primary colorectal tumors were found to harbor a loss of function mutation in the PPARγ gene emphasizing the putative role of this receptor as a tumor suppressor in humans (12).

The TGF-β family of growth factors regulate a plethora of biological processes including embryonic development, wound healing, angiogenesis, proliferation, and differentiation of cells (reviewed in Ref. 13). This latter function has been well defined in the colon, where TGF-β is a potent inhibitor of colonic epithelial cell growth (14, 15). Loss of normal TGF-β responsiveness occurs commonly during the development of colorectal cancers associated with microsatellite instability and genetic lesions that disrupt the TGF-β pathway have been identified, including loss of function mutations in the TGF-β type II receptor (16, 17), SMAD4 (18), and SMAD2 (19). Under these circumstances continued expression of TGF-β paradoxically leads to enhanced tumor growth through stimulation of angiogenesis, extracellular matrix production, and immune suppression (20).

Thus, although both PPARγ and TGF-β are key regulators of epithelial cell biology, the molecular mechanisms by which either pathway induces growth inhibition and differentiation are incompletely understood. Here we have identified transforming growth factor simulated clone-22 (TSC-22) as a target...
gene of both pathways in intestinal epithelial cells. Functional studies with wild-type and dominant negative forms of TSC-22 suggest that TSC-22 is an important downstream component of PPARγ and TGF-β signaling during intestinal epithelial cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**

The MOSER S (M-S) (21) and MOSER R (M-R) (22) colon carcinoma lines were a gift of M. Brattain (University of Texas Health Sciences, San Antonio, TX). The FET cell line was a gift of W. Grady (Vanderbilt University, Nashville, TN) and the CBS cell line was a gift of H. Moses (Vanderbilt University). COS7 cells were purchased from ATCC (Rockville, MD). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 0.1% bovine serum albumin, 0.1% Me2SO, 1μM TGFs, and 1μM TGF-β3 Pathways Software (Research Genetics Inc., Huntsville, AL) to identify studies with wild-type and dominant negative forms of TSC-22 gene of both pathways in intestinal epithelial cells. Functional total RNA (20μg) was fractionated on a 1.2% agarose-formaldehyde gel and electrophoretically transferred back and the cells were allowed to grow for another 72 h.

**Northern Hybridization Analysis**

Northern blot analysis was performed as described previously (23). Although representative Northern blot images are shown, all Northern blot experiments were repeated a minimum of two times. Briefly, total RNA (20 μg) was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences). Filters were prehybridized for 4 h at 42 °C in Ultrahyb (Ambion, Austin, TX). Hybridization was conducted in the same buffer in the presence of 32P radiolabeled cDNA of a partial fragment of human TSC-22. Blots were washed 4× for 15 min at 50 °C in 2× SSC, 0.1% SDS and once for 30 min in 1× SSC, 0.1% SDS. Membranes were then exposed to a PhosphorImager screen and images of mRNA intensities for Fig. 4 (A–D) were done using Optiquant Software (Hewlett-Packard).

**Western Blot Analysis**

Exponentially growing cells were harvested in ice-cold 1× phosphate-buffered saline and cell pellets were lysed in RIPA buffer. Centrifuged lysates (50 μg) from each cell line were fractionated on a 4–20% gradient SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% powdered milk. The primary antibody was then added and incubated at room temperature for 2 h or overnight at 4 °C. The following primary antibodies were used: monoclonal anti-hemagglutinin (HA) antibody clone HA.11 (1:1000; Babco, Richmond, CA), monoclonal anti-FLAG antibody clone M2 (1:500; Sigma), monoclonal anti-keratin 20 antibody clone K-20 (1:1000; Roche Molecular Biosciences, Indianapolis, IN) at a lipid:DNA ratio of 3.5:1 and with a DNA concentration of 1.5 μg/ml in Opti-MEM (Invitrogen). 6 h post-transfection, regular media was added back and the cells were allowed to grow for another 48 h prior to harvest.

**Immunoprecipitations**

Anti-HA affinity matrix (100 μl; Roche Molecular Biosciences) was added to 500 μg of COST cell lysate and the cells were incubated in NET-N buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, and 10% glycerol) at 4 °C for 4 h. Beads were then washed 3× with NET-N buffer. Protein was eluted by the addition of 1× SDS loading buffer followed by a 5-min incubation at 100 °C.

**Stable Cell Lines**

Four different vectors were utilized to generate eight unique pools of stably transfected cells: 1) pcDNA3.1Zeo (pools designated as M-S VEC clones A and B); 2) pcDNA3.1Zeo (M-S SVEC DN clones A and B); 3) pcDNA3.1Zeo (M-S TSC DN clones A and B); and 4) pcDNA3.1Zeo (M-S TSC DCON clones A and B).

**Transfection—**M-S cells were transfected with the appropriate vector using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) at a lipid:DNA ratio of 3:1 and a DNA concentration of 5 μg/ml in Opti-MEM. After 6 h of transfection, normal media was added back and the cells were allowed to grow for another 72 h.

**Selection—**Cells were then split into media containing 200 μg/ml zeocin (Invitrogen, La Jolla, CA) and selected for 2 weeks. Resistant clones were combined into two unique pools and expanded. Zeocin was maintained in the media prior to all experiments at a dose of 100 μg/ml.

**Cell Growth Measurements**

Cells were plated at a density of 5 × 10^4 and replaced the next day with Dulbeccco’s modified Eagle’s medium containing 10% charcoal-stripped fetal bovine serum and rosiglitazone or TGF-β. Cells were counted at the indicated times using a Coulter counter. Each experiment was done in triplicate.

**In Situ Hybridization**

In situ hybridization was performed as described previously (24). Sense or antisense 35S-labeled cRNA probes were generated from human TSC-22. The probes had specific activities at 2 × 10^6 disintegrations per minute (dpm/μg). Sections hybridized with the sense probes did not exhibit any positive autoradiographic signals and served as negative controls.
RESULTS

Cellular Response of Colorectal Cancer Cells to PPARγ and TGF-β1—We noticed a striking similarity in the cellular response to either TGF-β1 or PPARγ ligands in colorectal cancer cells with intact PPARγ and TGF-β1 signaling pathways. For example, in the M-S, CBS, and FET colon carcinoma lines, exposure to the high affinity PPARγ ligand rosiglitazone (25) or TGF-β1 results in accumulation of cells in the G1 phase of the cell cycle (data not shown) and a decrease in cell growth (Fig. 1A). Moreover, in all three cell lines, addition of either agent results in an increase in the p21 (Fig. 1B) and keratin 20 (data not shown). Elevated levels of the cyclin-dependent kinase inhibitor p21 (26, 27) and keratin 20 (28) are associated with intestinal epithelial differentiation in various model systems and p21 is a well characterized TGF-β-regulated gene (29).

Because both PPARγ and TGF-β1 regulate such a wide spectrum of physiological processes, the genomic response to either pathway is complex. This makes it difficult to identify those target genes that play functionally important roles in cell growth and differentiation. These data led us to hypothesize that one way to identify relevant target genes of PPARγ and TGF-β1 in the setting of colon epithelial cell growth and differentiation is to focus on the common subset of downstream target genes regulated by both pathways.

Identification of TSC-22 as a PPARγ and TGF-β1 Target Gene in Colon Epithelial Cells—Microarray analysis was utilized to determine the genomic response of one of these lines, the M-S cells, after exposure to rosiglitazone or TGF-β1 for 12 h. A subset of genes was commonly regulated by either pathway, including several members of the keratin and carcinoembryonic antigen superfamilies. However, time course experiments suggested that these genes were not likely to be direct

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**Fig. 1.** The PPARγ ligand rosiglitazone or TGF-β1 induces growth inhibition and increases in protein levels of p21 in a panel of colon epithelial cell lines. A, M-S, FET, or CBS cell lines were treated with vehicle (0.1% Me2SO + 0.1% bovine serum albumin), 1 μM rosiglitazone, or 2 ng/ml TGF-β1 and cells were counted at days 2, 4, and 6 post-treatment. Each data point represents the mean of two independent experiments, each done in triplicate. Error bars = S.E. B, M-S, FET, and CBS cell lines were treated with vehicle, 1 μM rosiglitazone, or 2 ng/ml TGF-β1 for 4 days after which total protein lysates were probed for levels of p21 by immunoblot.

**Fig. 2.** TSC-22 is a downstream target of both PPARγ and TGF-β1 in colon epithelial cells. M-S, FET, and CBS cell lines were treated with vehicle, 1 μM rosiglitazone, or 2 ng/ml TGF-β1 for 12 h after which total RNA was collected and probed for TSC-22 levels by Northern blot (20 μg RNA/lane).
targets of PPARγ or TGF-β and may represent end points (rather than effectors) of the differentiation process. One promising candidate rapidly induced by either treatment was TSC-22, a gene originally identified as a TGF-β1-stimulated gene in osteoblast cells (30). Northern blot analysis confirmed that either PPARγ or TGF-β1 could induce TSC-22 in the M-S, CBS, and FET cell lines (Fig. 2). We further became interested in studying this gene when in situ hybridization of TSC-22 in the normal colon demonstrated that its expression was enriched in the postmitotic epithelial compartment of the normal human colon, where the most differentiated enterocytes reside (Fig. 3).

**Transcriptional Regulation of TSC-22 by PPARγ and TGF-β1**—The M-S cells were chosen as a system to study TSC-22 regulation by PPARγ or TGF-β1 and to test the hypothesis that TSC-22 is a regulator of intestinal epithelial differentiation. The induction of TSC-22 by either PPARγ or TGF-β1 was both time- and dose-dependent (Fig. 4, A–D). Following treatment with either rosiglitazone or TGF-β1, anti-TSC-22 antiserum detected an appropriately sized polypeptide, which was absent in untreated cells (Fig. 4E). We conclude that TSC-22 protein levels increase as well. TSC-22 has been shown to be a direct target gene of TGF-β1 (30). We were interested in determining whether it is also a target for PPARγ as well. Cells were pretreated with the protein synthesis inhibitor cycloheximide or the RNA polymerase II inhibitor DRB followed by rosiglitazone. DRB co-treatment blocked the ability of PPARγ to induce TSC-22 suggesting that the ability of PPARγ to increase steady-state RNA levels of the gene was dependent on de novo transcription rather than through an increase in mRNA stability (Fig. 5). Furthermore, whereas cycloheximide slightly induced TSC-22, co-treatment of rosiglitazone and cycloheximide led to a superinduction (Fig. 5), suggesting that de novo protein synthesis was not required for PPARγ to induce TSC-22. This result suggested that PPARγ was not inducing TSC-22 by first increasing the levels of TGF-β1 and in fact rosiglitazone was not able to induce TGF-β1 in the parental M-S cells (data not shown).

As TSC-22 has not previously been shown to be a target of PPARs, we wanted to determine the specificity and selectivity of this induction. Cells were treated with two structurally distinct PPARγ ligands (the thiazolidinedione-based agonist rosiglitazone or the tyrosine-based agonist GW7845 (31)), an irreversible PPARγ antagonist (GW9662 (32)), or rosiglitazone plus GW9662. TSC-22 was induced by both PPARγ agonists and the induction by rosiglitazone could be blocked by co-treatment with the PPARγ antagonist (Fig. 6). Cells were also
domains within TSC-22 (Fig. 8A). Finally, they were able to demonstrate that titration of a 22, RD 1 and 2, which were in large part responsible for this DNA-binding domain and identified two domains within TSC-22, RD 1 and 2, which were in large part responsible for this transcriptional repressor activity when fused to a heterologous leucine zipper domain. They also showed that TSC-22 has demonstrated that TSC-22 could form homodimers via the transferase pull-down and mammalian two-hybrid assays, they mine whether expression of TSC-22 in the M-S cells could recapitulate any of the phenotypic changes induced by PPAR (LG100268), or a combination of rosiglitazone and LG100268. Despite the fact that M-S cells express both PPARα and PPARδ (data not shown), neither of these other two PPAR isoforms was able to regulate TSC-22 expression (Fig. 6). The Ability of PPARγ to Induce TSC-22 Is Independent of TGF-β1—To further clarify the issue of whether PPARγ signaling in the colon was dependent on the TGF-β pathway, the M-R cell line was utilized. The M-R line is a subclone of the parental M-S cells that are relatively refractory to the growth inhibitory effects of TGF-β (22). PPARγ, but not TGF-β1, could induce expression of TSC-22 in the M-R cells (Fig. 7A). Finally, activators of PPARγ inhibited the growth of either cell line by equivalent amounts, whereas the M-R cells (as has been previously reported) were relatively resistant to TGF-β1 induced growth inhibition (Fig. 7B).

Overexpression of Wild-type TSC-22 Inhibits Colon Epithelial Cell Growth and Induces Elevated Levels of p21—The functional role of TSC-22 in mediating any of the biological effects induced by either PPARγ or TGF-β is unknown. To address this issue we focused on two experimental strategies: 1) to determine whether expression of TSC-22 in the M-S cells could recapitulate any of the phenotypic changes induced by PPARγ or TGF-β1, and 2) to determine whether inhibition of normal TSC-22 function blocks the phenotypic changes induced by either treatment. Kester et al. (35) have defined 4 functional domains within TSC-22 (Fig. 8A). Using both glutathione S-transferase pull-down and mammalian two-hybrid assays, they demonstrated that TSC-22 could form homodimers via the leucine zipper domain. They also showed that TSC-22 has transcriptional repressor activity when fused to a heterologous DNA-binding domain and identified two domains within TSC-22, RD 1 and 2, which were in large part responsible for this effect. Finally, they were able to demonstrate that titration of a mutant TSC-22 in which both RD 1 and 2 were deleted could inhibit the repressor activity of the wild-type protein and thus act as a dominant-negative (dn) inhibitor. Based on these results, we constructed three different HA-tagged TSC-22 constructs for stable introduction of this gene into the M-S cells: TSC-22 wt, TSC-22dn (in which both RD 1 and 2 were deleted), and TSC-22dncon (which, in addition to both RD domains being treated with a PPARα selective ligand (GW7647 (33)), a dual PPARα/δ ligand (GW2433 (34)), an RXR specific ligand (LG100268), or a combination of rosiglitazone and LG100268. Despite the fact that M-S cells express both PPARα and PPARδ (data not shown), neither of these other two PPAR isoforms was able to regulate TSC-22 expression (Fig. 6). The induction of TSC-22 by PPARγ is not dependent on an intact TGF-β1 signaling pathway. A, M-R cells (a naturally identified TGF-β1 resistant clone) were treated with 1 μM rosiglitazone or 2 ng/ml TGF-β1 for 12 h. TSC-22 mRNA levels was detected by Northern blotting (20 μg of total RNA/lane). B, M-S and M-R cells were treated with vehicle (0.1% Me2SO) or 2 ng/ml TGF-β1 for 6 days after which time the number of viable cells were counted. Values are expressed as percent of vehicle-treated cells. Each experiment was performed in triplicate and the values represent the mean of two independent experiments. Error bars = S.E.
deleted, contains mutations in two highly conserved leucine residues within the leucine zipper domain (Fig. 8A). Co-immunoprecipitation experiments confirmed that TSC-22dn, but not TSC-22dncon, could dimerize with TSC-22 wt (Fig. 8B). This last construct was made to help properly interpret any potential artifacts because of expression of TSC-22dn that were independent of its ability to dimerize and inhibit the function of the wild-type protein.

Each of these three constructs (plus empty vector) was used to generate eight unique pools of stably transfected cells. Each of the pooled cell lines was found to express relatively equivalent protein levels of the integrated cDNA (Fig. 8C and D). The two different pools of M-S cells expressing TSC-22 wt were found to have significantly reduced growth rates compared with vector-transfected cells (Fig. 9A). They also displayed higher levels of p21 protein but showed no difference in the levels of keratin 20 protein (Fig. 9B).

**Overexpression of Dominant Negative TSC-22 Partially Inhibits PPARγ Ligand and TGF-β1-induced Growth Inhibition and p21 Induction**—We next tested the ability of TSC-22dn and TSC-22dncon to block the ability of PPARγ or TGF-β1 to inhibit the growth of M-S cells. In the two different pools of cells expressing TSC-22dn, the ability of PPARγ or TGF-β1 to inhibit growth was reduced by ~60% (Fig. 10A). Importantly, no differences in the inhibitory activity of either PPARγ or TGF-β1 were seen when comparing vector and TSC-22dncon-transfected cells (Fig. 10A). Northern blot analysis confirmed that PPARγ and TGF-β1 could still induce TSC-22 in these cell lines (data not shown). Finally, the ability of rosiglitazone or TGF-β1 to induce p21, but not keratin 20, was greatly diminished in M-S cells expressing TSC-22dn but not TSC-22dncon (Fig. 10B).

**DISCUSSION**

The complex mechanisms by which the undifferentiated stem cells of the intestine give rise to differentiated cells with specialized functions remain incompletely understood. Some of the key pathways that govern this process in the intestine have been identified from understanding genetic lesions found in colorectal cancer (36). For example, a majority of human colorectal cancers contain a loss of function mutation in the tumor suppressor gene adenomatous polyposis coli (37). Subsequent studies have suggested a critical role for this gene in exerting strict growth controls such that the renewing population of intestinal epithelial cells is kept at a constant number. Likewise, loss of function mutations have been identified in genes involved in the TGF-β signaling pathway and PPARγ. Further studies have shown that activation of either of these pathways in cell culture models can lead to growth inhibition and the induction of markers of differentiation. These experiments emphasize the biological relevance of these two pathways in intestinal epithelial cell biology. In this study, we used microarray technology to identify TSC-22 as a target gene of both PPARγ and TGF-β1 in intestinal epithelial cells. We further show using wild-type and dominant negative forms of TSC-22 that this gene plays an important role in the ability of either PPARγ or TGF-β1 to inhibit cell growth.

There are several reasons why the results presented here are likely to be biologically relevant. First of all, TSC-22 is localized to the most differentiated epithelia in the normal human colon.
PPARγ and TGF-β Pathways Inhibit Intestinal Cell Growth

TSC-22 is highly conserved during evolution, with the human protein sequence being 98.5% identical to the mouse and rat proteins (40). TSC-22 has been shown to have a solution structure similar to members of the bZIP family of transcription factors (41). Similar to bZIP proteins, TSC-22 contains a leucine zipper domain and, analogous to the basic domain in bZIP family members, has a highly conserved sequence known as the TSC domain or box. In addition, TSC-22 is also homologous to the Drosophila melanogaster gene shortsighted (shs) or bunched, which is required for normal development of oocytes, larval peripheral nervous system, and adult eye and wing (42–44). Recent evidence also implicates an important role for TSC-22 in the developing feather tract (45). There is also evidence suggesting that TSC-22 can negatively regulate the growth of cancer cells. For example, antisense expression of TSC-22 in a salivary gland carcinoma line inhibits the growth of these cells in vivo (46) and overexpression of TSC-22 appears to sensitize carcinoma cells to certain anti-cancer chemotherapeutic drugs (47).

Evidence to date suggests that TSC-22 is part of a family of proteins that act as transcriptional repressors. As mentioned earlier, TSC-22 has been shown to homodimerize and exhibit transcriptional repressor activity when fused to a heterologous DNA-binding domain (35). In fact, our results with the TSC-22Δn protein in which both repressor domains were deleted provide functional evidence in a biological system for the importance of these domains. This notion is further supported by the finding that the TSC-22 Drosophila homolog, bunched, is a powerful repressor of the enhancer trap reporter A359 (44). It is also possible that TSC-22 may modify gene expression through protein-protein interactions. Recently, a TSC-22 homologue (termed THG-1) was cloned and found capable of forming heterodimers with TSC-22 (35). (THG-1 was not found to be expressed in the M-S cells.) Exactly how either a TSC-22 homodimer complex or a TSC-22-THG-1 heterodimer complex can regulate the expression of a particular target gene is unknown.

One possible target gene of TSC-22 may be p21. However, at this point it is unclear whether TSC-22 increases p21 levels directly or indirectly. Although our work provides evidence that TSC-22 is involved in the signaling pathway by which both PPARγ and TGF-β induce p21, there are other mechanisms by which activation of either pathway may modulate p21 expression. For example, TGF-β has been shown to activate p21 proximal promoter activity via two consensus Sp1-binding sites (48, 49). How (or if) TSC-22 may act to enhance basal Sp1 transcriptional activity is unknown. Clearly, a major goal of future work will be to identify genes that are directly regulated by TSC-22 and determine the mechanism(s) by which TSC-22 modulates the expression of these genes. Determining TSC-22 target genes in the intestine will lead to much better understanding of the exact role of this protein during colon epithelial differentiation.

In summary, the data reported here point to a previously unidentified role for the putative transcriptional repressor TSC-22 as a regulator of intestinal epithelial cell differentiation. A large percentage of advanced colorectal tumors lose their responsiveness to growth inhibition induced by TGF-β. It will be of interest to determine what percentage of these colorectal tumors also contain mutations in PPARγ. If this is not found to be a common occurrence, then our present studies predict that treatment with activators of PPARγ may provide a way to bypass the loss of normal TGF-β signaling that occurs during the progression of these tumors.

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![Figure 10: Dominant negative TSC-22 blocks the ability of PPARγ or TGF-β to induce p21 and inhibit cell growth.](image-url)
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