Transforming Growth Factor-β Promotes Inactivation of Extracellular Thyroid Hormones via Transcriptional Stimulation of Type 3 Iodothyronine Deiodinase

Stephen A. Huang, Michelle A. Mulcahey, Alessandra Crescenzi, Mirra Chung, Brian W. Kim, Carmen Barnes, Wichert Kuijt, Helen Turano, John Harney, and P. Reed Larsen

Thyroid hormone is a critical mediator of cellular metabolism and differentiation. Precise tissue-specific regulation of the concentration of the active ligand, T₃, is achieved by iodothyronine monodeiodination. Type 3 iodothyronine deiodinase (D3) is the major inactivating pathway, preventing activation of the prohormone T₄ and terminating the action of T₃. Using nontransformed human cells, we show that TGF-β stimulates transcription of the hDio3 gene via a Smad-dependent pathway. Combinations of Smad2 or Smad3 with Smad4 stimulate hDio3 gene transcription only in cells that express endogenous D3 activity, indicating that Smads are necessary but not sufficient for D3 induction. TGF-β induces endogenous D3 in diverse human cell types, including fetal and adult fibroblasts from several tissues, hemangioma cells, fetal epithelia, and skeletal muscle myoblasts. Maximum stimulation of D3 by TGF-β also requires MAPK and is synergistic with phorbol ester and several mitogens known to signal through transmembrane receptor tyrosine kinases but not with estradiol. These data reveal a previously unrecognized interaction between two pluripotent systems, TGF-β and thyroid hormone, both of which have major roles in the regulation of cell growth and differentiation.

First Published Online July 21, 2005

Abbreviations: aFGF, Acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; D1–D3, types 1–3 iodothyronine deiodinase; E2, estradiol-17β; EGF, epidermal growth factor; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSMM, human skeletal muscle myoblasts; R-Smad, receptor-associated Smad; TSS, transcription start site.

Molecular Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
dependent proliferation and differentiation in an anatomically and temporally precise manner. During *Xenopus laevis* metamorphosis, thyroid hormone promotes cell proliferation and axon projection in the ventral ciliary marginal zone of the retina. These same thyroid hormone-dependent events are prevented in the dorsal ciliary marginal zone by the local expression of D3, permitting asymmetric retinal growth and the normal transition to binocular vision (8). In fact, the entire tadpole metamorphic process is triggered and finely regulated by combinations of T₄ activation by D2 and T₃, inactivation by D3 in various tissue microenvironments (9, 10). Highly regulated D3 expression has also been documented in human fetal liver and brain, implying a similar role in primate development (11, 12).

Despite these data implying the programmed regulation of D3 expression, little is known of the molecular mechanisms that control this. Due to the paucity of D3-expressing human cell lines, data regarding D3 regulation has derived mostly from the study of rodent cells or immortalized cell lines (1, 13). In these models, D3 is up-regulated by thyroid hormone, retinoids, serum, 12-O-tetradecanoylphorbol 13-acetate (TPA), epidermal growth factor (EGF), acidic (aFGF) and basic (bFGF) fibroblast growth factor (14–18). In many cases, the molecular mechanism for these effects is not defined nor is it understood how D3 might be induced during conditions such as embryonic implantation, tumorigenesis, or ischemia. We used primary cultures of human fibroblasts in the present study and, to our knowledge, these experiments are the first to employ nontransformed human cells which retain not only basal D3 activity but also the capacity for D3 regulation. TGF-β is found in several human tissues known to express D3, including the fetal epithelium, gliomas, vascular anomalies, and the uterine endometrium (2, 19–26). Our data show that the Dio3 gene is transcriptionally induced by TGF-β via a Smad and MAPK-dependent pathway. Regulation by TGF-β can synergize with, or be modulated by, the action of other ligands such as TPA, EGF, and the fibroblast growth factors, suggesting the potential to be both constitutive and locally regulated.

### RESULTS

**TGF-β Stimulates D3 in Fibroblasts from Diverse Developmental Stages and Anatomic Sites**

With the goal of identifying D3-expressing human cells, we screened primary collagenase-dispersed cultures of placental cells (27). Those expressing basal D3 activity were fibroblasts by morphology and immunocytochemistry in that they expressed CD90 (thy-1) and were negative for endothelial and muscle markers including vWF, P1H12, and smooth muscle actin. Endogenous D3 activity in human placental fibroblasts increased after a 48-h incubation with 5 ng/ml TGF-β1 and 10⁻⁷ M TPA (Table 1). We then examined a series of commercially available human fibroblasts from other tissues. Those from fetal pulmonary tissue expressed the highest basal D3 activity. After a 24-h incubation with 10 ng/ml TGF-β1, 10⁻⁷ M TPA, and 2 ng/ml bFGF, D3 activity in fibroblasts from fetal lung, fetal skin, and adult uterine endometrium was comparable to that of term human placental tissue sonicate (Table 1).

In subsequent experiments, we focused on the fetal lung fibroblast AG04526 to understand the mechanisms for this potent D3 induction. Both TGF-β1 and phorbol ester (TPA) increased D3 activity and their effects were synergistic (Fig. 1A). In contrast, EGF and the fibroblast growth factors induced no significant D3 activity alone but greatly amplified the response to TGF-β1 with stimulated activities approximating the high levels found in infantile hemangiomas (Fig. 1A) (3, 4). To focus on the mechanism of D3 stimulation by TGF-β, subsequent experiments were performed in simplified media (MEM 10% fetal bovine serum (FBS)) without other growth factors. In lung fibroblasts, all three TGF-β isoforms induced endogenous D3 activity (Fig. 1B). When T₃ was added to BSA-containing media to a final free concentration of 13 pm (about three times that in euthyroid human serum), 5 ng/ml TGF-β1 increased inner-ring deiodination of T₃ from 6.4% ± 0.4% to 71.7% ± 0.1% over 24 h. Iopanoic acid, an inhibitor of D3-mediated deiodination, blocked this (Fig. 1, C–E) (28). This confirms that the endogenous D3 activity induced by TGF-β1 in fibroblasts is sufficient to rapidly degrade extracellular T₃. Inner-ring

### Table 1. Inducible D3 Expression in Primary Fibroblasts from Fetal and Adult Human Tissues

<table>
<thead>
<tr>
<th>Cell Origin</th>
<th>D3 Activity (fmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Placental fibroblasts</td>
<td>Term placenta</td>
</tr>
<tr>
<td>2. AG02101</td>
<td>Endometrium of 27-yr female</td>
</tr>
<tr>
<td>3. AG04525</td>
<td>Skin of 17-wk male fetus</td>
</tr>
<tr>
<td>4. AG04526</td>
<td>Lung of 17-wk male fetus</td>
</tr>
</tbody>
</table>

Placental fibroblasts (cell 1) were propagated in EGM-2MV endothelial media 10% FBS supplemented with 2 ng/ml bFGF and then treated with 5 ng/ml TGF-β1 and 10⁻⁷ M TPA for 48 h. Other fibroblast cultures (cells 2–4) were grown in MEM 10% FBS and then exposed to EGM-2MV containing 10% FBS, 2 ng/ml bFGF, 10 ng/ml TGF-β1, and 10⁻⁷ M TPA for 24 h.
Deiodinase activity in lung fibroblasts was confirmed to be authentic D3 by its resistance to propylthiouracil and a nanomolar Michaelis constant (Km) for T3 (1.0 nM T3) (Fig. 1F). No significant D1 or D2 activity was present either in the basal state or after TGF-β1 exposure [undetectable D1 and maximal velocity (Vmax) < 0.01 fmol T4/min-mg for D2].

**D3 Stimulation by TGF-β Is Transcriptional and Rapid, but Transient**

D3 activity in lung fibroblasts was increased 31-fold by TGF-β1 stimulation, reaching 73 fmol/min at 10 h (Fig. 1G). Despite the continued presence of TGF-β1, D3 activity decreased to one third of this peak value by 24 h. D3 mRNA was detectable by Northern blot within 5 h after TGF-β1 exposure and peaked at 10 h but returned to undetectable levels by 24 h (Fig. 1G). In all Northern blot experiments, the D3 cDNA probe hybridized to a single RNA band of approximately 2.3 kb that comigrated with D3 mRNA from placenta. To determine whether this dynamic excursion was due to the cessation of Dio3 gene transcription, actinomycin D was added 10 h after exposure to TGF-β1 and cells harvested for RNA 3 h later. The D3:GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA ratio at 13 h decreased by 55% and 67%, respectively, in actinomycin D- and control-treated cells, indicating the termination of hDio3 gene transcription by 10 h and a short mRNA half-life (Fig. 1H). Pulse-chase analysis of transiently expressed human D3 has documented a protein half-life of approximately 12 h, ex-
plaining the persistence of activity despite the rapid disappearance of D3 mRNA (29).

**Smad Proteins Transactivate the hDio3 Promoter**

To confirm that TGF-β stimulates Dio3 gene transcription, lung fibroblasts were transfected with the hDio3 promoter-reporter construct 4.3hDio3-luc, containing 4327 bp of the 5′-flanking region plus 224 bp of the 5′-untranslated region (28). 3TP-Lux was used as a positive control for TGF-β responsiveness (30). Exogenous TGF-β1 stimulated 4.3hDio3-luc 4.7-fold at 16 h compared with 1.9-fold for 3TP-Lux. To determine whether the major TGF-β second messengers, the Smad proteins, were required for this effect, dominant-negative Smads were coexpressed and reduced or prevented the increase in luciferase activity in both 4.3hDio3-luc and 3TP-Lux (Fig. 2A). Cotransfection of either receptor-associated Smad (R-Smad), Smad2, or Smad3, alone produced up to a 6.3-fold induction of 4.3hDio3-luc. When combined with the common Smad4, they induced a 25- or 61-fold transactivation of 4.3hDio3-luc (Fig. 2B).

To localize the sequences required for Smad responsiveness, we transfected a series of hDio3 promoter-reporter constructs containing variable lengths (1–4327 bp) of the hDio3 5′-flanking region and the entire 5′-untranslated region. Responsiveness to Smad2+4 cotransfection localized to sequences −160 to −360 bp relative to the transcription start site (TSS) (Fig. 2C). Transcription Element Search System analysis of this 200-bp hDio3 sequence showed that the consensus Smad binding element, CAGAC, was not present, but six Sp1 binding elements were identified (Fig. 2D) (31). An additional construct containing hDio3 sequences +134 to +224 bp relative to the TSS was used as a control and expressed low luciferase levels with or without Smad cotransfection. Smad coexpression also stimulated 3TP-Lux in transfected lung fibroblasts, but 2- to 3-fold less than 4.3hDio3-luc (Table 2). This pattern was also observed in the D3-expressing NCLP6E monkey hepatocarcinoma line, but not in HepG2 cells or the choriocarcinoma JEG-3 cells that lack endogenous D3 activity (Table 2). This indicates that Smads are necessary but not sufficient for hDio3 stimulation by TGF-β.

**TGF-β Stimulation Synergizes with Other Kinase Pathways**

Like TGF-β1, TPA increased D3 activity and mRNA in lung fibroblasts (Fig. 1A and Northern blot data not...
shown). For both TGF-β1 and TPA, the transcriptional induction of D3 decreased after 10 h despite the continued presence of ligand. To determine whether this occurred at the level of the signaling pathway or the Dio3 gene itself, fibroblasts were sequentially exposed to TGF-β1 and/or TPA (Fig. 3, A and B). These experiments demonstrated that the resistance to induction was ligand specific, not due to a repressed state of the Dio3 gene. It also confirmed that the actions of TGF-β1 and TPA on hDio3 occur by distinct mechanisms. Because Smad activity has been shown to also be regulated by kinase pathways, pharmacologic kinase inhibitors were tested for the ability to block D3 induction by TGF-β. TGF-β stimulation of D3 activity in fetal lung fibroblasts was attenuated by specific inhibitors of both the p38 (SB203580) and ERK (U0126)

### Table 2. Correlation of Smad Induction of 4.3hDio3-luc and 3TP-Lux in Various Cell Types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Endogenous D3</th>
<th>4.3hDio3-luc Smad2+4</th>
<th>4.3hDio3-luc Smad3+4</th>
<th>3TP-Lux Smad2+4</th>
<th>3TP-Lux Smad3+4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal lung fibroblasts</td>
<td>+</td>
<td>108</td>
<td>33</td>
<td>62</td>
<td>12</td>
</tr>
<tr>
<td>NCLP6E (hepatocarcinoma)</td>
<td>+</td>
<td>242</td>
<td>33</td>
<td>43</td>
<td>12</td>
</tr>
<tr>
<td>HepG2 (hepatoma)</td>
<td>–</td>
<td>1</td>
<td>2</td>
<td>14</td>
<td>83</td>
</tr>
<tr>
<td>JEG-3 (choriocarcinoma)</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

The cell types listed above were cotransfected with plasmids encoding wild-type Smads and either 4.3hDio3-luc or 3TP-Lux.

**Fig. 3.** TGF-β1 Dio3 Induction and Other Protein Kinase Signaling Pathways

A and B, Lung fibroblasts were exposed to two stimuli over a 96-h period, at 0 and 48 h. Prior exposure to either TGF-β1 (A) or TPA (B) resulted in an attenuated second response to the same ligand but full induction with the alternate stimulator. C, Induction of D3 activity by TGF-β1 was blocked by inhibitors of ERK MAPK (U0126) and p38 MAPK (SB203580). D, In lung fibroblasts transiently expressing ERα, E2 stimulated 4.3hDio3-luc 10.7-fold. Dominant-negative Smad3 or Smad4 cotransfection decreased 4.3hDio3-luc induction but had no effect on E2 stimulation of the ERETK-luc control.
The p38 MAPK inhibitor SB203580 also blocked induction of D3 mRNA by TGF-β1, suggesting that this is a transcriptional effect which was confirmed by its inhibition of 4.3hDio3-luc reporter stimulation by Smad cotransfection (data not shown). Inhibitors of ERK have been shown to block the increase in D3 mRNA induced by either TPA or by fibroblast growth factors, suggesting that a similar mechanism is operative for U0126 (32).

Estradiol Stimulates hDio3 Transcription

Thyroid hormone requirements increase during pregnancy coincident with the rise in serum estradiol, and estradiol replacement alone also causes a persistent increase in levothyroxine requirements (33, 34). Because D3 is expressed in the uterine epithelium as well as in the placenta, these results suggest that increased iodothyronine inactivation by estradiol-stimulated D3 may contribute to these phenomena (2). To determine whether estrogen can stimulate Dio3 gene transcription, lung fibroblasts were transfected with estrogen receptor (ER) α and 4.3hDio3-luc. ERETK-luc was used as a control for estrogen responsiveness. Overnight exposure to 10⁻¹⁰ M estradiol-17β (E2) produced a 10.8-fold increase in luciferase activity compared with a 3.3-fold increase in ERETK-luc (Fig. 3D). Estradiol induction of 4.3hDio3-luc but not ERETK-luc was attenuated by dominant-negative Smads, indicating that human D3 is transcriptionally responsive to estradiol and that Smad activation is required for maximal hDio3 stimulation in these cells. No increase in Dio3 gene transcription occurred in the absence of ERα expression. However, unlike with TPA or the fibroblasts growth factors, no synergism was found between TGF-β and E2 (data not shown).

TGF-β1 Induces Endogenous D3 in Hemangioma Cells, Fetal Epithelia, and Human Skeletal Myocytes

To determine whether TGF-β stimulation was implicated in other human tissues known to express D3, additional cell types were examined. The D3 activity in large infantile hemangiomas can cause consumptive hypothyroidism, and TGF-β message has recently been detected in infantile hemangiomas and other vascular lesions (25, 26). Accordingly, dispersed cells prepared from the cutaneous hemangiomas of two infants were cultured and exposed to TGF-β1. This increased D3 activity 3.8- or 7.6-fold (Fig. 4A). Immunohistochemical studies have shown high D3 expression in the epithelial surfaces of the human fetus (2). Because TGF-β is the prototypical inducer of epithelial-mesenchymal transition during embryonic development, fetal epithelia derived from amniocentesis

---

**Fig. 4.** TGF-β Induces Endogenous D3 Activity in Infantile Hemangioma Cells, Fetal Epithelial Cells, and Skeletal Myoblasts

A, Dispersed hemangioma cells from two patients were stimulated with TGF-β1. Due to the limited number of cells from each tumor, responsiveness to TPA was not tested. B, Each column represents an individual amniocentesis sample stimulated with TGF-β1 and/or TPA. C, HSMM increase D3 expression in response to TGF-β1 and/or TPA.
were treated and found to express D3 after stimulation with TGF-β1 and/or TPA (Fig. 4B) (2, 21, 35).

As mentioned, D3 is expressed in the ischemic skeletal muscle of patients who died in an intensive care unit. Because TGF-β mediates the response of muscle to injury and is also a downstream target of hypoxia-inducible-factor 1, human skeletal muscle myoblasts (HSMM) from adult donors were analyzed (36, 37). Similar to the pattern observed in lung fibroblasts and fetal epithelia, TGF-β1 and TPA stimulated D3 activity synergistically (Fig. 4C).

**DISCUSSION**

Thyroid hormone controls critical developmental and metabolic events in vertebrates, and the specific monodeiodination of iodothyronine molecules regulates thyroid status both systemically and at the tissue-specific level. One of the goals of our studies has been to identify factors which regulate human D3. TGF-β is found in several D3-expressing tissues and was shown here to induce endogenous D3 in diverse human cell types, including fetal and adult fibroblasts, hemangioma cells, fetal epithelia, and skeletal muscle myoblasts (Table 1 and Figs. 1 and 4) (2, 19–26). During embryonic development, secondary epithelia transdifferentiates into mature epithelia or, under the influence of TGF-β and other paracrine factors, undergoes epithelial-mesenchymal transition to produce the various cells types of connective tissue (including astrocytes, adipocytes, muscle cells, and fibroblasts) (21). Viewed in this context, our results suggest that D3 expression in fetal epithelia can be retained through the process of epithelial-mesenchymal transition or reactivated after terminal differentiation by the action of TGF-β. The fibroblast D3 activity induced by TGF-β is robust and exceeds the specific activities previously documented in normal human tissues, suggesting a possible role for D3 in inflammation and fibrosis (Table 1 and Fig. 1) (21, 38). Similarly, the stimulated D3 activity in cultured myoblasts exceeds that reported in critically ill patients and further supports the hypothesis that inducible D3 contributes to the low serum T₃ characteristic of the euthyroid sick syndrome (Fig. 4C) (5). Because Smads are expressed in both the implantation site and nonpregnant endometrium, it seems likely that TGF-β can explain D3 expression in these tissues and, together with the novel finding that the hDio3 gene is transcriptionally responsive to estradiol, may contribute to the increased thyroid hormone requirements observed in hypothyroid women during pregnancy and hormone replacement therapy (Fig. 3D) (2, 19, 20, 33, 34). The precise role of D3 in these conditions remains to be defined but, similar to its established role in pregnancy, local hypothyroidism induced by TGF-β could facilitate the expression of oncofetal genes by inhibiting the differentiating effects of thyroid hormone or promote the survival of normal cells during ischemia/inflammation by reducing their metabolic requirements (Fig. 5).

The TGF-β family, like thyroid hormone, can affect many tissues by controlling cell differentiation, migration, growth, and neoplastic transformation through effects on the transcription of its target genes (38–41). The classic TGF-β signaling cascade is a linear pathway involving two cell surface receptor kinases that, when activated, phosphorylate one or more Smad proteins, causing them to enter the nucleus and activate target gene transcription (40–42). Smad activity can also be modulated by several kinase pathways (41). All three TGF-β isoforms increased D3 expression in fibroblasts and this increase was amplified synergistically by phorbol ester and several mitogens (EGF, aFGF, bFGF) known to signal through transmembrane receptor tyrosine kinases (Fig. 1, A and B). Dominant-negative Smad expression inhibited TGF-β1 induction of D3 and R-Smads combined with Smad4 caused massive stimulation of the Dio3 promoter (Fig. 2, A and B). We found that the sequences required for Smad activation of the hDio3 gene lie between –360 and –160 bp relative to the TSS (Fig. 2C). This region does not contain the common canonical Smad binding element (CAGAC), but long GC-rich sequences have been shown to complex with Smads and this region is 84% GC-rich (Fig. 2D) (40, 42, 43).

Interestingly, the effect of TGF-β1 to stimulate Dio3 transcription occurs rapidly (within 5 h) but is short lived with the transcriptional stimulation already terminated within approximately 10 h of exposure (Fig. 1G). The half-life of D3 mRNA is quite short, about 3 h, but D3 activity persists for nearly 48 h after TGF-β1 exposure due to the 12 h half-life of the protein (Fig. 1H). The induction of D3 by TGF-β1 in human fibroblasts was attenuated by specific inhibitors of both ERK (U0126) and p38 MAPK (SB203580), indicating cross talk with both the mitogen-activated (ERK) and stress-activated (p38) branches of the MAPK system (Fig. 3C) (17, 32). Both TGF-β and TPA were capable of stim-
Huang et al. • TGF-β Stimulates Type 3 Deiodinase


ulating D3 message and activity individually, and their combined effect was synergistic (Fig. 1A). That these two pathways for D3 activation are independent is illustrated by the fact that TGF-β and TPA cause isoligand resistance but do not block stimulation of D3 expression by the other ligand (Fig. 3, A and B). This indicates that resistance is not due to repression of the hDio3 gene itself. Interestingly, there was no effect of TPA on even the longest (4.3 kb) 5'-hDio3 flanking region promoter reporter construct. We also found no TPA response of a 3.4-kb rat Dio3 promoter, and others have noted only a 2-fold response to TPA of a 519-bp mDio3 promoter construct (16). The latter study showed that inclusion of a downstream enhancer sequence about 6 kb 3’ to the TSS of the mDio3 gene (and conserved in the human gene) confers a 2-fold TPA induction when linked in cis to a heterologous promoter (16). This could be the explanation for the potent synergistic response to TGF-β and TPA because it has also been shown that R-Smads can also form complexes with the jun-fos heterodimer on genes containing AP-1 response elements (40, 45, 46).

Adding further to the complexity of Dio3 induction by Smads are the data showing that, in the D3-expressing human lung fibroblasts and the monkey hepatocarcinoma cell line NCLP6E, Smads stimulate both the Dio3 promoter and the Smad-responsive 3TP-Lux promoter (Table 2) (18). On the other hand, despite a robust stimulation of 3TP-Lux by Smads in HepG2 cells, Dio3 is virtually unaffected. Similarly, there is little or no stimulation of Dio3 in non-D3-expressing JEG-3 cells despite a 3TP-Lux response to the same stimulus. This indicates that Smad activation is necessary but not sufficient to induce hDio3 transcription in response to TGF-β and that there are other as-yet-identified mechanism or perhaps cell type-specific transcription factors required for Dio3 induction. This is consistent with the mechanism of other known Smad target genes because transcriptional activation via a Smad binding element alone is not feasible due to the low affinity of the Smad MH1 domain (40, 47). High-affinity selective interaction with DNA requires association with other cell type-specific binding proteins, and this may explain a prior report of poor D3 induction after TGF-β1 stimulation in rodent brown adipocytes (15). In addition to whatever this cell type-specific mechanism may be, many TGF-β-responsive genes, including the cyclin-dependent kinase inhibitors p15INK4b and p21, require functional cooperation of Sp1 with Smad proteins for transcriptional activation (31, 48, 49). Interestingly, there are six Sp1 binding sites within the 200-bp hDio3 sequence required for Smad responsiveness (Fig. 2D), consistent with its involvement in TGF-β Dio3 induction.

Because these are the first nontransformed human cells in which Dio3 gene transcription has been studied, there are many aspects of the TGF-β thyroid hormone interrelationship to be explored. These include identification of the DNA-binding partners required for Smad activation of hDio3, with the above data suggesting Sp1 as a likely candidate. Several lines of investigation to study the isoligand D3 resistance observed in human fibroblasts are also suggested by precedents in the TGF-β literature, including the expression of inhibitory Smads and the proteasomal degradation of activated Smads (41). Our results have identified an unexpectedly potent mechanism of D3 stimulation by TGF-β. Because TGF-β is found in several tissues known to express D3, this provides new insight into the pathways of hDio3 regulation. Conversely, it also reveals a novel Smad-dependent mechanism by which stromal cells can modify the thyroid status of their microenvironment and we speculate that the local deiodinative inactivation of thyroid hormone may be an important mechanism by which the effects of TGF-β are generated.

MATERIALS AND METHODS

Materials

Recombinant TGF-β1, 2, and 3 were purchased from R&D Systems (Minneapolis, MN). Phorbol-12-myristate-13-acetate (TPA) was purchased from Alexis Biochemicals (Lausen, Switzerland). EGF, aFGF, and bFGF were purchased from Chemicon International (Temecula, CA). Kinase inhibitors (UO126 and SB203580) were purchased from EMD Biosciences (San Diego, CA). FBS, E2, iopanoic acid, actinomycin D, and sodium selenite were purchased from Sigma-Aldrich (St. Louis, MO). Fibroblast cultures from collagenase-digested human placenta were provided by Dr. Carmen Barnes. Additional primary (finite) fibroblast cultures were obtained from the Coriel Cell Repository (Camden, NJ). The Clonetics Skeletal Muscle Myoblast Cell System normal HSMMs were purchased from Cambrex Corp. (Baltimore, MD). Primary mixed cell cultures from discarded surgical specimens of infantile hemangiomas were a gift from Dr. Joyce Bischoff (Vascular Biology Program, Childrens Hospital Boston). Endothelial cells were removed from the primary cultures using Ulex europeus-l-coated magnetic beads and the unbound fibroblast-like cells were cultured as previously described (50). Amniocytes were acquired from the Brigham and Women’s Hospital clinical cytology laboratory. Studies of human tissues were approved by the Institutional Review Boards of the Brigham and Women’s Hospital and Childrens Hospital Boston.

Reporter and Expression Plasmids

An hDio3 gene reporter, designated 43hDio3 luc, containing 4327 bp of the 5′-flanking region plus 224 bp of the 5′-untranslated region was created from a clone isolated from a human female placental lambda EMBL3 SP6/T7 library subcloned into the pGL3 basic luciferase reporter vector (Promega, Madison, WI) using standard procedures. Additional hDio3 reporter constructs were generated from this parent construct using the Erase-a-base System (Promega, Madison, WI) or standard PCR subcloning techniques and then characterized by sequencing. Smad2, Smad3, and Smad4 in eukaryotic expression vectors in pcDNA were a gift from Dr. Malcolm Whitman (Harvard Medical School, Boston, MA) (51). Dominant-negative C-terminal truncated Smad3 and Smad4 expression vectors were gifts from Dr. Rik Derynck (University of California at San Francisco, San Francisco, CA) (51). 3TP-Lux is a promoter-reporter construct containing...
three tetradecanoxy phorbol acetate response elements and a TGF-β-responsive segment of the human plasminogen activator inhibitor-1 promoter often employed as a positive control for TGF-β responsiveness (30). 3TP-Lux was a gift from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) (30). A PERE-TK-driven luciferase reporter construct (ERETK-luc) and a eukaryotic ERα expression plasmid were gifts from Dr. Myles Brown (Dana Farber Cancer Institute, Boston, MA) (52).

**Tissue Culture**

With the exception of the initial screening experiment (Table 1 legend), fibroblasts were propagated in MEM supplemented with 10% FBS, glutamine, and gentamicin. Primary fibroblast cultures were analyzed at passage 12 or earlier. Amniocentesis cells were grown in MEM with 10% FBS, and cultures from each patient were exposed to drug or vehicle rather than amplified by further passaging. These cells originate from fetal epithelium and retain epithelial markers under the conditions used (35). EGM-2MV endothelial media (Cambrex Corp.) with 10% FBS was used to propagate hemangioma cells and to screen Coriel fibroblasts cultures for inducible D3 activity (Table 1). Cells exposed to E2 were grown in phenol-red-free DMEM supplemented with 10% charcoal-stripped FBS. With the exception of HSMM and hemangioma cultures, all media were purchased from Invitrogen (Carlsbad, CA). Media for all cell types was supplemented with 10⁻⁷ M sodium selenite.

**Deiodination Assays**

Cellular sonicates were prepared for enzyme analysis using 0.1 M phosphate and 1 mM EDTA at pH 6.9 with 10 mM dithiothreitol and 0.25 mM sucrose. D1 and D2 assays were performed as previously described using 3,5'[125I]T4 or 3,3'-[125I]T3, 0–5 ng/ml TGF-

**Northern Blotting**

Total RNA was isolated with Trizol reagent (Invitrogen). Northern blots were prepared according to standard methods and probed with a 1.9-kb fragment representing the full-length of the hDio3 cDNA (28). D3 Northern blotting was performed using 15 μg of total RNA, exposed for 3 d on a phosphorimager. Blots then were stripped and reprobed with a GAPDH cDNA probe to adjust for differences in sample loading and transfer.

**Acknowledgments**

We thank Drs. Antonio C. Bianco (Thyroid Section of the Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women’s Hospital) and Joyce Bischoff (Vascular Biology Program, Childrens Hospital Boston) for helpful insights and comments on this manuscript.

Received April 29, 2005. Accepted July 14, 2005.

Address all correspondence and requests for reprints to: Stephen A. Huang, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Room 642, Boston, Massachusetts 02115.

E-mail: stephen.huang@childrens.harvard.edu.

This work was supported by Grants DK60494, DK44128, DK07699, DK064643, and DK07529 from the National Institutes of Health, the Cancer Research Institute Oliver R. Grace Jr. Fellowship, Postdoctoral Fellowship No. PF0311101CSM from the American Cancer Society, the Lawson Wilkins Abbott Clinical Scholar Award, the Charles A. King Trust Postdoctoral Research Fellowship Award, the Charles A. Janeway Child Health Research Center Award, and the Clinical Scientist Development Award from the Doris Duke Charitable Foundation.

**REFERENCES**


**Northern Blotting**

Total RNA was isolated with Trizol reagent (Invitrogen). Northern blots were prepared according to standard methods and probed with a 1.9-kb fragment representing the full-length of the hDio3 cDNA (28). D3 Northern blotting was performed using 15 μg of total RNA, exposed for 3 d on a phosphorimager. Blots then were stripped and reprobed with a GAPDH cDNA probe to adjust for differences in sample loading and transfer.
Xenopus laevis retina during metamorphosis is controlled by type III deiodinase. Neuron 24:871–878


43. Larbre E, Silvestri C, Hoodless PA, Wrana JL, Attisano L 1998 Smad2 and Smad3 positively and negatively regulate TGF-β-dependent transcription through the forkhead DNA-binding protein FAST. Mol Cell 2:109–120

48. Feng XH, Lin X, Derynck R 2000 Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-β. EMBO J 19:5178–5193