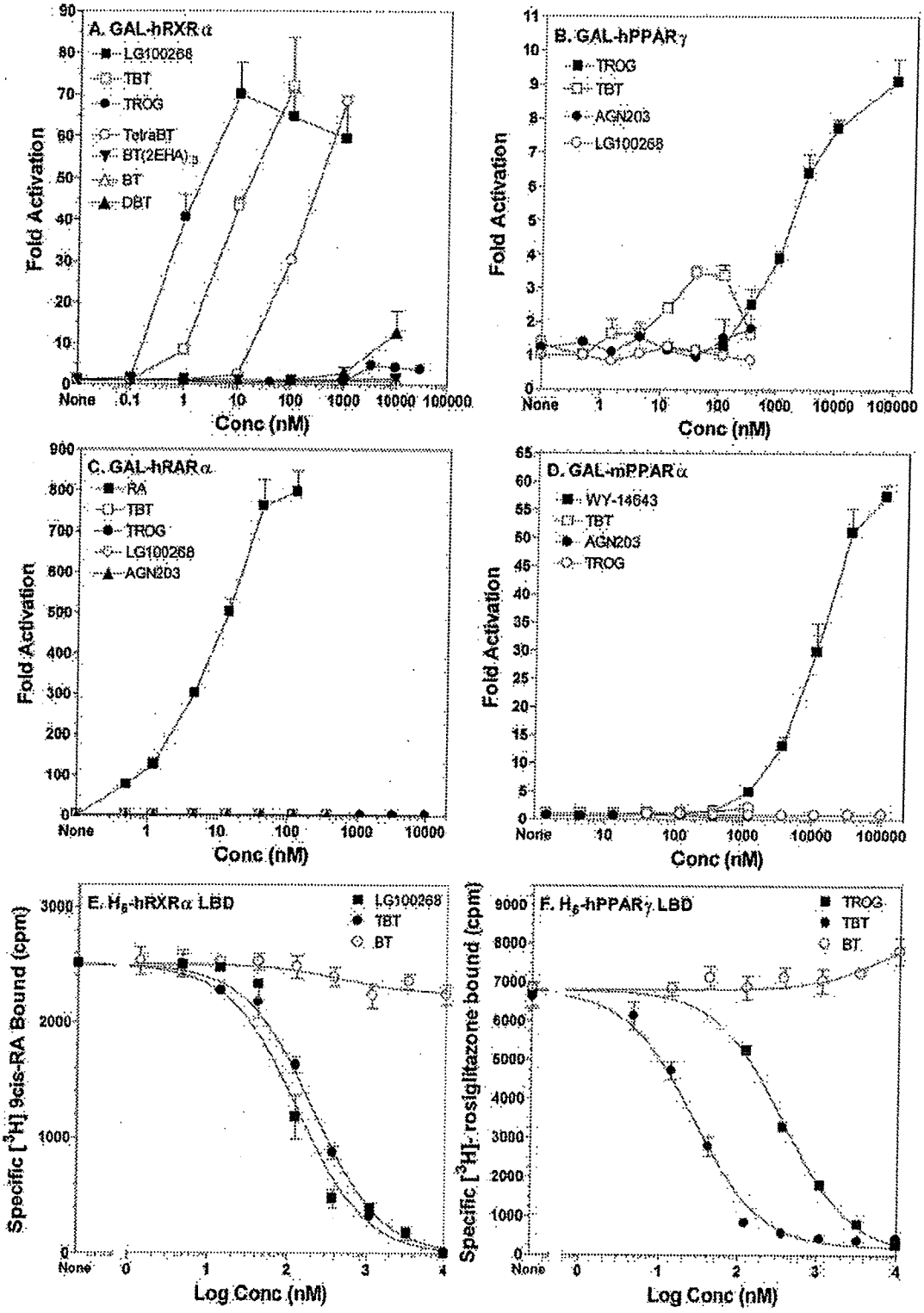


**Fig. 1.** Organotins Are Agonist Ligands of RXRα and PPARγ

Organotins are high-affinity ligand agonists of RXRα and PPARγ. A–D, Activation of GAL4-hRXRα, -hPPARγ, -hRARα, or -hPPARα in transiently transfected Cos7 cells by organotins and receptor-specific ligands. Data represent reporter luciferase activity normalized to β-galactosidase and plotted as the average fold activation ± SEM (n = 3) relative to solvent-only controls from representative experiments. E and F, Competition binding curves of histidine-tagged RXRα or PPARγ LBDs with TBT. Data shown are from a representative experiment analyzed in GraphPad Prism 4.0 and $K_i$ values deduced (Table 3). Conc, Concentration; DBT, dibutyltin chloride; TROG, troglitazone.

**Table 1.** TBT Activates RXRs and RXR-Permissive Heterodimers

| GAL4-NR LBD | Fold Activation at 60 nM TBT | Permissive RXR Heterodimer |
|---|---|---|
| RXRα (*Homo sapiens*) | 60 | Yes |
| RXRα (*X. laevis*) | 25 | Yes |
| RXRγ (*X. laevis*) | 7.0 | Yes |
| NURR1 (*H. sapiens*) | 7.0 | Yes |
| LXR (*H. sapiens*) | 2.1 | Yes |
| PPARα (*Mus musculus*) | 0.7 | Yes |
| PPARγ (*H. sapiens*) | 5.3 | Yes |
| PPARδ (*H. sapiens*) | 1.7 | Yes |
| RARα (*H. sapiens*) | 0.7 | No |
| TR&β (*H. sapiens*) | 0.4 | No |
| VDR (*H. sapiens*) | 0.5 | No |
| SXR (*H. sapiens*) | 1.0 | No |

Data are fold activation at 60 nM TBT relative to solvent-only controls of transiently transfected Cos7 cells after 24 h ligand treatment. SXR, Steroid and xenobiotic receptor; TR, thyroid hormone receptor.

molar $EC_{50}$ values. Monobutyltin gave no significant activation whereas dibutyltin was moderately active in the micromolar range (Fig. 1A and Table 2). Tetrabutyltin was 20-fold less potent than TBT, whereas the branched side-chain butyltin tris(2-ethylhexanoate) [BT(2-EHA)$_3$] was inactive (Table 2). Although activation by dialkyltins is weaker than that of TBT, it is potentially significant due to their widespread use in the manufacture of polyvinyl chloride plastics and greater solubility than TBT.

The effect of the hydrocarbon chain length was very pronounced, suggesting an important structure-activity relationship. A reduction in hydrophobicity from butyl to ethyl side chains raised the $EC_{50}$ value by almost 1000-fold into the micromolar range. Trimethyltin was weakly active only above 100 μM (Table 2). Substitution of the halide component had no significant effect on the $EC_{50}$ values for TBT, probably due to the lability of the halide atom through exchange in aqueous tissue culture media where chloride ions are prevalent.

**Table 2.** Organotin $EC_{50}$ Values for Nuclear Receptor LBDs

| Ligand | GAL4-NR LBD Transactivation ($EC_{50}$ Values, nM) | | |
|---|---|---|---|
| | hRXRα | hRARα | hPPARγ |
| LGD268 | 2–5 | na | na |
| AGN195203 | 0.5–2 | na | na |
| 9-*cis* RA | 15 | na | na |
| all-*trans* RA | na | 8 | na |
| Butyltin chloride | na | na | na |
| Dibutyltin chloride | 3000 | na | na |
| TBT | 3–8 | na | 20 |
| Tetrabutyltin | 150 | ND | ND |
| Di(triphenyltin)oxide | 2–10 | na | 20 |
| Butyltin tris(2-ethylhexanoate) | na | ND | ND |
| Troglitazone | na | na | 1000 |
| Tributyltin fluoride | 3 | ND | ND |
| Tributyltin bromide | 4 | ND | ND |
| Tributyltin iodide | 4 | ND | ND |
| Triethyltin bromide | 2800 | ND | ND |
| Trimethyltin chloride | >10000 | ND | ND |

na, Not active; ND, not determined. $EC_{50}$ values were determined from dose-response curves of GAL4-NR LBD construct activation in transiently transfected Cos7 cells after 24-h ligand exposure.

## TBT Is a Potent Ligand of Both RXRα and PPARγ

Many, if not most, natural and synthetic nuclear receptor agonists act as ligands that specifically interact with their cognate receptor LBDs. We therefore performed equilibrium competition binding experiments with purified histidine-tagged human RXRα ($H_6$-RXRα) and PPARγ ($H_6$-PPARγ) LBDs to determine whether the potent and specific activation of these receptors by TBT was due to direct ligand-receptor interaction (Fig. 1, E and F).

The equilibrium binding curves indicate that TBT is a high-affinity, competitive ligand for 9-*cis* RA-bound RXRα. The inhibition equilibrium dissociation constant was calculated by the Chang-Prusoff method [inhibition constant ($K_i$) = dissociation constant ($K_d$)] as 12.5 nM (10–15 nM; 95% confidence interval) (Table 3). By comparison, the value obtained for the synthetic RXR agonist LG100268 was 7.5 nM, which compared favorably with its published value of approximately 3 ± 1 nM (27). Therefore, the identification of TBT as an RXR ligand expands the molecular definition of known rexinoids (agonists able to activate RXR) to include this structurally unique class of organotin compounds.

Somewhat surprisingly, we also observed potent specific competitive binding by TBT for rosiglitazone bound to human PPARγ LBD (Fig. 2B). The deduced $K_i$ of 20 nM (17–40 nM; 95% confidence interval) was slightly higher than for RXRα but significantly better than the $K_i$ for the PPARγ agonist troglitazone, which

**Table 3.** TBT Binding Constants ($K_d$) for hRXRα and hPPARγ LBDs

| Ligand | Receptor Competitive Inhibition Binding Constants $K_i$ (nM ± 95% CI) | | |
|---|---|---|---|
| | $H_6$-RXRα | $H_6$-PPARγ | Published |
| TBT | 12.5 (10–15) | 20 (17–40) | |
| LG100268 | 7.5 (6–10) | ND | 3 ± 1[a] |
| Troglitazone | ND | 300 (270–335) | 300 ± 30[b] |

Competition binding curves were determined at constant $^3$H-specific ligand concentrations [20 nM 9-*cis*-RA, $K_d$ = 1.4 nM (87) or rosiglitazone, $K_d$ = 41 nM (88)] with increasing cold competitor ligands over the range indicated in Fig. 1, E and F. Data were analyzed in GraphPad Prism by nonlinear regression of a competitive one-site binding equation (Chang-Prusoff method) to determine $K_i$ values ± 95% confidence intervals (n = 3). CI, Confidence interval; ND, not determined.
[a] RXRα:LG100268 $K_d$ = 3 ± 1 nM (27).
[b] PPARγ:troglitazone $K_d$ = 300 ± 30 nM (28).

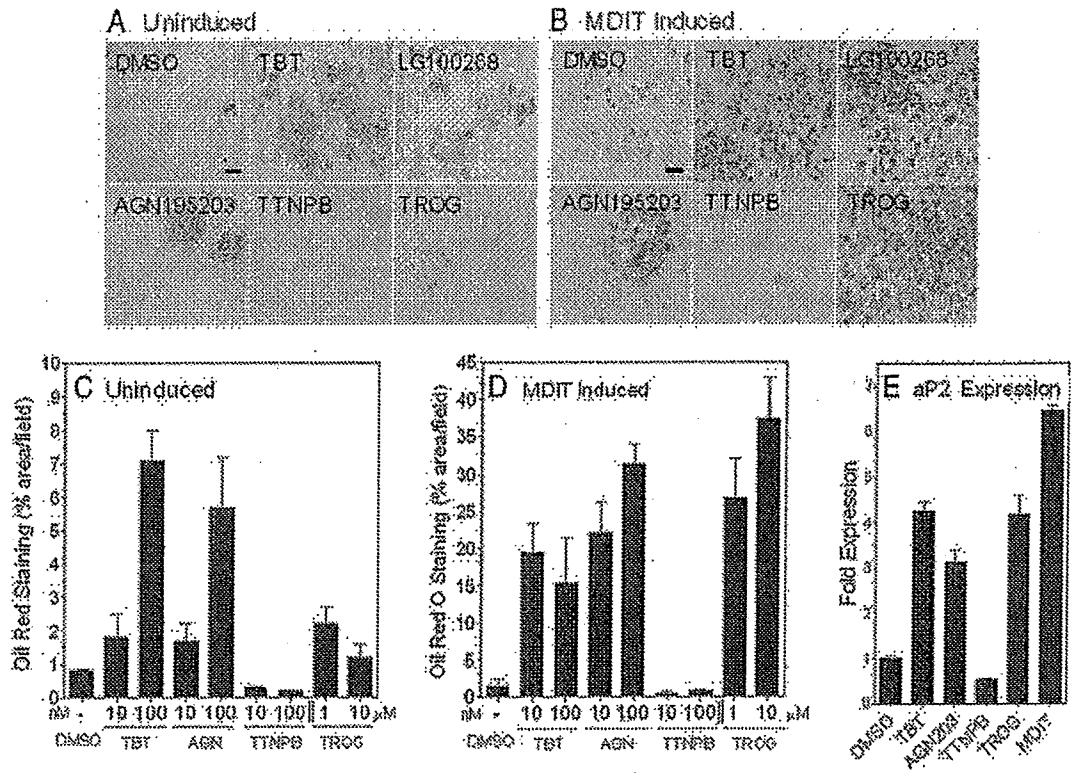


**Fig. 2.** Tributyltin Induces Adipogenesis in 3T3-L1 Cells

Uninduced (A) and MDIT-induced (B) 3T3-L1 cultures grown for 1 wk in the presence of vehicle (DMSO), or ligands were analyzed for mature adipocyte differentiation by Oil Red O staining. *Scale bar* represents 100 μm. C and D, The percentage area stained was determined by automated analysis of random fields (n = 9) from high-contrast dissecting scope photographs of monolayers analyzed in ImageJ; 1–100 nM of TBT, AGN195203, and TTNPB or 1–10 μM troglitazone. E, Quantitative real-time PCR (QRT-PCR) of adipocyte-specific fatty acid binding protein aP2 (aP2/Fabp4) expression levels in postconfluent 3T3-L1 cells treated with the indicated ligands for 24 h. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase controls and plotted as average fold induction ± SEM (n = 3).TROG, Troglitazone.

yielded a $K_i$ of 300 nM, consistent with its published $K_d$ (28). The $K_d$ values for TBT binding to RXRα (12.5 nM) and PPARγ (20 nM) are also in close agreement with $EC_{50}$ values obtained from transient transfection assays using GAL4-RXRα and GAL4-PPARγ constructs (Table 2).

Taken together, these data show that organotins such as TBT, although structurally distinct from previously described natural or synthetic ligands, can interact with RXRα and PPARγ, via direct ligand binding to induce productive receptor-coactivator interactions and promote transcription in a concentration-dependent manner. Organotins are therefore potent nanomolar receptor activators on par with synthetic RXR and PPARγ ligands such as LG100268, AGN195203, and thiazolidinediones.

### TBT Promotes Adipogenesis in the Murine 3T3-L1 Cell (Embryonic Murine Preadipocyte Fibroblast Cell Line) Model

Numerous studies have demonstrated the critical role played by RXRα:PPARγ signaling in regulation of mammalian adipogenesis (29–31). In the murine 3T3-L1 preadipocyte cell model, adipogenic signals induce early key transcriptional regulators such as CCAAT/enhancer binding proteins (C/EBPs) β and δ that lead to mitotic clonal expansion of growth-arrested preadipocytes and induction of the late differentiation factors C/EBPα and PPARγ (32–34). The combination of C/EBPα expression together with PPARγ signaling efficiently drives terminal adipocyte differentiation and lipid accumulation. We therefore tested whether TBT signaling through RXR:PPARγ could promote adipogenesis in the murine 3T3-L1 differentiation assay and compared its effect to other RXR-specific or PPARγ ligands (Fig. 2). Undifferentiated 3T3-L1 cells were cultured for 1 wk in the presence of ligands either with or without a prior 2-d treatment with MDIT (an adipogenic-sensitizing cocktail of 3-isobutyl-1-methylxanthine, dexamethasone, insulin, and $T_3$) (35). Cells were then scored for lipid accumulation using Oil Red O staining to determine the degree of terminal adipocyte differentiation. TBT was as effective as LG100268 or AGN195203 in promoting dif-

ferentiation in the absence of MDIT treatment, increasing the number of differentiated adipocytes about 7-fold over solvent-only controls (Fig. 2, A and C). The PPARγ agonist troglitazone was a weak inducer in the absence of MDIT. Prior treatment with MDIT increased the response to TBT, LG100268, and AGN195203 a further 3- to 5-fold (Fig. 2, B and D). MDIT treatment also boosted the response to troglitazone to equivalent levels as expected from published studies showing that combination treatment with PPARγ ligands promotes efficient adipocyte differentiation (36–38). In contrast, the RAR agonist TTNPB inhibited the differentiation of 3T3-L1 cells, consistent with previously published data that showed RAR signaling blocks adipogenesis during the early stages of differentiation *in vitro* and can modulate adiposity and whole body weight *in vivo* (39–41). The differential response of 3T3-L1 cells to receptor-selective retinoids indicates that TBT favors RXR homodimer or permissive RXR-heterodimer rather than RXR:RAR signaling in this cell model.

Adipocyte differentiation by TBT was accompanied by direct transcriptional effects on RXR:PPARγ targets such as adipocyte-specific fatty acid-binding protein (aP2) mRNA. The aP2 promoter contains response elements sensitive to C/EBP factors and RXRα:PPARγ signaling (42). Quantitative real-time PCR analysis showed aP2 levels were elevated by TBT treatment approximately 5-fold at 24 h (Fig. 2E) and 45-fold at 72 h (data not shown). LG100268, troglitazone, and MDIT treatment also increased aP2 expression at these time points whereas the RAR agonist TTNPB was inhibitory, consistent with the observed cellular responses.

### TBT Induces Adipogenic Regulators and Markers of RXRα:PPARγ Signaling *in Vivo*

The ability of organotins to regulate RXRα:PPARγ target genes and key modulators of adipogenesis and lipid homeostasis *in vivo* has not been previously examined. Therefore, we next asked whether TBT could perturb expression of critical transcriptional mediators of adipogenesis such as RXRα, PPARγ, C/EBPα/β/δ, and sterol regulatory element binding factor 1 (Srebf1) as well as known target genes of RXRα:PPARγ signaling from liver, epididymal adipose tissue, and testis of 6-wk-old male mice dosed for 24 h with TBT [0.3 mg/kg body weight (b.w.)], AGN195203 (0.3 mg/kg b.w.), troglitazone (3 mg/kg b.w), or vehicle (corn oil) administered by ip injection. TBT either had no effect or weakly repressed RXRα and PPARγ transcription in liver (Fig. 3, A and B). A more pronounced decrease was observed for RXRα, PPARγ, C/EBPα, and C/EBPδ in adipose tissue and testis (Fig. 3, B and C). In contrast, TBT, AGN195203, and troglitazone significantly induced expression of the early adipogenic transcription factor C/EBPβ in liver and testis, whereas it was more weakly induced in adipose tissue. Induction was strongest in testis where TBT and troglitazone increased expression greater than 10-fold and AGN195203 increased expression 60-fold compared with vehicle controls (Fig. 3C). In addition to C/EBPβ, the proadipogenic transcription factor Srebf1 was also significantly increased in adipose tissue by all three receptor ligands and weakly induced in liver.

We also observed coordinate changes in several well-characterized direct target genes of RXR:PPARγ signaling. Fatty acid transport protein (Fatp) acts as a key control point for regulation of cellular fatty acid content. The Fatp promoter contains a functional PPAR response element shown to be sensitive to RXR:PPARγ signaling in 3T3-L1 adipocytes and white fat (43–46). Fatp mRNA levels were up regulated 2- to 3-fold in liver and epididymal adipose tissue but not testis by TBT, AGN195203, and troglitazone (see Fig. 5, A and B). Similarly, the PPARγ target phosphoenolpyruvate carboxykinase 1 (PEPCK/Pck1) (47), the rate-limiting step in hepatic gluconeogenesis and adipose glyceroneogenesis, was up-regulated in liver and adipose tissues by TBT or troglitazone treatment.

Signaling through RXR:PPARγ, RXR:LXR, and ADD1/Srebf1 in hepatocytes has been shown to modulate fatty acid synthesis through transcriptional control of acetyl-coenzyme A carboxylase (Acac), the rate-limiting step in long-chain fatty acid synthesis (48, 49), as well as fatty acid synthase (Fasn) (50–53). Hepatic expression of both Acac and Fasn was unregulated between 1.5–2.5-fold by TBT, AGN195203, and troglitazone. Therefore, the coordinate increased expression of Fatp, Pck1, Acac, and Fasn in liver suggests that TBT stimulates fatty acid uptake and triglyceride synthesis. Similar changes have been reported in the induction of hepatic steatosis by overactive PPARγ signaling (49, 54).

Taken together, these data show that TBT exposure induces lipogenic RXR:PPARγ target gene expression, in adipose tissue and liver, and modulates associated early adipocyte differentiation factors such as C/EBPβ and Srebf1. We inferred from these data that organotins are potential adipogenic agents *in vivo*.

### Developmental Exposure to TBT Disrupts Lipid Homeostasis and Adipogenesis in Vertebrates

Based on its molecular pharmacology, ability to induce 3T3-L1 adipocyte differentiation, and *in vivo* transcriptional responses, we reasoned that TBT would disrupt normal endocrine control over lipid homeostasis and impact adipogenesis, particularly when exposure occurred during sensitive periods of development. We therefore tested this hypothesis in two vertebrate model systems, mouse and *X. laevis*, during embryogenesis.

Pregnant C57BL/6 mice were injected daily from gestational d 12–18 with TBT (0.05 or 0.5 mg/kg body weight ip) dissolved in sesame oil or vehicle alone. Pups were then killed at birth, and histological sections were prepared from liver, testis, mammary gland, and inguinal adipose tissue. Sections were stained

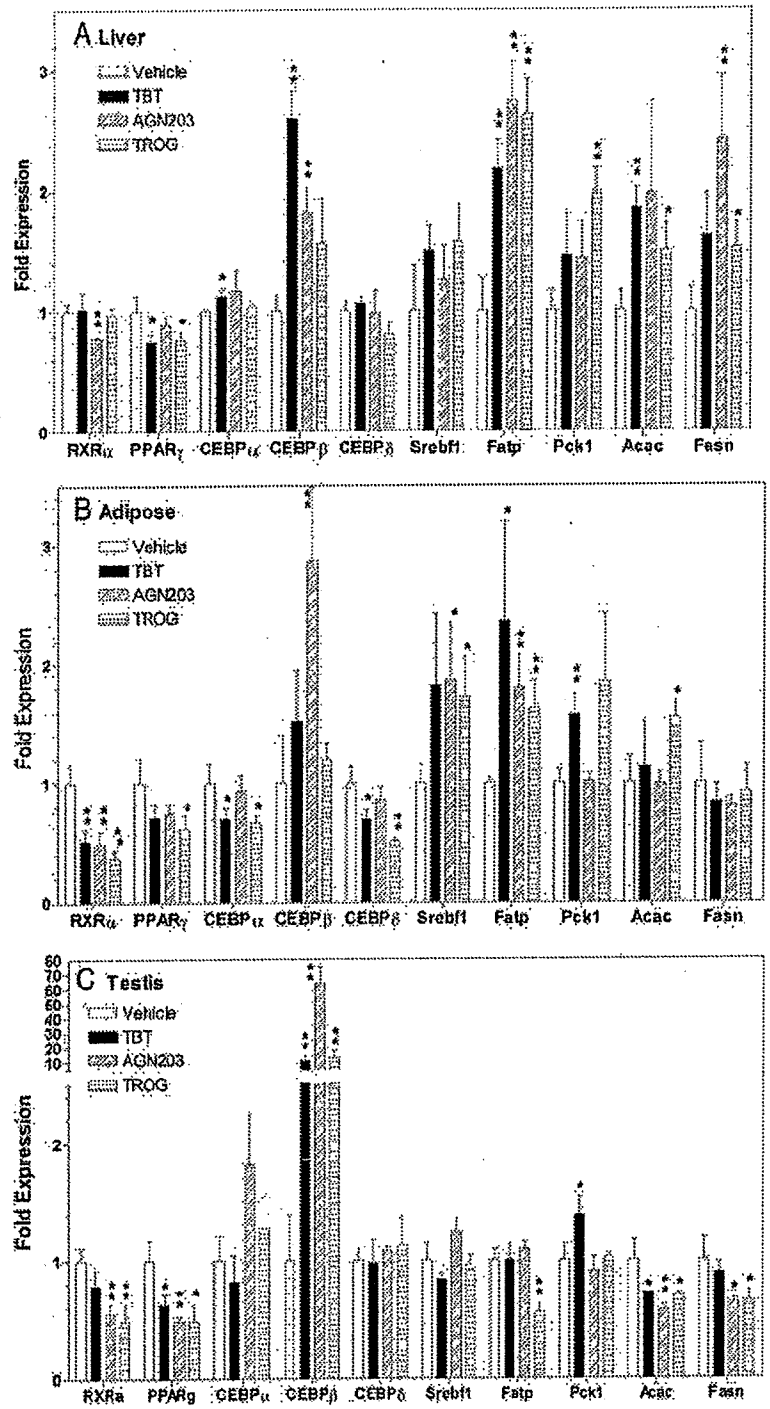


**Fig. 3.** *In Vivo* Induction of Adipogenic Modulators and RXR:PPARγ Target Genes

C57BL/6 male mice (three animals per treatment) were dosed with TBT (0.3 mg/kg b.w.), AGN195203 (0.3 mg/kg), troglitazone (3 mg/kg b.w.), or vehicle (corn oil) only by ip injection. Animals were killed after 24 h and dissected and cDNA was prepared from liver, epididymal fat pad, or testis for quantitative real-time PCR analysis. Expression levels were normalized to histone Hist2h4 and shown as the average fold change ± SEM (n = 3) compared with vehicle (corn oil) controls. Control *vs.* ligand treatments were analyzed by the unpaired Student's *t* test: *, $P < 0.1$; **, $P < 0.05$. TROG, Troglitazone.

with Oil Red O to assess changes in total tissue lipid accumulation. TBT exposure caused a disorganization of hepatic (Fig. 4, A and B) and gonadal (Fig. 4, C and D) architecture and significantly increased Oil Red O staining in treated animals *vs.* controls. Liver sections exhibited signs of steatosis consistent with the mis-

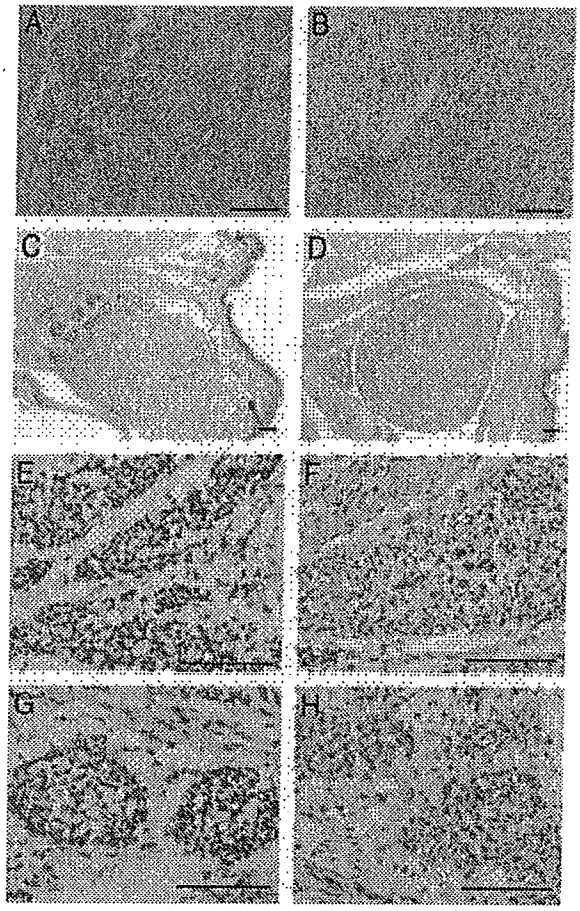


**Fig. 4.** *In Utero* Exposure to TBT Increases Adiposity in Mouse Liver, Testis, and Adipose Depots

Histological sections (12 μm) of newborn mouse liver (A and B), testis (C and D), inguinal adipose (E and F) and mammary adipose (G and H) stained with Oil Red O and counterstained with hematoxylin following *in utero* exposure to vehicle only (sesame oil) (A, C, E, and G) or 0.5 mg/kg b.w. TBT (B, D, F, and H) given s.c. daily from E12–18. *Scale bar*, 100 μm.

regulation of fatty acid uptake and synthesis observed using molecular markers. In addition, Oil Red O positive staining in mammary and inguinal adipose (Fig. 4, E–H) tissues was dramatically elevated, reflecting either an increase in lipid accumulation or an increase in mature adipocytes.

To determine whether exposure induced long-term changes in growth or adipose tissue, we followed mice from birth to adulthood after *in utero* exposure to TBT as indicated above. At birth, mice were cross-fostered to unexposed dams, and total body weight was recorded until 10 wk of age (Fig. 5A). Growth curves for male and female pups showed a slight trend for lower total body weight consistent with published observations (9) but were not statistically significant at 10 wk [control *vs.* TBT: male, 26.00 g ± 0.70 (n = 9) *vs.* 25.53 g ± 0.39 (n = 10), $P = 0.583$; female, 21.22 g ± 0.41 (n = 10), *vs.* 20.24 g ± 0.24 (n = 10), $P = 0.0529$]. Males were killed at 10 wk and epididymal fat pads were weighed (Fig. 5B). Adipose mass in TBT-treated males was increased significantly by 20% over controls [control *vs.* TBT: 0.30 g ± 0.020 (n = 9) *vs.* 0.36 g ± 0.018 (n = 10), $P = 0.0374$]. These data support the conclusion that TBT can increase body adiposity without overtly increasing total body weight. Similar lipid accumulation and changes in adipose tissue mass have also been observed after TZD or rexinoid treatment (55–57).

We had previously observed that TBT activated *Xenopus* RXRs (Table 1) and reasoned that the strong conservation in vertebrate nuclear receptor signaling pathways should result in consistent responses to organotin and RXR/PPARγ ligands across diverse vertebrate species. We therefore tested chronic exposure to environmentally relevant low doses of TBT (1–10 nM), the RXR-specific ligands LG100268 and AGN195203 (10–100 nM), troglitazone (0.1–1 μM), and estradiol (1–10 nM) on developing *X. laevis* tadpoles from stage 48 to metamorphs. To determine the effectiveness of these doses in *X. laevis* tadpoles, we used aromatase expression as a molecular marker because activity and expression are sensitive to endocrine disruption by organotins and RXR/PPARγ ligands in mammals (17, 18). *Xenopus* aromatase expression was similarly repressed 2- to 3-fold by 10 nM TBT, AGN195203, LG100268, or 1 μM troglitazone at stage 56 tadpoles (Fig. 6A) and at all subsequent stages. Despite significant ligand-induced aromatase down-regulation, neither sex ratios nor the time required to reach metamorphosis was altered (data not shown). *Xenopus* liver and kidney also exhibited no gross structural abnormalities at the doses given.

However, consistent with the testis and adipose results from mice presented above, we observed a dose-dependent increase in ectopic adipocyte formation posterior to the fat bodies in and around the gonads of both sexes after TBT or RXR/PPARγ ligand exposure (Fig. 6B). In contrast, estradiol-treated animals did not show increased adipocyte formation compared with controls. At 10 nM TBT, 10 nM AGN195203, or 1 μM troglitazone, ectopic adipocytes were observed in approximately 45–60% of animals. At the highest dose of TBT in males, testicular tissue was interspersed with, or replaced by, adipocytes along the anterior-posterior axis (Fig. 6, D, E, and G).

The concordant changes observed in *Xenopus* aromatase expression, gonadal adipocyte formation, and increased murine adiposity after exposure to TBT, RXR and PPARγ ligands are therefore consistent with a common mechanism of action through RXR:PPARγ activation, supporting the conclusion that endocrine disruption via nuclear receptor transcriptional regulation is a novel and key feature of organotin toxicity.

## DISCUSSION

We have shown above that TBT is a potent inducer of adipogenesis, *in vitro* and *in vivo*, by acting as a novel,

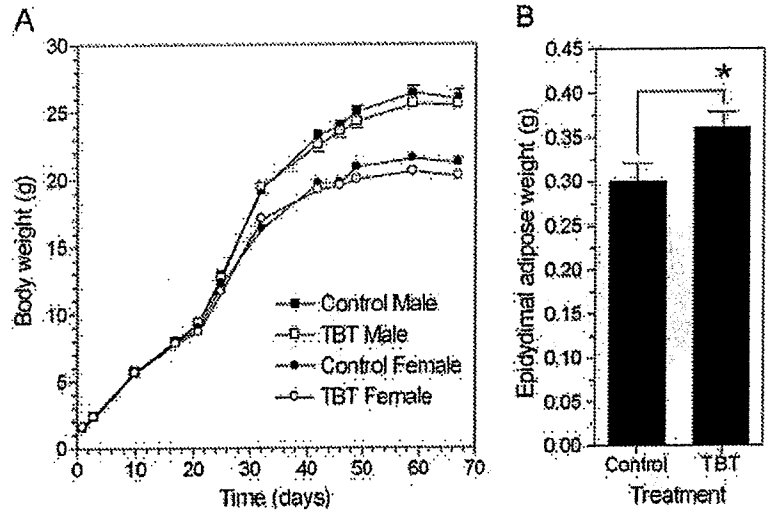


**Fig. 5.** *In Utero* Exposure to TBT Increases Adipose Mass But Not Body Weight in Adult Mice

A, Growth curves of C57BL/6 male and female pups exposed to control (sesame oil) or TBT *in utero* (E12–18). Data are mean ± SEM (n = 10). B, Epididymal fat pad weights from control or TBT-treated males at 10 wk. *, Epididymal adipose mass from exposed males was approximately 20% greater [control *vs.* TBT: 0.30 g ± 0.020 (n = 9) *vs.* 0.36 g ± 0.018 (n = 10); *, $P = 0.0374$). Data represent mean ± SEM (n = 9–10).

high-affinity xenobiotic ligand for RXRα and PPARγ. The ability of organotins to bind and activate these receptors, in particular the RXRs, which exhibit very restricted ligand specificity, is unexpected given the radically different chemical composition and three-dimensional molecular structure of organotins when compared with known natural and synthetic nuclear receptor ligands. Typically, RXR ligands comprise a carboxylic acid functional group and a three-dimensional molecular shape that mimics 9-*cis* RA. Structure-activity profiles indicate distinct structural preferences for organotins but also a relatively broad accommodation for agonist activity that is not easily reconciled with the classical ligand-binding model. Organotins may therefore interact somewhat differently than previously described RXR/PPARγ ligands with receptor LBDs to induce productive conformational changes required for coactivator recruitment. However, the binding data indicate that TBT is a potent and efficacious ligand for both RXRs and PPARγ that interacts, at least partially, with the same receptor-binding sites of other high-affinity ligands and promotes the necessary cofactor interactions required for agonist activation. In the study of Kanayama *et al.* (24), TBT was only effective in coactivator recruitment assays with PPARγ above 10 μM *in vitro*. However, in accord with our findings, they show that TBT activated PPARγ significantly at nanomolar concentrations in transfection assays. This may reflect a limitation of preference in the cofactor used *in vitro*. Alternatively, the lower maximal activation observed with TBT on PPARγ in cells (~30% at 100 nM TBT cf troglitazone) is consistent with one of two possibilities: either nonspecific cellular toxicity at high levels or activation as a partial agonist.

The ability of TBT to act as a dual ligand for permissive heterodimers such as RXRα:PPARγ, which can be activated by specific ligands for either receptor, also raises the possibility for additive or synergistic effects that might increase the potency of these compounds *in vivo* at low doses for this specific signaling pathway. Of note is that receptor activation is observed at nanomolar concentrations, whereas other mechanisms of toxicity and endocrine disruption, *e.g.* direct inhibition of aromatase activity, typically occur in the micromolar range. Furthermore, the activation of other permissive RXR heterodimeric partners, *e.g.* LXR and NURR1, suggests that organotins may act more widely to disrupt multiple nuclear receptor-mediated hormonal signaling pathways.

The biological consequences of organotin activation of the RXR:PPARγ signaling pathway are predictable and should follow known aspects of RXR/PPARγ biology. The RXR:PPARγ pathway plays a key role in adipocyte differentiation and energy storage, and is central to the control of whole-body metabolism (58). PPARγ activation increases the expression of genes that promote fatty acid storage and represses genes that induce lipolysis in adipocytes in white adipose tissue (59). PPARγ such as the thiazolidinediones can modulate insulin sensitivity due to these effects on the adipocyte, reversing insulin resistance in the whole body by sensitizing the muscle and liver tissue to insulin (60). However, a consequence of this increase in whole-body insulin sensitivity is that fat mass is increased through the promotion of triglyceride storage in adipocytes. Evidence is also mounting that depot-specific remodeling and adipocyte numbers increase after thiazolidinedione treatment (55–57). Therefore, PPARγ agonists comprise a class of phar-

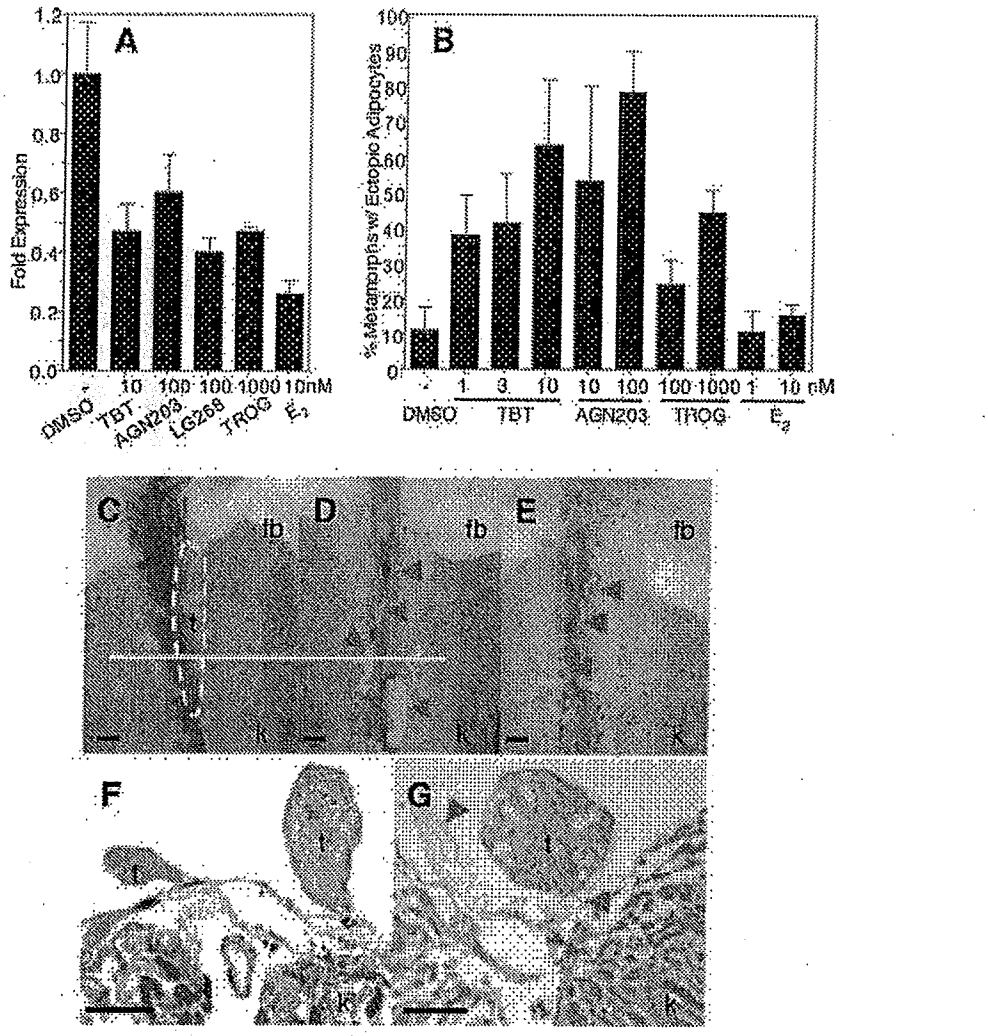


**Fig. 6.** Endocrine Disruption of RXR:PPARγ Signaling and Ectopic Induction of Adipocytes in *X. laevis* by TBT

A, Expression levels of *Xenopus* aromatase (XCYP19) were determined in tadpoles (stage 56) by quantitative real-time PCR after 24-h exposure to vehicle only (DMSO) or the indicated ligands. Expression was normalized to *Xenopus* EF1α and expressed as average fold change in expression ± SEM (n = 9) relative to vehicle controls. B, *X. laevis* tadpoles were dosed weekly under static renewal conditions with indicated ligands from stage 48 (before gonadogenesis) until stage 64 (metamorphic climax). Metamorphs (stage 66) were scored for ectopic adipocyte patches on gonads and urogenital ducts. Data are shown as the percentage of metamorphs exhibiting ectopic adipocyte patches posterior to the fat bodies; mean ± SD from triplicate tanks. C–E, Dissecting microscope photographs of kidneys (k), testis (t), and fat bodies (fb) from DMSO control, 10 nM TBT, and 1 μM troglitazone-treated male metamorphs. Multiple ectopic adipocyte patches (*red arrows*) are present posterior to the fat bodies along the anterior-posterior axis of gonads in TBT (D)- and troglitazone (E)-treated animals but not controls (C). Histological sections of kidneys and gonads from the same control (F) and 10 nM TBT (G)-treated males at the level indicated by the *white line* in C and D. Gonadal and connective tissue was either completely replaced by, or interspersed with, adipocytes (*red arrows*) in TBT-treated animals. Sections were developed with Mallory's trichrome stain. *Scale bars*, 100 μm.

maceutical therapies for type 2 diabetes that can also promote obesity by increasing fat storage. Likewise, RXR ligands also act as insulin-sensitizing agonists in rodents (61), underscoring the permissive nature of the PPARγ:RXR heterodimer and the potential effects on diabetes and obesity of both PPARγ and RXR agonists.

Our data are consistent with recent studies that organotins can mediate some of their endocrine disruption effects by transcriptional regulation through nuclear receptors, in particular RXR:PPARγ signaling (17–19, 24). Consequently, TBT exposure can promote adipocyte differentiation in the same manner as other RXR or PPARγ ligands *in vitro* using the standard murine 3T3-L1 cell model and *in vivo* through increased adiposity after intrauterine organotin exposure in newborn mice. It is currently unknown whether the increased adiposity *in vivo* results from an increase

in adipocyte precursor cell number, enhanced adipocyte differentiation from the same number of precursors, an increase in adipocyte size without an increase in number, or some combination of these.

The prevailing epidemiological data ascribe high-density caloric and/or fatty diets coupled with decreased physical activity as the root causes for the rise in obesity rates in the general population (62). The contribution of genetic components is less clear. Although genetic variation contributes to an individual's propensity to develop obesity, the rapid worldwide increase in obesity suggests that interaction with the modern environment exposes inherent genetic differences. The Barker hypothesis postulates that *in utero* fetal nutritional status is a potential risk factor for metabolic syndrome diseases (63–67). In this view, developmental metabolic programming of a thrifty phenotype limits the range in adaptive responses to the environment, *e.g.* diet and exercise, in later life (68). Experimental evidence from animal models lends support to this hypothesis (69). Plausible mechanisms include imprinting of obesity-sensitive hormonal pathways or changes in cell type and number, *e.g.* adipocytes, established during development.

Others, however, argue that the environment plays another role in obesity. Because the increase in obesity rates parallels the rapid growth in the use of industrial chemicals over the past 40 yr, it is plausible and provocative to associate *in utero* or chronic lifetime exposure to chemical triggers present in the modern environment with this epidemic. Hence, an "obesogen" model predicts the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. Several recent studies serve as proof-of-principle for such a hypothesis. Environmental estrogens such as bisphenol A and nonylphenol, for instance, can promote adipocyte differentiation or proliferation in murine cell lines (70, 71). Furthermore, epidemiological studies link maternal smoking during pregnancy to an elevated risk of childhood obesity (72–76).

Seen in this context, we propose that organotins such as TBT and its congeners are chemical stressors or obesogens that activate RXR:PPARγ signaling to promote long-term changes in adipocyte number and/or lipid homeostasis after developmental or chronic lifetime exposure.

## MATERIALS AND METHODS

### Plasmids and Transfections

pCMX-GAL4 and pCMX-VP16 plasmid fusion constructs to nuclear receptor LBDs and coactivators [GAL4-hRARα, hRXRα, -xRXRα/γ, -hPPARγ, -mPPARα, -human steroid and xenobiotic receptor (SXR), -NURR1, -VDR, -LXR, -hACTR, -hPPAR-binding protein (PBP), -human steroid receptor coactivator-1 (SRC-1), human transcriptional intermediary factor 2 (TIF2)] have been previously described (77–82). Transfections were performed in Cos7 cells (transformed green monkey kidney fibroblast cell line) essentially as described elsewhere (83) using MH200-x4-TK-Luc as reporter and normalized to pCMX-β-galactosidase controls. Briefly, Cos7 cells were seeded at 5000 cells per well in 96-well tissue culture plates in 10% fetal bovine serum/DMEM and transfected for 8 h with 11 μg/plate of DNA/calcium phosphate precipitate mix (MH200x4-TK-Luc-CMX-β-galactosidase-nuclear receptor/coactivator effector(s) at a ratio of 5:5:1). Cells were washed free of precipitate with PBS and media were replaced with serum-free insulin, transferrin, lipid, bovine serum albumin supplemented (ITLB)/DMEM (84) plus ligands for an additional 24 h before assays for luciferase and β-galactosidase activity. All transfection data points were performed in triplicate, and all experiments were repeated at least three times.

### Quantitative Real-Time PCR Analyses

Total cellular RNA from C57BL/6 mouse and *X. laevis* tissues was isolated with Trizol reagent and reversed transcribed with oligo dT and Superscript II (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Triplicate cDNA samples (50 ng/reaction) were analyzed by quantitative real-time PCR on a DNA Engine Opticon thermal cycler [MJ Research (Watertown, MA)/Bio-Rad Laboratories (Hercules, CA)] using SYBR Green chemistry (PerkinElmer Life Sciences, Wellesley, MA). Fold changes in expression levels were calculated after normalization to histone Hist2h4 using the Δ-Δ cycle threshold method (85). Gene-specific primers were as follows. Hist2h4 forward (F): 5′-CCCGTGGTGTGCTGAAGGTGTT-3′; reverse (R), 5′-GAATTGAAGCGGCGGCGTCTA-3′; RXRα F: 5′-CGGCTGCTCAGGGTACTTGTGTTT-3′; R, 5′-CGGCTGCTCAGGGTACTTGTGTTT-3′; PPARγ F: 5′-TGGGTGAAACTCTGGGAGATTC-3′; R, 5′-AATTTCTTGTGAAGTGCTCATAGGC-3′; C/EBPα F: 5′-CCAAGAAGTCGGTGGACAAGA-3′; R, 5′-CGGTCATTGTCACTGGTCAACT-3′; C/EBPβ F: 5′-GCCCGCCGCCTTTAGACC-3′; R, 5′-CGCTCGTGCTCGCCAATG-3′; C/EBPδ F: 5′-AACCCGCGGCCTTCTACGAG-3′; R, 5′-ACGGCGGCCATGGAGTCAAT-3′; aP2 F: 5′-GAATTCGATGAAATCACCGCA-3′; R, 5′-CTCTTTATTGTGGTCGACTTTCCA-3′; FATP F: 5′-AGCCGCTTCTGGGATGACTGTGT-3′; R, 5′-ACCGAAGCGCTGCGTGAACTC-3′; ACS F: 5′-CCCAGCCAGTCCCCACCAG-3′; R, 5′-CACACCACTCAGGCTCACACTCGT-3′; FASN F: 5′-TCGGGTGTGGTGGGTTTGGTGAAT-3′; R, 5′-ACTTGGGGGCGTGAGATGTGTTGC-3′;ACAC F: 5′-G GATGGCAGCTCTGGAGGTGTATG-3′; R, 5′-TGTCCTTAAGCTGGCGGTGTTGTA; Pck1 F: 5′-CTGGCAGCATGGGGTGTTTGTAGG-3′; R, 5′-TGCCGAAGTTGTAGCCGAAGAAGG-3′; Srebf1 F: 5′-GCCCCTGCCCACCTCAAACCT-3′; R, 5′-ACTGGCACGGGCATCCTTCCTC-3′; *Xenopus* EF1α F: 5′-GATCCCAGGAAAGCCAATGTGC 3′; R, 5′-CCGGATCCTGCTGCCTTCTTCT-3′; *Xenopus* CYP19 (aromatase) F: 5′-GTCTGGATTAATGGCGAGGAAACA-3′; R, 5′-CTGATGAAGTATGGCCGAATGACC-3′.

### Ligand Binding

Histidine-tagged RXRα LBD ($H_6$-RXRα LBDs) was expressed and purified from pET15b(+) vector in BL21(DE3) pLysS bacteria cultures after induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 C (86). Purified $H_6$-PPARγ was purchased from Invitrogen. Proteins were bound to 96-well Nickel Chelate Flashplates (PerkinElmer Life Sciences) at 100 μg/ml overnight at 4 C and washed five times with 200 μl/well Flashplate Assay Buffer (20 mM HEPES, pH 7.9; 100 mM KCl, 0.1% cholamidopropyldimethylammonio-2-hydroxy-1-propanesulfonate, 0.1 mM dithiothreitol). Competition assays typically used 1–5 nM [$^3$H]-9-*cis*-RA (PerkinElmer Life Sciences) or 10–50 nM [$^3$H]rosiglitazone (American Radiochemicals, Inc., St. Louis, MO) plus cold competitor ligands in Flashplate Assay Buffer at concentrations indicated in the figures. Plates were incubated at room temperature, pro-

tected from light, and read after 4 h on a Packard Topcount scintillation counter (Packard Instruments, Meriden, CT). Specific bound counts/min were determined by subtraction of counts/min from uncoated wells at each ligand concentration. Data were analyzed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA) using a one-site competition binding equation to determine $K_i$ values for competitor ligands; $K_d$ values of 1.4 and 41 nM for 9-*cis*-RA and rosiglitazone for their respective receptors were used in the calculations (87, 88).

### 3T3-L1 Cell Assays

3T3-L1 (American Type Culture Collection, Manassas, VA) cells were maintained as subconfluent cultures by passage every 3 d from cultures seeded at 5000 cells/cm$^2$ in 8% calf serum/DMEM. For differentiation assays, cells were seeded at $15 \times 10^3$ cells per well into 24-well tissue culture plates in 8% fetal bovine serum/DMEM, after which cultures were grown for 2 d and then treated with the indicated RXR, RAR, and PPAR ligands either with or without MDIT (100 μM 3-isobutyl-1-methylxanthine, 100 nM dexamethasone, 0.1 ng/ml insulin, and 2 nM $T_3$ thyroid hormone) induction cocktail. Media and ligand treatments were renewed every 2 d. After 1 wk, cells were scored for adipocyte differentiation by Oil Red O staining for lipid droplet accumulation. Cultures were washed with PBS, fixed with 10% formaldehyde for 15 min, washed with distilled water, and stained with filtered Oil Red O solution (4 g/liter, 60% isopropanol) for 15 min. Excess stain was removed by washing three times with distilled water. Three random fields from each well were photographed under phase contrast and analyzed in ImageJ. Images were converted into high-contrast black and white images to visualize lipid droplets and scored as the percentage area per field. Data are shown as the mean ± SEM from three wells per treatment. The method was validated by extraction of Oil Red O from stained cells into 100% isopropanol and quantitated by absorbance at 540 nm on a spectrophotometer.

### *In Vivo* Animal Exposure Experiments

C57BL/6J mice were housed under a 12-h light, 12-h dark cycle. Pregnant mice were dosed by ip injection with TBT [0.05 or 0.5 mg/kg body weight (b.w.)] or vehicle (sesame oil) from embryonic d 12 (E12) every 24 h until the day before delivery. Neonates were killed at the day of delivery and analyzed. The samples were embedded in optimal cutting temperature embedding compound and sectioned (12 mm) using a cryostat. Sections were fixed on slides with 4% paraformaldehyde for 10 min and rinsed in PBS. The slides were then sequentially washed with distilled water and 60% of isopropanol and stained with Oil Red O (4 g/liter, 60% isopropanol). After washing with 60% isopropanol and distilled water, the slides were counterstained with hematoxylin. Sections were evaluated and photographed using a Zeiss microscope (Carl Zeiss, Thornwood, NY).

For long-term growth studies, pups were cross-fostered to unexposed C57BL/6 dams after birth, and litter sizes were kept constant at eight pups per dam (control, two male + two female; TBT treated, two male + two female). Animals were weaned at 3 wk of age and maintained on standard rodent chow. Total body weight was followed until 10 wk of age. Males were then killed, and epididymal fat pads were dissected and weighed.

*X. laevis* tadpoles were sorted at stage 48 (89) and maintained in 1-liter glass tanks in 20% Holtfreter's buffered salt solution (90) at a density of 10 tadpoles per tank on a diet of ground Tetramin Fish Flakes and spirulina. Compounds prepared in dimethylsulfoxide (DMSO) as $10^5$-fold stock solutions were tested on triplicate tanks and dosed by static renewal after weekly water changes. Metamorphs at stage 64 were transferred to individual containers and fed frozen brine shrimp for 2 wk until stage 66. Froglets were euthanized with 250 mg/liter MS222 in 20% Holtfreter's solution and then scored for gonadal abnormalities and interrenal/gonadal adipocyte formation under a dissecting microscope. Kidneys with attached gonads and livers were fixed in 10% formalin-PBS, embedded in paraffin, and sectioned at 15 μm thickness. Sections were developed with Mallory's trichrome stain.

All animal experiments were approved and performed in accordance with Institutional Animal Care and Use Committee protocols.

## Acknowledgments

We thank Drs. I. Blitz, K. Cho, C. Zhou, and T. Osborne for critical reading and comments on the manuscript, Dr. C. Li (Expression Technologies) for the $H_6$-RXRα LBD construct, and Dr. R. Chandraratna (Allergan Pharmaceuticals, Irvine, CA) for AGN203 and LG268).

Received September 8, 2005. Accepted March 30, 2006.

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This work was supported by grants from the U.S. Environmental Protection Agency (STAR R830686) and National Institutes of Health (GM-60572) (to B.B.); from the Ministries of Education, Culture, Sports, Science and Technology, Environment and Health Labor and Welfare, Japan (to T.I.); and from the University of California Toxic Substance Research and Training Program (UC-37579) (to F.G.).

F.G., H.W., Z.Z., L.M., K.A., R.C., D.M.G., J.K., T.I. have nothing to declare. B.B. is a named inventor on U.S. patents US 5,861,274, US 6,200,802, and US 6,815,168.

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**Journal of Pathology**
*J Pathol* 2006; **209**: 522–531
Published online 25 April 2006 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1993

**Original Paper**

# *PTOV1*: a novel testosterone-induced atherogenic gene in human aorta

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Received: 25 October 2005
Revised: 8 February 2006
Accepted: 4 March 2006

## Abstract

**There are gender differences in the development of atherosclerosis, possibly owing to differences in sex steroid hormone action and/or metabolism. One of the atherogenic effects of testosterone is thought to be androgen receptor (AR)-mediated vascular smooth muscle cell (VSMC) proliferation. However, the detailed mechanism of this effect, particularly the identity of the genes associated with VSMC proliferation, remains largely unknown. Therefore, we first employed microarray analysis and, subsequently, quantitative RT-PCR to analyse RNA expression in AR-positive human VSMCs treated with testosterone in order to detect testosterone-induced genes associated with cell proliferation. We further examined whether the genes identified were involved in cell proliferation using small interfering RNA (siRNA) transfection. Expression of the gene products was then evaluated in human aorta with various degrees of atherosclerosis in order to evaluate the clinical relevance of the findings. Both microarray and quantitative RT-PCR analyses demonstrated marked induction of the human prostate overexpressed protein 1 (*PTOV1*) gene by testosterone in the cell lines: this gene was recently identified as a novel androgen-induced gene involved in prostate tumour cell proliferation. Inhibition of *PTOV1* by transfection of its corresponding siRNA suppressed testosterone-induced cell proliferation. In human aorta, PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundantly detected in male aorta with mild atherosclerotic changes compared with female aorta or male aorta with severe atherosclerotic changes. These findings indicate that the *PTOV1* gene is androgen-responsive in VSMCs and that it may play an important role in androgen-related atherogenesis in the human aorta, particularly early atherosclerosis in the male aorta, through regulating proliferation of neointimal VSMCs.**


**Keywords: vascular smooth muscle cells; androgen receptor; testosterone; cell proliferation; PTOV1**

## Introduction

There is an important, well-documented, gender difference in coronary heart disease risks, with earlier onset of disease and excess mortality in male subjects [1–3]. Athero-protective effects of oestrogens on vascular structure and function have been proposed as one of the most important mechanisms accounting for this gender difference [4]. On the other hand, an association between androgens and atherosclerosis continues to be disputed. Androgens have been considered to reduce the incidence of ischaemic myocardial disease in men, but they have also been reported to exert atherogenic effects on the human cardiovascular system through promoting plaque formation and enhancing monocyte adhesion to endothelial cells [5–8]. It has been demonstrated that testosterone exerts direct atherogenic effects by promoting cell proliferation through an initial interaction with the androgen receptor (AR) in vascular smooth muscle cells (VSMCs) *in vitro* [9]. However, unlike oestrogens, the possible effects of testosterone on atherogenesis and/or anti-atherogenesis have not been extensively studied. It is therefore important to study the detailed mechanisms of these direct effects of testosterone on the human cardiovascular system.

In this study, we first screened for testosterone-induced genes involved in the proliferation of VSMCs using microarray analysis in cell lines derived from AR-positive human VSMCs. We then confirmed the results by employing other *in vitro* studies. As testosterone induced marked overexpression of *PTOV1* in

these assays, we subsequently examined the levels of expression of PTOV1 protein in VSMCs in samples of the human abdominal aorta obtained at autopsy.

## Materials and methods

### Vascular smooth muscle cells

Two types of human dedifferentiated VSMCs, ie HUVS-112D (derived from human umbilical cord), and T/G HA-VSMC (derived from human aorta) were commercially obtained from American Type Culture Collection (Manassas, VA, USA) [10,11]. We examined whether these cells expressed AR using an RT/real-time PCR with a light Cycler System using DNA binding dye SYBR Green I, and immunoblotting with AR polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), as reported previously [10,12].

### GeneChip microarray assay

The VSMCs above were cultured until a sub-confluent state was achieved. The medium was then replaced with fetal bovine serum (FBS)-free and phenol red-free medium to arrest cell proliferation. After 24 h, the medium was replaced again with phenol red-free and FBS-free medium in the presence of testosterone (10 nM) or vehicle (0.1% ethanol). After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for microarray analysis. Isolated total RNA was labelled as described in the Affymetrix (Santa Clara, CA, USA) GeneChip Expression Analysis Technical Manual (revision 3), as previously described [10]. The ratios represent the values up- or down-regulated by 10 nM testosterone treatment compared with control. We independently repeated the same experiment twice. Genes for which the average ratios increased more than 1.5-fold in both experiments using 10 nM testosterone treatment were considered up-regulated via AR when compared with control values [13]. When studying the potential functions of these genes, we used the homepage of the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature, accessed 28 March 2006) for further examination of whether any had been previously reported to be involved in cell proliferation and to be associated with androgen effects. In this study, among the genes that were found to be significantly induced by testosterone treatment by microarray analysis, we regarded a gene that was up-regulated, and was known to be associated with both cell proliferation and androgenic effects, as a target gene.

### Quantitative real-time PCR

After achieving sub-confluence and following growth arrest states of the VSMCs as described above, the medium was replaced again with phenol red-free and FBS-free F12-K medium with testosterone (10 nM), testosterone (10 nM) with flutamide, an AR-blocker (100 nM), or vehicle. After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis, described previously [10]. mRNA levels for the target gene *PTOVI* were determined in each VSMC as a ratio relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and evaluated as a ratio (%) compared with that of each control cDNA. The analyses with real-time PCR were repeated three times. Table 1 summarizes the primers used [14].

### siRNA preparation, transfection, and cell count assay

Small interfering RNAs (siRNAs) corresponding to *PTOVI* (Table 2) were synthesized based on results of a previous report, and were transfected into the VSMCs [15]. These VSMCs were seeded in a 24-well plate at an initial concentration of 50 000 cells/well with F-12K medium containing 2% FBS and were cultured until a sub-confluent status was achieved. The medium was then replaced with phenol red-free and FBS-free medium to arrest cell proliferation. After 24 h, transfection experiments of siRNA for endogenous gene targeting (10 nM or 100 nM) were carried out using RNAiFect™ transfection reagent (Qiagen, Valencia, CA, USA). After transfection, the cells were incubated in phenol red-free medium containing 2% dextran-coated, charcoal-stripped FBS with testosterone (10 nM) or vehicle (0.1% ethanol) for 24 h. We measured the number of cells in each sample as described above with Cell Counting Kit-8 system (Wako, Tokyo, Japan) after incubation for 48 h. We also examined the number of cells treated with

**Table 1.** Primer sequences used in RT-PCR analysis

| cDNA | Sequence | Size (bp) |
|---|---|---|
| *AR* | Forward 5′-CTCACCAAGCTCCTGGACTC-3′<br>Reverse 5′-CAGGCAGAAGACATCTGAAG-3′ | 246 |
| *GAPDH* | Forward 5′-TGAACGGGAAGCTCACTGG-3′<br>Reverse 5′-TCCACCACCCTGTTGCTGTA-3′ | 307 |
| *PTOVI* | Forward 5′-CACCATCCCTCCATGTTGCTG-3′<br>Reverse 5′-TCTTCATTGGCCTCATCCCC-3′ | 250 |

**Table 2.** Sequences used in siRNA transfection analysis

| cDNA | Sequence |
|---|---|
| *PTOVI* | Sense r(CAACAAGUUUCUGGCAUGG)dTdT<br>Antisense r(CCAUGCCAGAAACUUGUUG)dTdT |
| Negative control | Sense r(UUCUCCGAACGUGUCACGU)dTdT<br>Antisense r(ACGUGACACGUUCGGAGAA)dTdT |

The target gene in this study (*PTOVI*) was determined by microarray analysis. The sequences of *PTOVI* siRNAs are based on a previous report [15].

transfection of negative control siRNA with scrambled sequences (Table 2), and treated with testosterone (10 nM) or vehicle. In order to evaluate transfection efficiency, we examined relative *PTOV1* mRNAs levels in these cells at 24 h after transfection of the specific siRNAs. The mRNA levels in each VSMC were calculated as a ratio relative to *GAPDH*, and were normalized to the ratio after transfection of negative control siRNA (10 nM).

### Quantitative RT-PCR analysis of *PTOV1* mRNA expression in human aorta

Samples of human abdominal aorta were collected at autopsy from patients without a history of hormone replacement therapy. Autopsies were performed on 32 subjects (16 male, 16 female; mean 60.7 ± 3.3 years) in Tohoku University Hospital (Sendai, Japan) within 2 h post mortem. The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. Aortic specimens were tentatively classified into the following four groups according to the sex of the deceased patient and degree of atherosclerosis, as previously described: group A = male, mild atherosclerosis, corresponding to groups I–III in the American Heart Association (AHA) classification; group B = male, advanced atherosclerosis, corresponding to groups IV–VI in the AHA classification; group C = female, mild atherosclerosis; and group D = female, advanced atherosclerosis) [10,11]. The distribution of the cases among these groups was as follows: A, 8 cases (mean 44.3 ± 10.6 years); B, 8 cases (mean 71.3 ± 3.7 years); C, 8 cases (mean 52.0 ± 3.9 years); and D, 8 cases (mean 75.0 ± 2.1 years), respectively. For RT/real-time PCR analysis, these specimens were treated according to our previous report [10]. The mRNA levels for *PTOV1* and *AR* in each sample are given as a ratio relative to *GAPDH*, and evaluated as a ratio (%) compared with that of each control cDNA.

### Immunohistochemical analysis for PTOV1 protein expression in human aorta

Details of immunohistochemical procedures have been previously described [10,11]. We used immunostaining with diaminobenzidine (DAB) for immunohistochemical analysis of PTOV1 protein (using a monoclonal anti-human PTOV1 antibody; Novocastra Laboratories, Newcastle, UK) and AR (using a monoclonal antibody for human AR; Dako Corporation, Carpinteria, CA, USA). We also used double immunostaining with DAB and Vector Blue as colorimetric reagents, with a combination of monoclonal antibodies for α-smooth muscle actin (α-SMA; Dako Corporation), for macrophages (PG-M1, Dako Corporation), and for leukocytes (human leukocyte common antigen antibody (LCA; Dako Corporation) in adjacent tissue sections. After determining the areas for evaluation by simultaneous observation using a multi-headed light microscope, three authors (YN, TS, and HS) independently evaluated immunoreactivity. Scoring of immunoreactivity was performed based on our previous reports with some modifications [10,16]. When PTOV1 protein was immunolocalized to the cytoplasm, the relative immunoreactivity in each specimen was classified into the following three groups: 2 = more than 50% positive cells; 1 = more than 10% and less than 50% positive cells; and 0 = negative or less than 10% positive cells, respectively [16]. When PTOV1 protein immunoreactivity was detected in the nuclei, the relative immunoreactivity in each specimen was evaluated by the percentage of immunoreactivity, ie the labelling index (LI) [10]. When inter-observer differences were >5%, the three aforementioned authors re-evaluated these discrepant immunostained slides simultaneously using a multi-headed light microscope, and the mean value was obtained.

### Statistical analysis

Values for all results were given as the mean ± standard error of the mean (SEM). Results of quantitative RT-PCR, cell count assay, and the relative immunoreactivity for protein in the nuclei were analysed using one-way analysis of variance followed by unpaired *t*-test for comparisons between two groups. Results of immunohistochemistry of cytoplasmic protein were analysed using the $\chi^2$-test. Statistical differences between immunoreactivity for PTOV1 protein and AR were evaluated using Spearman's rank correlation. A $p$ value $<0.05$ was considered significant in this study.

## Results

### Characterization of two VSMC cell lines

By RT-PCR analysis, both HUVS-112D cells and T/G HA-VSMC cells expressed *AR* mRNA (Figure 1A). In addition, AR protein was demonstrated in both of these cell lines by immunoblotting analysis (110 kD) (Figure 1B). Relative levels of AR mRNA and protein expression in these cells were approximately 5–10% of those in LNCaP cells that were examined in parallel (data not shown).

### Gene chip microarray assay

Table 3 summarizes 11 genes that were up-regulated by testosterone treatment in both types of VSMC in duplicated microarray analysis. Among these genes, human prostate tumour overexpressed protein 1, ie *PTOV1* was detected in both of these VSMCs. Recently, *PTOV1* has been reported to be induced by androgen and to be involved in cell cycle regulation [14,15,17]. *AGTR2* was also reported to be associated with androgenic effects, but it is unknown whether *AGTR2* is involved in cell growth [18]. Therefore we focused our subsequent studies on *PTOV1* as

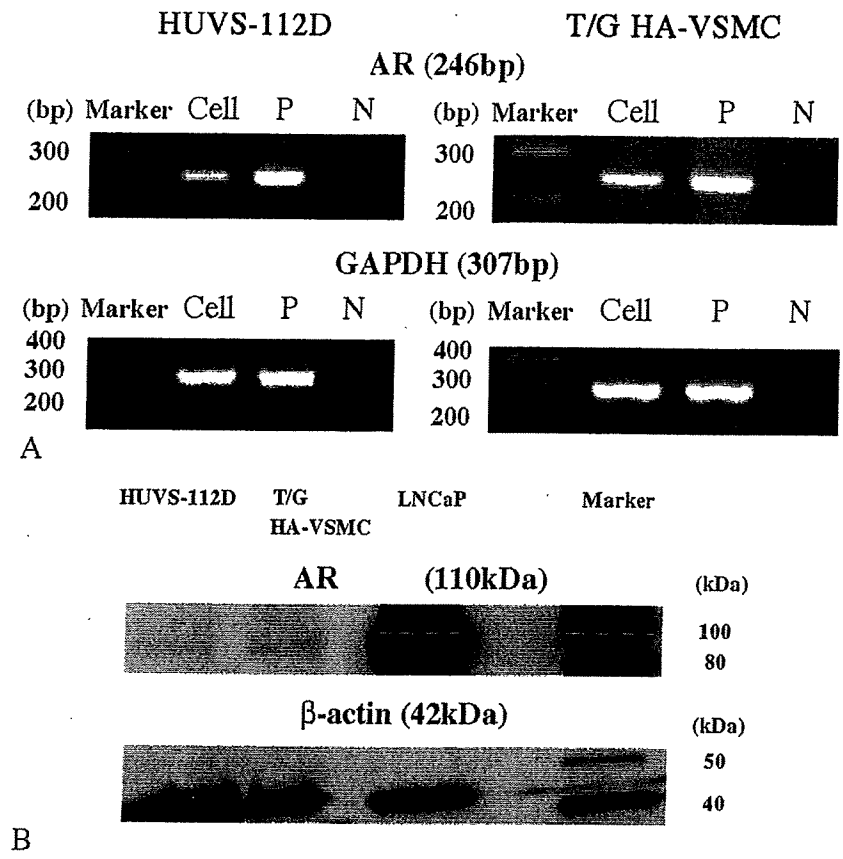


**Figure 1.** (A) Results of RT/real-time PCR analysis for *AR* and *GAPDH* in two cultured human VSMCs (HUVS-112D, and T/G HA-VSMC), positive controls, and negative controls. Cell = each type of cultured vascular smooth muscle cell; P = positive control (LNCaP prostate cancer cell line); N = negative control (no cDNA), respectively). (B) Immunoblotting analysis of AR and β-actin in HUVS-112D, T/G HA-VSMC, and LNCaP cells. Total protein was extracted, and 60 μg protein from each cell was loaded. Immunoblotting analysis demonstrated both AR (110 kD) and β-actin protein (42 kD) in all cells

**Table 3.** Ratios of gene expression determined by GeneChip microarray analysis after testosterone treatment of cultured VSMCs for 2 h

| Gene symbol | HUVS-112D | T/G HA-VSMC | Function | Association with androgen (reference) |
|---|---|---|---|---|
| *PAK7* | 3.9 | 3.6 | Neurite development | Unknown |
| *PIK3R4* | 1.7 | 3.3 | Cell signalling | Unknown |
| *CELSR1* | 1.9 | 3.1 | Cell adhesion | Unknown |
| *CACNA1G* | 2.5 | 2.9 | Calcium channel | Unknown |
| *AGTR2* | 2.2 | 2.5 | Regulator of aldosterone secretion | Koike *et al* [18] |
| *INVS* | 2.7 | 3.6 | Renal tubular development | Unknown |
| *GPR77* | 3.5 | 2.6 | Cell signalling | Unknown |
| *CASP10* | 3.3 | 2.8 | Apoptosis | Unknown |
| *AP4S1* | 2.1 | 2.5 | Formation of cell structure | Unknown |
| *T1A-2* | 1.7 | 2.7 | Membrane glycoprotein | Unknown |
| *PTOVI* | 1.8 | 2.0 | Cell growth/mitogenesis | Benedit *et al* [14] |

'Ratios' represent the mean ratios of expression levels of each gene mRNA in duplicate experiments compared with control.

an androgen-responsive gene possibly involved in the proliferation of human VSMCs.

### *PTOVI* mRNA expression in VSMCs after androgen treatment

Testosterone significantly increased *PTOVI* mRNA levels in AR-positive VSMCs compared with controls in both of these cell lines ($p < 0.05$) (Figure 2). However, testosterone with flutamide, an AR-blocker (100 nM), did not increase its mRNA expression in either of these cells ($p < 0.05$) (Figure 2).

### *PTOVI* siRNA transfection and cell proliferation assay

Quantitative RT-PCR analysis demonstrated that *PTOVI* mRNA levels were decreased in a dose-dependent manner in the cells transfected with *PTOVI* siRNAs (Figure 3A). After transfection of negative

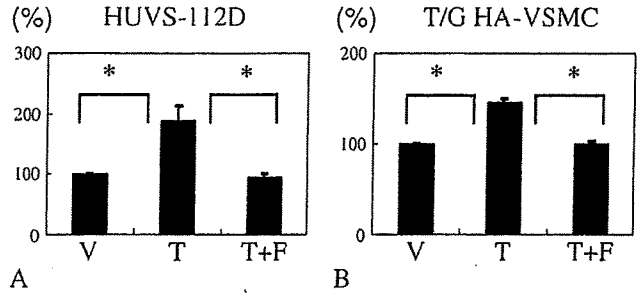


**Figure 2.** Results of RT/real-time PCR analysis for *PTOV1* in HUVS-112D (A) and T/G HA-VSMC cells (B) among cells treated with vehicle (V) (control), testosterone (T) alone (10 nM), and T (10 nM) with flutamide (F), an AR-blocker (100 nM), respectively after 2 h (*$p < 0.05$)

control siRNA (10 nM), testosterone promoted cell proliferation significantly in both of these cell lines ($p < 0.05$) (Figure 3B). However, testosterone with transfection of *PTOV1* siRNA (10 nM and 100 nM) did not increase cell proliferation in these two cell lines (Figure 3B).

## *PTOV1* mRNA expression in human aorta

The results of RT/real-time PCR analysis demonstrated the presence of specific single bands for *AR* and *PTOV1* in human aorta (Figure 4A). The relative abundance of *PTOV1* mRNA was significantly greater in male aorta with a mild degree of atherosclerotic changes (group A) than in those of other groups (groups B, C, and D)($p < 0.05$) (Figure 4B). The relative abundance of *AR* mRNA was also significantly greater in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe

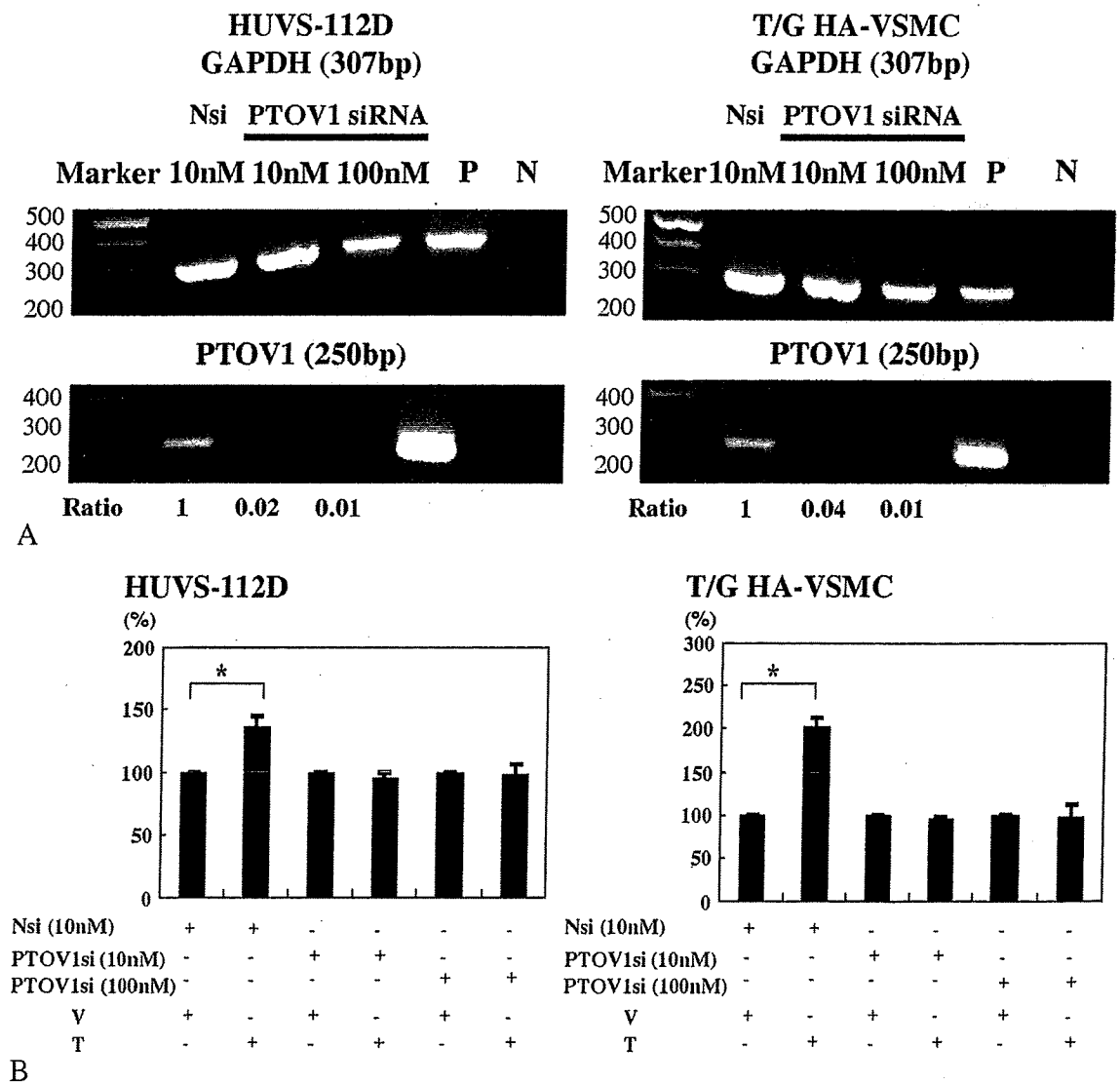


**Figure 3.** (A) Expression of *PTOV1* and *GAPDH* mRNAs at 24 h after transfection of *PTOV1* siRNA (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) in HUVS-112D and T/G HA-VSMC cells detected by real-time PCR, respectively. *GAPDH* mRNA expression was monitored as an internal control. The ratio of *PTOV1/GAPDH* was calculated and values were normalized to the ratio obtained from the negative control transfection of Nsi (10 nM). P = positive controls (LNCaP prostate cancer cell lines); N = negative controls (no cDNAs), respectively. (B) The relative levels of cell numbers in HUVS-112D and T/G HA-VSMC cells among cells treated with vehicle (V) (0.1% ethanol) and testosterone (T) alone (10 nM) after transfection of *PTOV1* siRNA (PTOV1si) (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) (*$p < 0.05$)

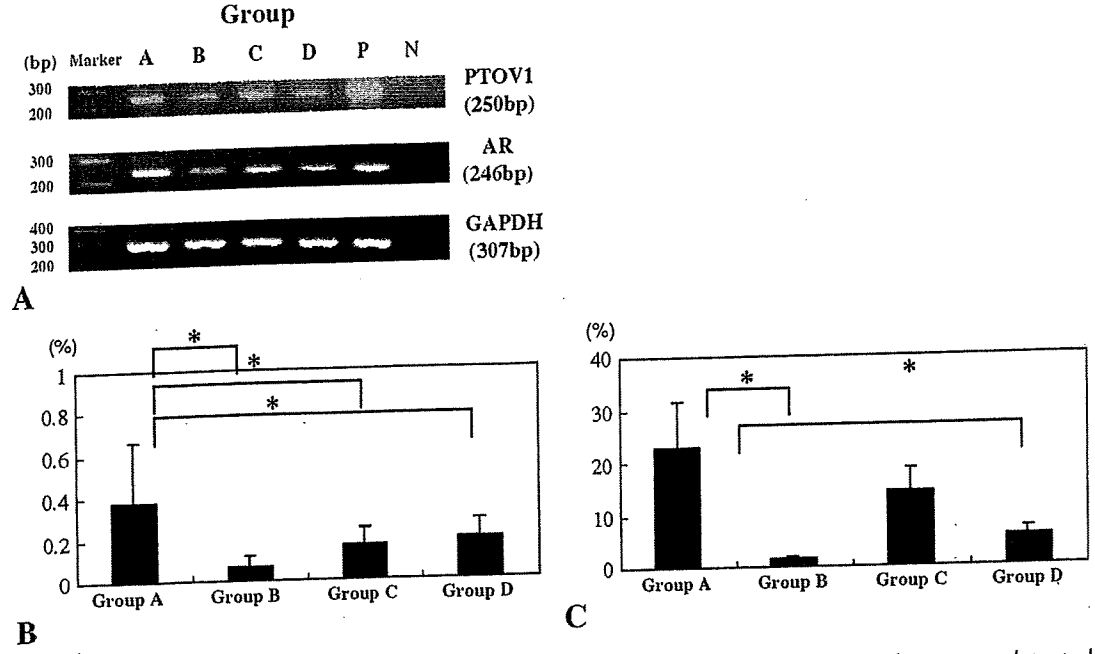


**Figure 4.** (A) Results of RT/real-time PCR analysis for *PTOV1* in human aortas. Bands for PCR products were detected as specific single bands (246 bp for *AR*, 250 bp for *PTOV1*, and 307bp for *GAPDH*). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these RT/real-time PCR gene products are illustrated. A = aorta from a 38-year-old man with mild atherosclerotic change; B = aorta from a 72-year-old man with severe atherosclerotic change; C = aorta from a 45-year-old woman with mild atherosclerotic change; D = aorta from a 76-year-old post-menopausal woman with severe atherosclerotic change; P = positive controls; N = negative controls. (B) Results for *PTOV1* mRNA expression levels (*$p < 0.05$). (C) Results for *AR* mRNA expression levels (*$p < 0.05$)

degree of atherosclerosis (group B) and in female aorta with a severe degree of atherosclerosis (group D) ($p < 0.05$) (Figure 4C).

### Immunohistochemistry for PTOV1 in human aorta

PTOV1 protein was expressed in both the nucleus and the cytoplasm of VSMCs in each group examined (Figures 5 and 6). AR protein was expressed in the nuclei of VSMCs in each group (Figures 5 and 6). However, none of the LCA- or PG-M1-positive cells demonstrated any PTOV1 immunoreactivity (Figure 5). The relative levels of PTOV1 immunoreactivity in the nuclei of neointimal VSMCs were significantly higher in male aorta with a mild degree of atherosclerotic change (group A) than in those of the other groups examined (groups B, C, and D) (Figure 6A). In addition, AR-positive cells in the neointima were also significantly more abundant in male aorta with a mild degree of atherosclerotic changes (group A) than in those of the other groups (groups B, C, and D) ($p < 0.05$) (Figure 6E). There was also a significant positive correlation between AR and PTOV1 immunoreactivity in the nuclei of VSMCs in the neointima ($p < 0.05$) (data not shown). AR-positive cells in the tunica media were significantly more abundant in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe degree of atherosclerosis (group B) and in female aorta with a mild degree of atherosclerosis (group C) ($p < 0.05$) (Figure 6F). However, there were no significant differences in PTOV1 immunoreactivity in the cytoplasm of cells in the neointima or in the nucleus and/or cytoplasm of cells in the tunica media among these groups (Figure 6B, C, and D).

## Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses all indicated that *PTOV1* is one of the genes induced by testosterone via AR-dependent pathways in cultured human VSMCs. In addition, siRNA analysis demonstrated that *PTOV1* is involved in AR-mediated VSMC proliferation. Results of both quantitative RT-PCR and immunohistochemical studies in human aorta obtained at autopsy further demonstrated that PTOV1, as well as AR, detected in the nuclei of neointimal VSMCs was abundant in relatively young male aorta at an early stage of atherosclerosis.

*PTOV1* has been known to be involved in stimulation of cell proliferation [14,15,17]. This gene is a mitogenic factor that shuttles between nucleus and cytoplasm in a cell cycle-dependent manner in prostate carcinoma cells [14,15,17]. In addition, *PTOV1* overexpression induced cell proliferation and facilitated entry of prostate carcinoma cells into the S phase [14,15,17]. Therefore, these findings all indicated that *PTOV1* may play a very important role in the proliferation of VSMCs. However, it is also true that other atherogenic effects on human VSMCs, such as promotion of PDGF-induced VSMC proliferation,

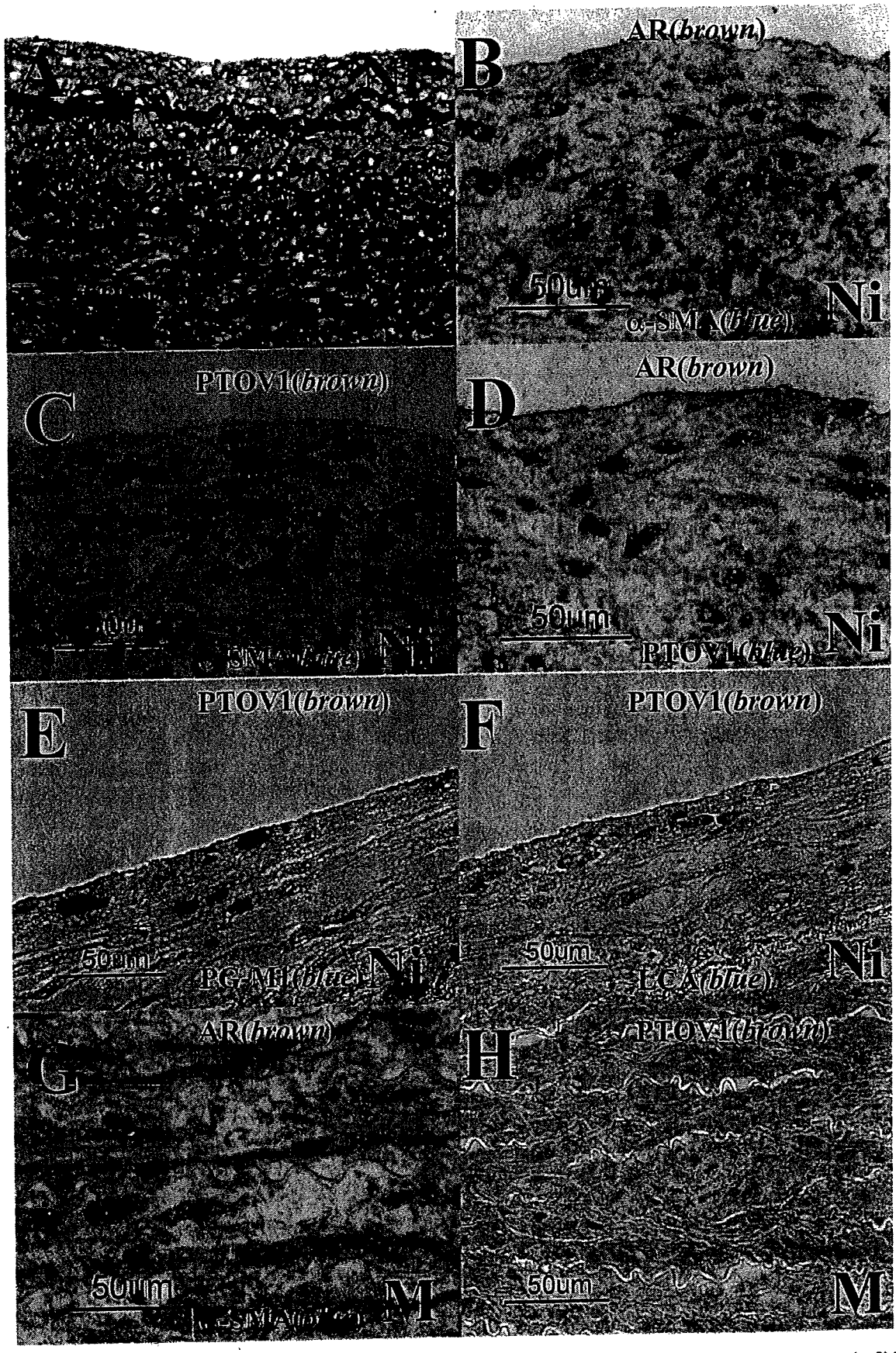


**Figure 5.** Modified Masson Goldner's stains (A), double-immunohistochemical staining for AR and α-muscle actin (α-SMA) (B), for PTOV1 and α-SMA (C), for AR and PTOV1 (D), for PTOV1 and PG-M1 (E), for PTOV1 and leukocyte common antigen (LCA) (F) in the neointima, double-immunohistochemical staining for AR and α-SMA (G) and immunohistochemical staining for PTOV1 (H) in the media of an abdominal aorta specimen obtained from a 38-year-old man with a mild degree of atherosclerosis (group A). Immunopositive cells appear brown as a result of DAB colorimetric reaction and blue as a result of Vector Blue colorimetric reaction. Double-immunopositive cells are confirmed. Ni = neointima; M = media