Supporting Information

Tributyltin engages multiple nuclear receptor pathways and suppresses osteogenesis in bone marrow multipotent stromal cells.

Amelia H. Baker[†], James Watt[‡], Cassie K. Huang[‡], Louis C. Gerstenfeld[§], and Jennifer J. Schlezinger[‡*]

[†]Department of Medicine, Boston University School of Medicine, Boston, MA, USA

[‡]Department of Environmental Health, Boston University School of Public Health, Boston, MA, USA

[§]Department of Orthopaedic Surgery, Boston University School of Medicine, Boston, MA, USA

*Corresponding Author: jschlezi@bu.edu

Table of Contents

Table S1. Primers used for mRNA expression analyses.

Target	Gene Symbol	Reference Number	Qiagen Catalog Number
Adipocyte			
CEBPα	*Cebpa*	NM_007678	QT00311731
PPARγ1/2	*Pparg*	NM_011146 NM_001127330	QT00100296
FABP4	*Fabp4*	NM_024406	QT00091532
Osteoblast/Bone			
Alkaline Phosphatase	*Alpl*	NM_007431	QT01740221
DMP1	*Dmp1*	NM_016779	QT01078210
Osterix	*Osx*	NM_130458	QT00293181
Osteocalcin	*Bglap*	NM_007541	QT00259406
Runx2	*Runx2*	NM_009820	QT00102193
Nuclear Receptor			
ABCA1	*Abca1*	NM_013454	QT00165690*
ETS1	*Ets1*	NM_001038642	QT01070923
LXRα	*Lxra*	NM_013839	QT00113729
LXRβ	*Lxrb*	NM_009473	QT00093443
MMP13	*Mmp13*	NM_008607	QT00111104
RARα (isoform 2)	*Rara*	NM_001176528	QT00125958
RARβ (isoforms 2,4)	*Rarb*	NM_011243	QT00151956
RARγ (isoform 2)	*Rarg*	NM_001042727	QT01554322
RXRα	*Rxra*	NM_011305	QT00112658
SREBP1c	*Srebf1*	NM_011480	*****
TGM2	*Tgm2*	NM_009373	QT00169792
Normalization			
18sRNA	*Rn18s*	X00686	QT01036875

*** -** Primers sequences also from Padovani et al. 2010.[1]

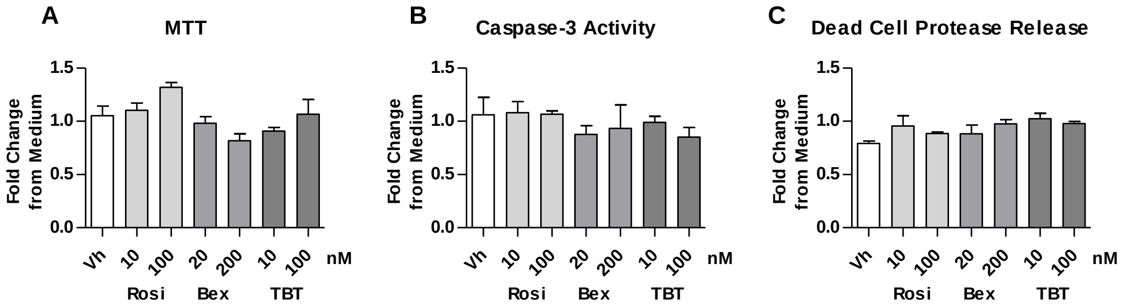

Figure S1. TBT does not cause overt toxicity at concentrations used *in vitro*. Primary bone marrow cells were isolated from 8-10 week old male C57BL/6J mice, plated, and allowed to adhere for 7 days. The MSC medium was replaced, and the cultures were treated with Vh (DMSO, 0.1%), rosiglitazone (Rosi), bexarotene (Bex) or TBT (10-200 nM) and incubated for 10 days. (A) Cellularity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) labeling for 3 hrs by standard methods. (B) Apoptosis assessed by measuring caspase-3 activity (Caspase-Glo® 3/7 Assay, Promega). (C) Necrosis was assessed by measuring dead cell protease release (CytoTox-Glo™ Cytotoxicity Assay, Promega). Absorbance and luminescence in experimental wells was normalized by dividing by that measured in medium wells, and data were reported as “Fold Change from Medium.” Data are presented as means ± SE of 4 independent bone marrow preparations. A 2hr treatment with 1 µM TBT was used as a positive control and induced significant caspase-3 activity and release of proteases (data not shown). There were no statistically significant differences (ANOVA).

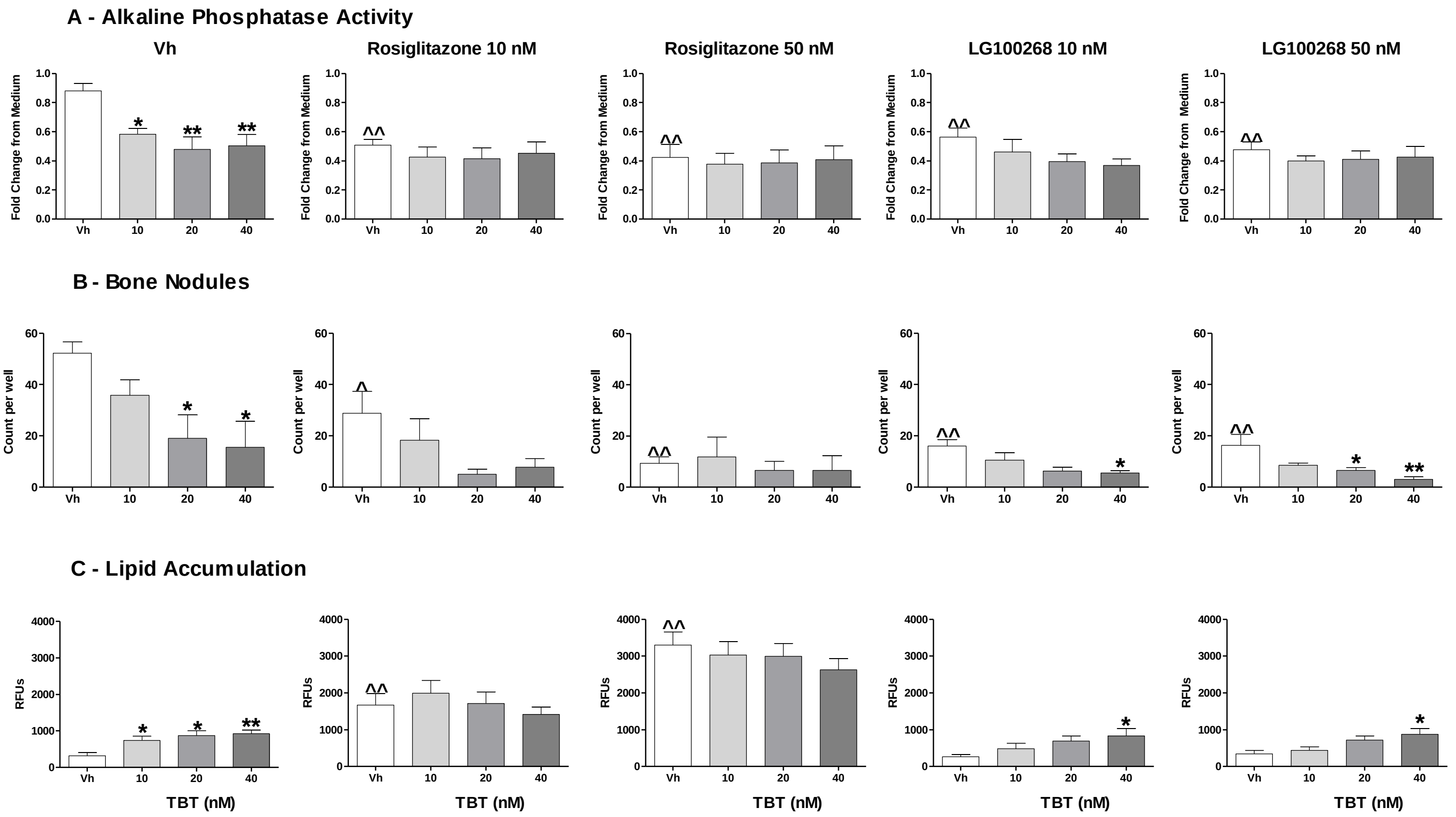

Figure S2. Effect of co-exposure to TBT and a PPARγ or an RXR ligand on BM-MSC differentiation. Primary bone marrow cells were isolated from 8-10 week old male C57BL/6 mice, plated, and allowed to adhere for 7 days. The medium was replaced with MSC medium containing the osteogenic additives, β-glycerol phosphate, ascorbate, insulin and dexamethasone. Naïve wells were left untreated. Cultures first were treated with Vh (DMSO, 0.1%) or TBT (10-40 nM) and then treated with Vh, rosiglitazone (10-50 nM) or LG100268 (10-50 nM), cultured for 10 days and analyzed for (A) alkaline phosphatase activity and (B) bone nodule number (Alizarin staining) and (C) lipid accumulation (Nile Red staining). Data are presented as means ± SE (n=4-7). Statistically different from Vh+Vh-treated (^$p<0.05$, ^^$p<0.01$, ANOVA, Dunnett's). Statistically different from Vh+agonist-treated (*$p<0.05$, **$p<0.01$, ANOVA, Dunnett's).

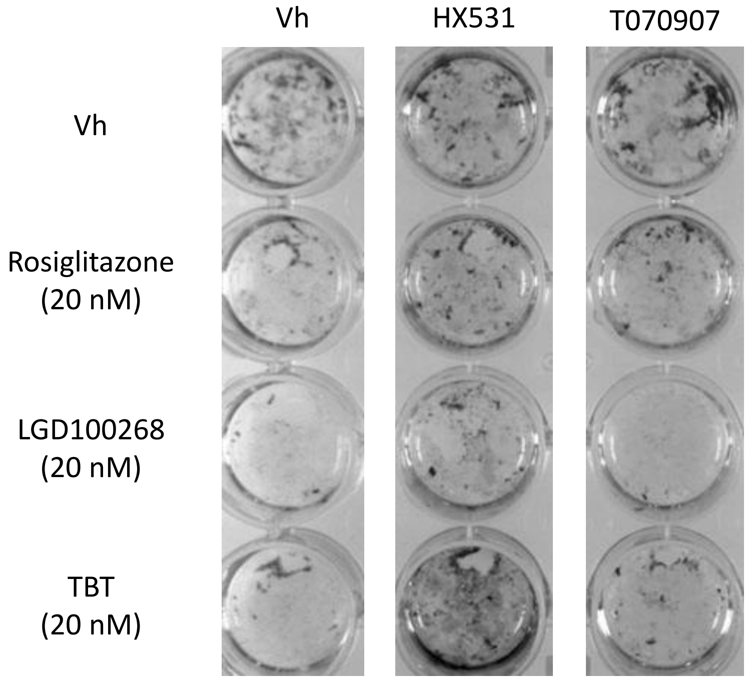

Figure S3. Representative experiment testing effect of RXR and PPARγ antagonism on TBT's effects on osteogenesis. Primary bone marrow cells were isolated from 8-10 week old male C57BL/6 mice, plated, and allowed to adhere for 7 days. The medium was replaced with MSC medium containing the osteogenic additives, β-glycerol phosphate, ascorbate, insulin and dexamethasone. Cultures first were treated with Vh, HX531 (RXR, 2 µM) or T0070907 (PPARγ, 2 µM) and then treated with Vh, rosiglitazone, LG100268 or TBT (20 nM), cultured for 10 days and stained with Alazarin Red. Quantification of alkaline phosphatase activity and bone nodule numbers from these experiments is presented in Figure 6. A representative culture is shown.

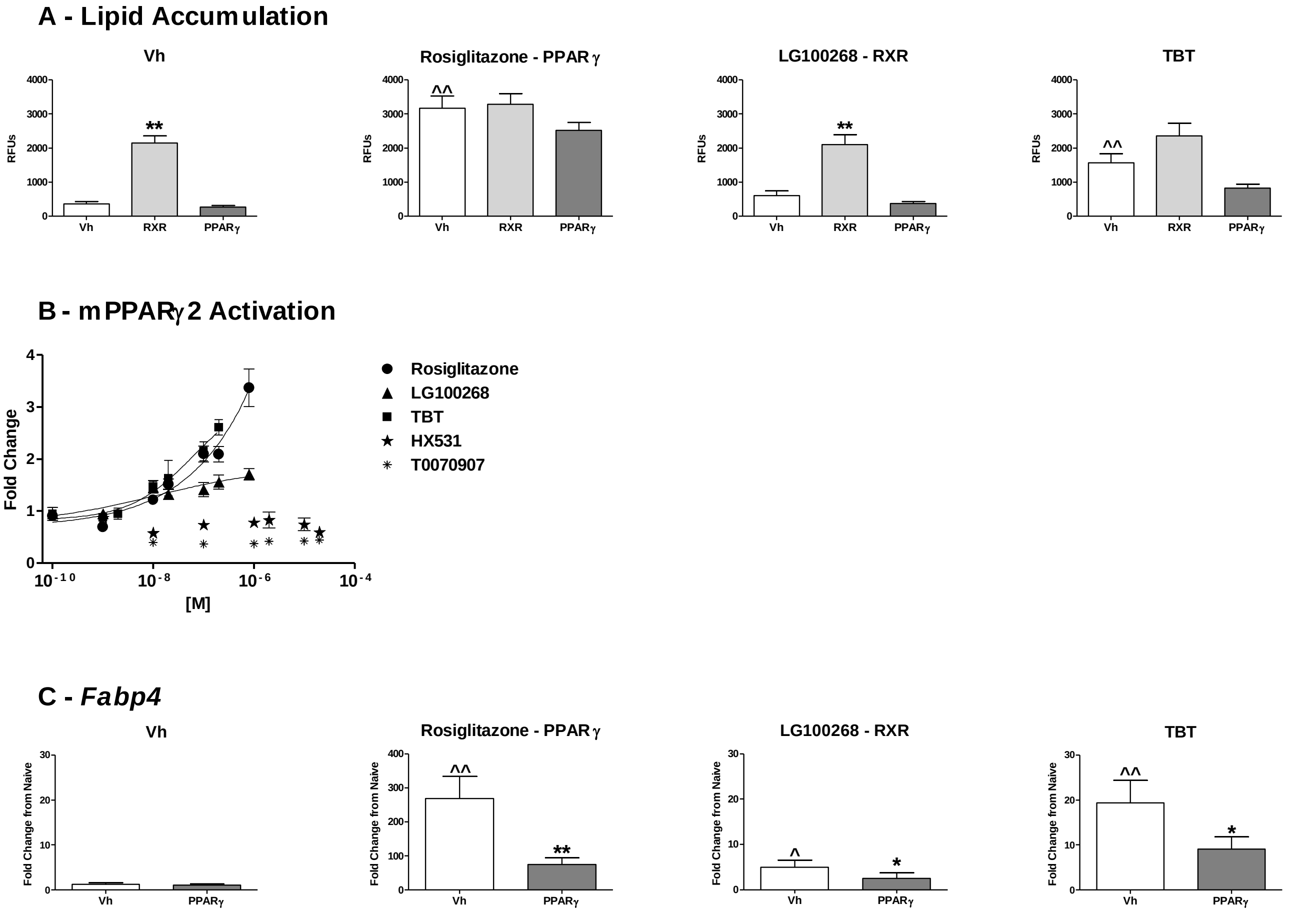

Figure S4. PPARγ and RXR antagonist effects on PPARγ activation and lipid accumulation. (A) Primary bone marrow cells were isolated from 8-10 week old male C57BL/6 mice, plated, and allowed to adhere for 7 days. The medium was replaced with MSC medium containing the osteogenic additives, β-glycerol phosphate, ascorbate, insulin and dexamethasone. Cultures first were treated with Vh (0.1%, DMSO), HX531 (RXR, 2 μM) or T0070907 (PPARγ, 2 μM) and then treated with Vh, rosiglitazone, LG100268 or TBT (20 nM), cultured for 10 days and stained with Nile Red. Data are presented as means ± SE (n=4-7). Statistically different from Vh+Vh-treated (^^p<0.01, ANOVA, Dunnett's). Statistically different from Vh+agonist-treated (*p<0.05, **p<0.01, ANOVA, Dunnett's). (B) Cos-7 cells were transfected with mouse PPARγ2 and a PPRE-luciferase reporter plasmid as previously described.[2, 3] Cultures were treated with Vh or the indicated compounds for 24 hrs. Luminescence normalized to constitutively expressed GFP fluorescence was divided by the normalized luminescence of untreated cultures to calculate fold change from untreated. Data are presented at means ± SE (n=3-4). (C) Primary bone marrow cultures were established, osteogenesis was initiated, and cultures were dosed as in A. At 48 hrs, RNA was isolated and analyzed for gene expression by RT-qPCR. Data are presented as means ± SE (n=5-6). Statistically different from Vh+Vh-treated (^p<0.05, ^^p<0.01, ANOVA, Dunnett's). Statistically different from Vh+agonist-treated (*p<0.05, **p<0.01, Student's T test).

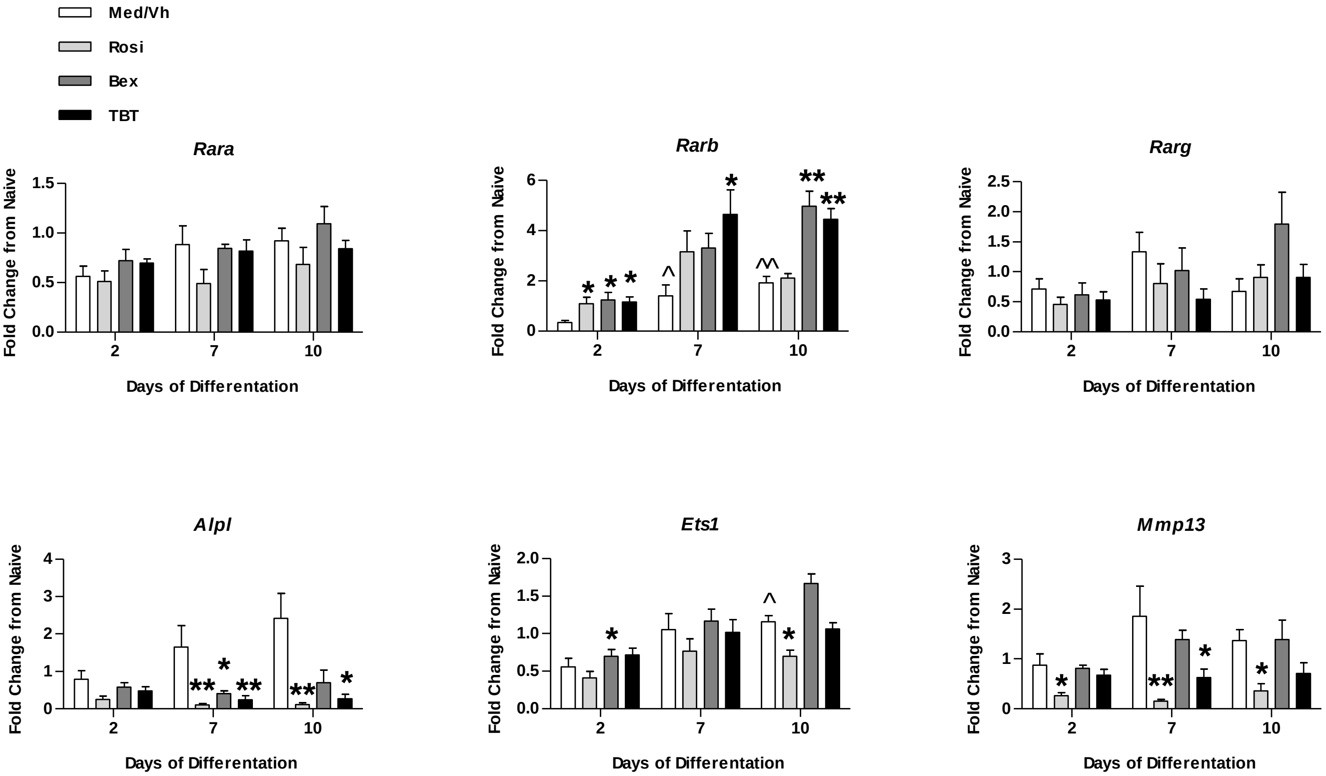

Figure S5. TBT does not stimulate expression of RAR-dependent genes. Primary bone marrow cells were isolated from 8-10 week old male C57BL/6 mice, plated, and allowed to adhere for 7 days. The medium was replaced with MSC medium containing the osteogenic additives, β-glycerol phosphate, ascorbate, insulin and dexamethasone. Naïve wells were left untreated. Cultures were treated with Vh (DMSO, 0.1%), rosiglitazone (Rosi), bexarotene (Bex) or TBT (10-100 nM), cultured for 2-10 days and analyzed for gene expression by RT-qPCR. Retinoic acid receptor (RAR) gene targets were chosen based on the fact that they are direct gene targets in the classical RAR pathway[4] and/or are relevant to bone biology, including *Rara*, *Rarb*, *Rarg*, alkaline phosphatase (*Alpl*),[5] E26 avian leukemia oncogene 1 (*Ets1*)[6] and matrix metallopeptidase 13 (*Mmp13*).[7] Statistically different from day 2, Vh-treated (^p<0.05, ^^p<0.01, ANOVA, Dunnett's). Data are presented as means ± SE (n=4-8). Statistically different from Vh-treated on the same day (*p<0.05, **p<0.01, ANOVA, Dunnett's).

References

(1) Padovani, A. M., Molina, M. F., and Mann, K. K. (2010) Inhibition of liver x receptor/retinoid x receptor-mediated transcription contributes to the proatherogenic effects of arsenic in macrophages in vitro. *Arterioscler. Thromb. Vasc. Biol*. *30*, 1228-1236.

(2) Watt, J., and Schlezinger, J. J. (2015) Structurally-diverse, ppargamma-activating environmental toxicants induce adipogenesis and suppress osteogenesis in bone marrow mesenchymal stromal cells. *Toxicology*. *331*, 66-77.

(3) Yanik, S. C., Baker, A. H., Mann, K. K., and Schlezinger, J. J. (2011) Organotins are potent activators of ppar{gamma} and adipocyte differentiation in bone marrow multipotent mesenchymal stromal cells. *Toxicol. Sci*. *122*, 476-488.

(4) Balmer, J. E., and Blomhoff, R. (2002) Gene expression regulation by retinoic acid. *J. Lipid Res*. *43*, 1773-1808.

(5) Hisada, K., Hata, K., Ichida, F., Matsubara, T., Orimo, H., Nakano, T., Yatani, H., Nishimura, R., and Yoneda, T. (2013) Retinoic acid regulates commitment of undifferentiated mesenchymal stem cells into osteoblasts and adipocytes. *J. Bone Miner. Metab*. *31*, 53-63.

(6) Raouf, A., and Seth, A. (2000) Ets transcription factors and targets in osteogenesis. *Oncogene*. *19*, 6455-6463.

(7) Jimenez, M. J., Balbin, M., Alvarez, J., Komori, T., Bianco, P., Holmbeck, K., Birkedal-Hansen, H., Lopez, J. M., and Lopez-Otin, C. (2001) A regulatory cascade involving retinoic acid, cbfa1, and matrix metalloproteinases is coupled to the development of a process of perichondrial invasion and osteogenic differentiation during bone formation. *J. Cell Biol*. *155*, 1333-1344.