

Table 1: The spike factors for various organs/tissues

Species	Organ/Tissue (adult, unless otherwise noted)	Spike Factor	total RNA/genomic DNA	SD
Mouse	Liver	0.2	211	46
Mouse	Lung	0.02	22	4
Mouse	Heart	0.05	-	-
Mouse	Thymus	0.01	8	2
Mouse	Colon Epithelium	0.05	105	30
Mouse	Kidney	0.1	-	-
Mouse	Brain	0.1	-	-
Mouse	Suprachiasmatic nucleus (SCN)	0.1	-	-
Mouse	Hypothalamus	0.1	63	4
Mouse	Pituitary	0.1	52	8
Mouse	Ovary	0.02	35	4
Mouse	Uterus	0.02	42	12
Mouse	Vagina	0.02	81	38
Mouse	Testis	0.15	56	7
Mouse	Epididymis	0.07	53	16
Mouse	Bone marrow	0.02	14	3
Mouse	Spleen	0.02	-	-
Mouse	Whole Embryo	0.15	97	36
Mouse	Fetal Telencephalon E10.5-16.5	0.1	48	9
Mouse	Neurosphere (E11.5-14.5)	0.03	42	10
Mouse	E9.5 embryo heart	0.15	58	15
Mouse	cell lines	0.2	-	-
Rat	Liver	0.2	-	-
Rat	Kidney	0.2	-	-
Rat	Uterus	0.04	56	5
Rat	Ovary	0.04	56	9
Human	Cancer Cell Lines	0.2	116	26
Xenopus	liver	0.03	-	-
Xenopus	embryo	0.15	-	-

temperature was set between 60° and 65°C with a product size of approximately 100 base pairs using an algorithm (nearest neighbor method, TAKARA BIO Inc., Japan), and the amplification co-efficiency (E) was set within the range 0.9 ± 0.1 ($E = 2^{\{-1/\text{slope}\}} - 1$) on a plot of \log_2 (template) against Ct value). For the GeneChip system, the signal/copy performance of each probe set depended on the strategy of designing the probes to keep the hybridization constant/melting temperature within a narrow range, ensuring that the dose-response performances of the probe sets were similar (cf. <http://www.affymetrix.com/technology/design/index.affx>). Failing this, any differences should at least be kept constant within the same make/version of the GeneChip. Taking into consideration the biases that lead to imperfections in estimating absolute copy numbers in each gene/probe set, we developed normalization methods to set up a common scale for Q-PCR and Affymetrix GeneChip systems.

The grade-dosed spike cocktail (GSC) and the "spike factor" for the Percellome method

A set of external spike mRNAs was used to transfer the measurement of cell number in the sample (as reflected by its DNA content) to transcriptome analysis. For the

spikes, we utilized five *Bacillus subtilis* mRNAs that were left open for users in the Affymetrix GeneChip series. The extent to which the *Bacillus* RNAs cross-hybridized with other probe sets was checked for the Affymetrix GeneChip system. The GSC was applied to Murine Genome U74Av2 Array (MG-U74v2) GeneChips with or without a liver sample. As shown in Figure 2, cross-hybridization between *Bacillus* RNAs and the murine gene probe sets was negligible [see Additional files 3 and 4]. Mouse Genome 430 2.0 Array (Mouse430-2), Mouse Expression Arrays 430A (MOE430A) and B (MOE430B), Rat Expression Array 230A (RAE230A), *Xenopus laevis* Genome Array and Human Genome U95Av2 (HG-U95Av2) and U133A (HG-U133A) Arrays sharing the same probe sets for these spike mRNAs showed no sign of cross-hybridization with the *Bacillus* probes (data not shown).

We prepared a cocktail containing in vitro transcribed *Bacillus* mRNAs in threefold concentration steps, i.e. 777.6 pM (for AFFX-ThrX-3_at), 259.4 pM (for AFFX-LysX-3_at), 86.4 pM (for AFFX-PheX-3_at), 28.8 pM (for AFFX-DapX-3_at) and 9.6 pM (for AFFX-TrpX-3_at). By referring to the amount of DNA in a diploid cell and employing a "spike factor" determined by the ratio of

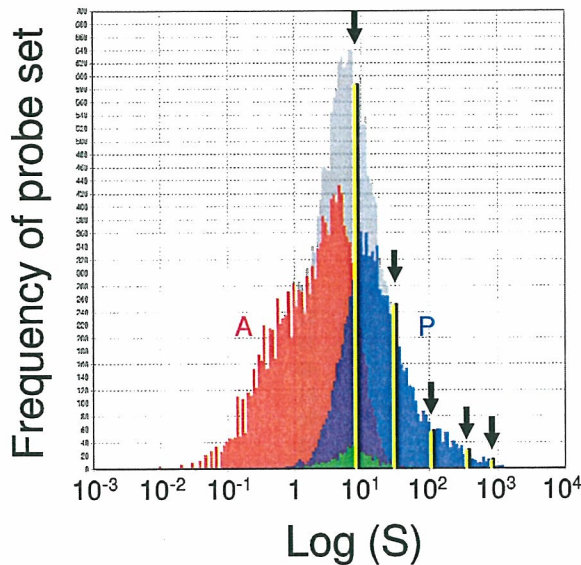


Figure 3
Positioning of GSC spike mRNAs in Affymetrix GeneChip dose-response range. A frequency histogram of the probe sets of Affymetrix GeneChip Mouse430-2 is shown. The histogram for all probe sets (gray) shows near-normal distribution. Blue columns are the "present" calls (P), red columns "absent" calls (A) and green "marginal" calls. The five yellow lines indicate the positions of the GSC spike mRNAs that are chosen to cover the "present" call range by a proper "spike factor".

total RNA to genomic DNA in a tissue type (Table 1), the spike mRNAs were calculated to correspond to 468.1, 156.0, 52.0, 17.3 and 5.8 copies per cell (diploid), respectively, for the mouse liver samples (spike factor = 0.2). The ratio of mRNAs in the cocktail is empirically chosen depending on the linear range of the measurement system and the available number of spikes. Here, we set the ratio to three to cover the "present" call probe sets of the Affymetrix GeneChip system (Figure 3).

We tested this grade-dosed spike cocktail (GSC) by Q-PCR and confirmed that the Ct values of the spike mRNAs were linearly related to the log concentrations (cf. Figure 4a), i.e. could be expressed as

$$Ct = \alpha \log C + \beta \quad \{1\}$$

The GSC was also tested by the GeneChip system and it was confirmed that the log of the spike mRNA signal intensities was linearly related to the log of their concentrations (cf. Figure 4b),

$$\log S = \gamma \log C + \delta \quad \{2\}$$

The linear relationship between the Ct values (Ct) and the log of RNA concentration (log C) was reasonable given the definition of Ct values (derived from the number of PCR cycles, i.e. doubling processes). The linear relationship between the log of GeneChip signal intensity (log S) and the log of RNA concentration (log C) was rationalized by the near-normal distribution of log S over all transcripts (cf. Figure 3).

Calculation of copy numbers of all genes/probe sets per cell

As described above, using a combination of DNA content and the spike factor of the sample, the GSC spike mRNAs become direct indicators of the copy numbers (C') per cell. When the samples were measured by Q-PCR or GeneChip analysis, the five GSC spike signals in each sample should obey function {1} for Q-PCR and function {2} for GeneChip with a good linearity. If the observed linearity was poor, a series of quality controls was performed and the measurement repeated. The coefficients of the functions were determined for each sample by the least squares method. Under the assumption that all genes/probe sets share the same signal/copy relationship, signal data for all genes/probe sets were fitted to the functions {1'} or {2'}, which are the individualized functions of {1} and {2} for each sample measurement (i).

$$Ct = \alpha_i \log(C') + \beta_i \quad \{1'\}$$

$$\log(S) = \gamma_i \log(C') + \delta_i \quad \{2'\}$$

(i = sample measurement no.)

The Q-PCR Ct values (Ct) and microarray signal values (S) of all mRNA species in the sample (i) are converted to copy numbers per cell (C') by the inverses of functions {1'} and {2'}, i.e. {3} and {4} below:

$$C' = B^{((Ct-\beta_i)/\alpha_i)} \quad \{3\}$$

for Q-PCR (Figure 4a);

$$C' = B^{((\log S-\gamma_i)/\delta_i)} \quad \{4\}$$

for GeneChips (Figure 4b),

where B is the logarithmic base used in {1} and {2} (see Materials and Methods for details).

Real world performance of the Percellome method

The correspondence between Q-PCR and GeneChip was tested using a sample set from 2,3,7,8-tetrachlorodiben-zodioxin (TCDD)-treated mice. Sixty male C57BL/6 mice

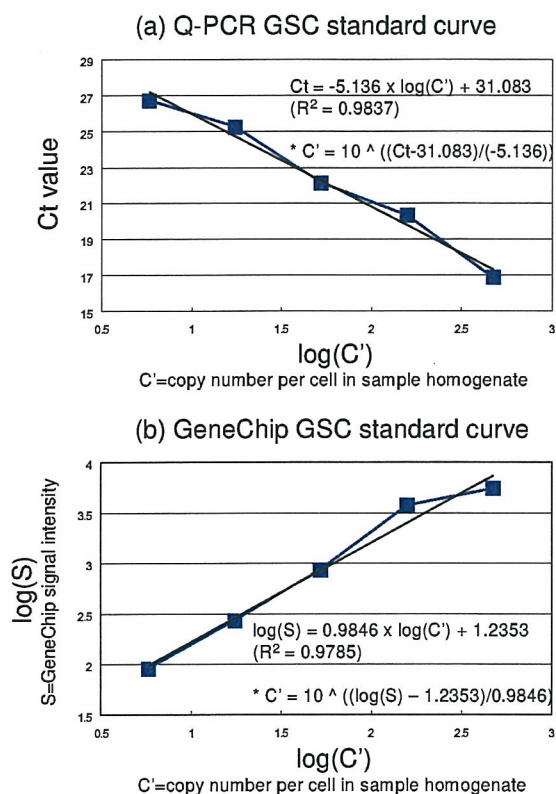


Figure 4
The dose-response linearity of the GSC spikes in Q-PCR and the Affymetrix GeneChip array system. Linear relationships are shown between (a) the Q-PCR Ct values and log of copy number ($\log(C')$), and (b) the GeneChip log signal intensity ($\log(S)$) and log of copy number ($\log(C')$) of the GSC mRNAs. The regression functions were obtained by the least squares method. The inverse functions (*) were further used to generate the copy numbers of all other genes/probe sets for Percellome normalization.

were divided into 20 groups of 3 mice each. TCDD was administered once orally at doses of 0, 1, 3, 10 and 30 $\mu\text{g}/\text{kg}$, and the livers were sampled 2, 4, 8 and 24 h after administration. Nineteen primer pairs were prepared for Q-PCR and the Ct values of the liver transcriptome were measured. The same 60 liver samples were measured using the Affymetrix Mouse430-2 GeneChip [see Additional files 5 through 8 and 9 through 12]. Q-PCR and GeneChip data were normalized against cell number by functions {3} and {4}, respectively. The averages and standard deviations (sd) of each group ($n = 3$) were calculated and plotted as three layers of isoborograms on to 5×4 matrix three-dimensional graphs (Figure 5). Together with another sample set (data not shown), a total of thirty-six primer pairs were compared, and there was a

correlation of up to 90% between the Q-PCR and GeneChip surfaces. It is notable that not only the average surfaces but also the +1sd and -1sd surfaces corresponded closely in shape and size. We infer that the differences resulted mainly from biological variations among the three animals in each experimental group rather than from measurement error (cf. Figure 7).

An important feature of Percellome normalization is its independence from the overall expression profile of the sample. When gene expression profiles differ among samples, Percellome normalization produces a robust transcriptome that is different from total-RNA dependent global normalization. As an example, Figure 6 shows the results of an experiment on the uterotrophic response of ovariectomized mice to estrogen treatment [12] [see Additional files 13 and 14]. The uteri of the vehicle control are atrophic because the ovaries, the source of intrinsic estrogens, are absent. The uteri of the treated groups are hypertrophic owing to estrogenic stimulus from the test compound administered. Global normalization (90 percentile) between the vehicle control group and the high-dose (1,000 mg/kg) group indicated that 4,600 of 12,000 probe sets showed 2-fold or greater increase, 470 were reduced by 0.5 or less, and 7,400 remained between these extremes. In contrast, analysis of Percellome-normalized data revealed that almost all the 12,000 probe sets showed a 2-fold or greater increase, including actin, GAPDH and other housekeeping genes. The hypertrophic tissues, consisting of cells with abundant cytoplasm, provide convincing evidence for the increases in various cellular components including housekeeping gene products.

Another important feature of Percellome normalization is the commonality of the expression scale across platforms. Batch conversion can be performed between results obtained from different platforms when the data are generated by the Percellome method. A practical strategy for such normalization is to prepare a set of samples from a target organ of interest with differences in gene expression, and measure them once by each platform. Data conversion functions with good linear dose-response relationships can be obtained individually for those genes/probe sets that are measured by both platforms (Figure 7).

Discussion

We have developed a novel method for normalizing mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration. For non-diploid or aneuploid samples, an average DNA content per cell should be determined beforehand for accurate adjustment. When there is significant DNA synthesis, a similar adjustment should be considered.

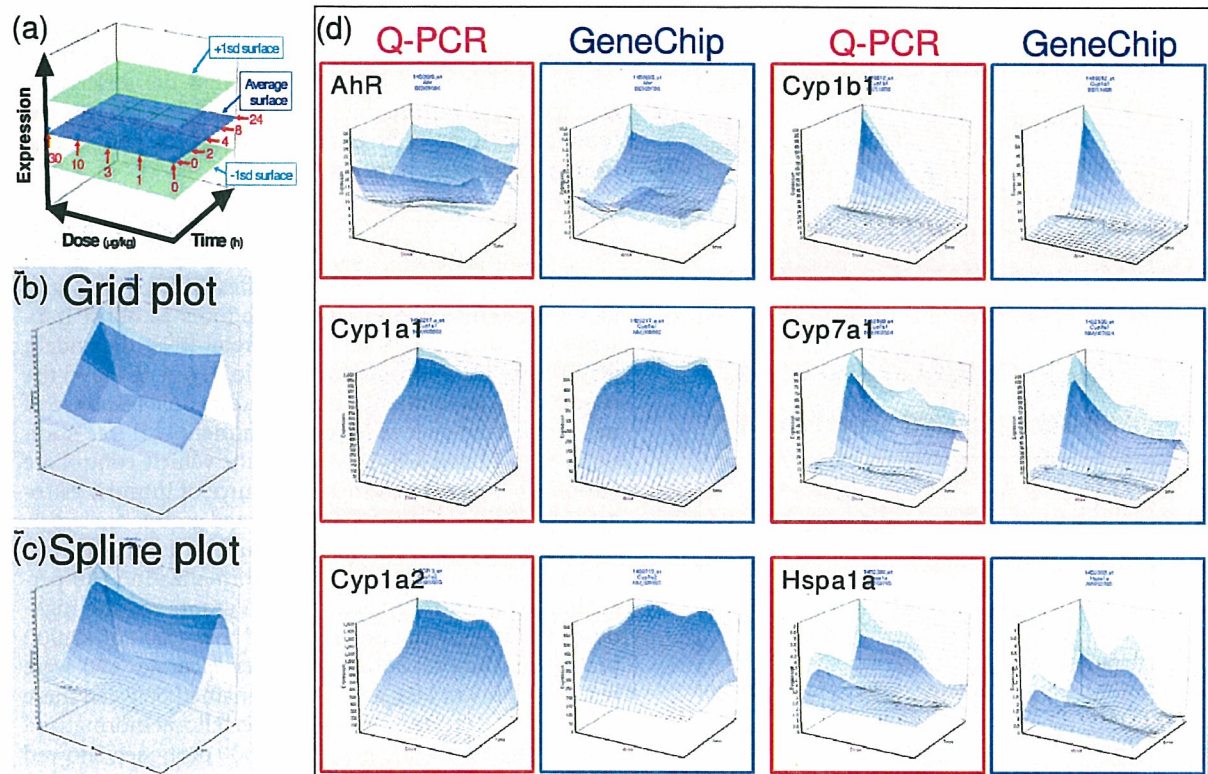


Figure 5

Correspondence between Q-PCR and GeneChip data. Sixty male C57BL/6 mice were divided into 20 groups of 3 mice each. 2,3,7,8-tetrachlorodibenzodioxin (TCDD) was administered once orally at doses of 0, 1, 3, 10 and 30µg/kg, and the liver was sampled 2, 4, 8 and 24 h after administration. The liver transcriptome was measured by the Affymetrix Mouse430-2 GeneChip. For Q-PCR, nineteen primary pairs were prepared and the Ct values of the same 60 liver samples were measured (19 genes and 5 spikes in duplicate, using a 96-well plate for 2 samples, total 30 plates). The Percellome data were plotted on to 3-dimensional graphs for average, +1sd, and -1sd surfaces as shown in (a). The scale of expression (vertical axis) is the copy number per cell. The 0 h data (*) are copied from the 2 h/dose 0 point for better visualization of the changes after 2 h. The surfaces are demonstrated as a grid plot (b) where the grid points indicate one treatment group (n = 3), and a smoothed spline surface plot (c) for easier 3D recognition ((b), (c)). *Gys2* (glycogen synthase 2, 1424815_at) showing a typical circadian pattern. (d) the smoothed plots of 6 representative genes/ probe sets generated by Q-PCR (red) and GeneChip (blue). AhR (arylhydrocarbon receptor, 1450695_at) showed imperfect correspondence. *Cyp1a1* (cytochrome P450, family 1, subfamily a, polypeptide 1, 1422217_a_at) and *Cyp1a2* (1450715_at) showed good correlations between Q-PCR and GeneChip except for the saturation in GeneChips above c. 400 copies per cell. *Cyp1b1* (1416612_at) and *Cyp7a1* (1422100_at) showed good correspondence. *Hspa1a* (heat shock protein 1A, 1452888_at) showed fair correspondence despite low copy numbers, near the nominal detection limit of the Affymetrix GeneChip system.

The smallest sample to which we have successfully applied the direct DNA quantification method with sufficient reproducibility is the 6.75 dpc (days post coitus) mouse embryo which consists of approximately 5,000 cells. This sample size is also approximately the lower limit for double amplification protocol to obtain sufficient amount of RNA for Affymetrix GeneChip measurement (cf. http://www.affymetrix.com/Auth/support/downloads/manuals/expression_print_manual.zip.) High-resolution technology such as laser-capture micro-

dissection (LCM) has become popular and the average sample size analyzed is getting smaller. An alternative method for LCM samples is to count the cell number in the course of microdissection. Although we have not yet applied Percellome method to LCM samples, we have applied the alternative method to cell culture samples to gain Percellome data. Stereological and statistical calculations should become available to correct the number of partially sectioned cells in the LCM samples. Another issue for small samples is the yield of RNA. Approximately

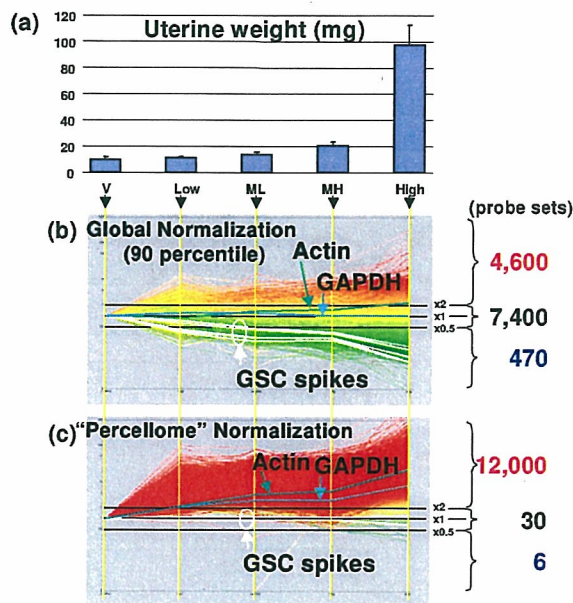


Figure 6
Uterotrophic response of ovariectomized female mice by an estrogenic test compound. (a) Shows the uterine weight, which increases in a dose-dependent manner; V, vehicle control; Low, low dose; ML, medium-low dose; MH, medium-high dose; High, high dose group. (b) Shows the line display of uterine gene expression (Affymetrix MG-U74v2 A GeneChips) normalized by global normalization (90 percentile), and (c) by the Percellome normalization. Averages of three samples per group were visualized (by K. A.). The five white lines are the GSC mRNAs. The green and blue lines are actin (AFFX-b-ActinMur/M12481_3_at) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, AFFX-GapdhMur/M32599_3_at), respectively. By global normalization, 7,400 probe sets remained unchanged and 4,600 probe sets increased more than two-fold in the H group compared to the V group, whereas almost all probe sets measured had increased. It is noted that housekeeping genes such as actin and GAPDH are significantly induced on a per cell basis.

30 ng of total RNA is retrieved from a single 6.75 dpc mouse embryo. This amount is sufficient for a double amplification protocol (DA) to prepare enough RNA for an Affymetrix GeneChip measurement. An inherent problem with the DA data is that the gene expression profile differs from that of the default single amplification protocol (SA). Consequently the DA percellome data differ from that of SA as if they were produced by a different platform. To bridge the difference, we applied the procedure that was used for data conversion between Q-PCR

and GeneChip (cf. Figure 7). A set of spiked-in standard samples including the LBM sample set (of sufficient concentration) were measured by the SA protocol and diluted versions to the limit measured by the DA protocol. These data provided us with information about whether DA was successful as a whole (by comparing 5' signal to 3' signals of selected probe sets) and which probe sets were properly amplified by DA (by checking the linearity of the diluted LBM data). For those probe sets that proved to be linearly amplified, conversion functions between DA and SA were generated. These details, along with embryo expression data will be published elsewhere.

Figures 5 and 7 indicate a close correspondence between the data generated by Q-PCR and GeneChip analyses. Since each of the 60 samples was normalized individually against each GSC signal, the high similarity between the two platforms indicates the robustness and stability of this spike system (cf. Figure 7, Cyp7a1 data). Although more spikes could potentially increase the accuracy of normalization, our experience is that five spikes are practically sufficient for covering the detection range of GeneChip microarrays and Q-PCR, as long as they are used in combination with the "spike factor". The overall benefits of using a minimum number of external spikes include lower probability of cross-hybridization, a reduced number of wells and spots occupied by the spikes in the Q-PCR plates and small scale microarrays, and less effort in preparation, QC and supply.

The Percellome data can be truly absolute when all mRNA measurements including GSC spikes are strictly proportional to the original copy numbers in the sample homogenate. As noted earlier, this condition is not guaranteed by any platform despite linearity of response. Therefore, the Percellome-normalized values have some biases for each primer pair/probe set, depending on the steepness of the dose-response curves. An advantage of Percellome normalization is that, as long as such biases are consistently reproduced within a platform, the data can be compared directly among samples/studies on a common scale. Consequently, when a true value is obtained by any other measure, all the data obtained in the past can be simultaneously batch-converted to the true values.

This batch-conversion strategy can be extended to data conversion between different versions and different platforms, as long as the data are generated in copy numbers "per cell". We have shown an example between Affymetrix GeneChip and Q-PCR for limited numbers of probe sets (cf. Figure 7). Custom microarrays that accept our GSC for Percellome normalization are in preparation by Agilent Technologies (single color) and GE Healthcare (CodeLink Bioarray).

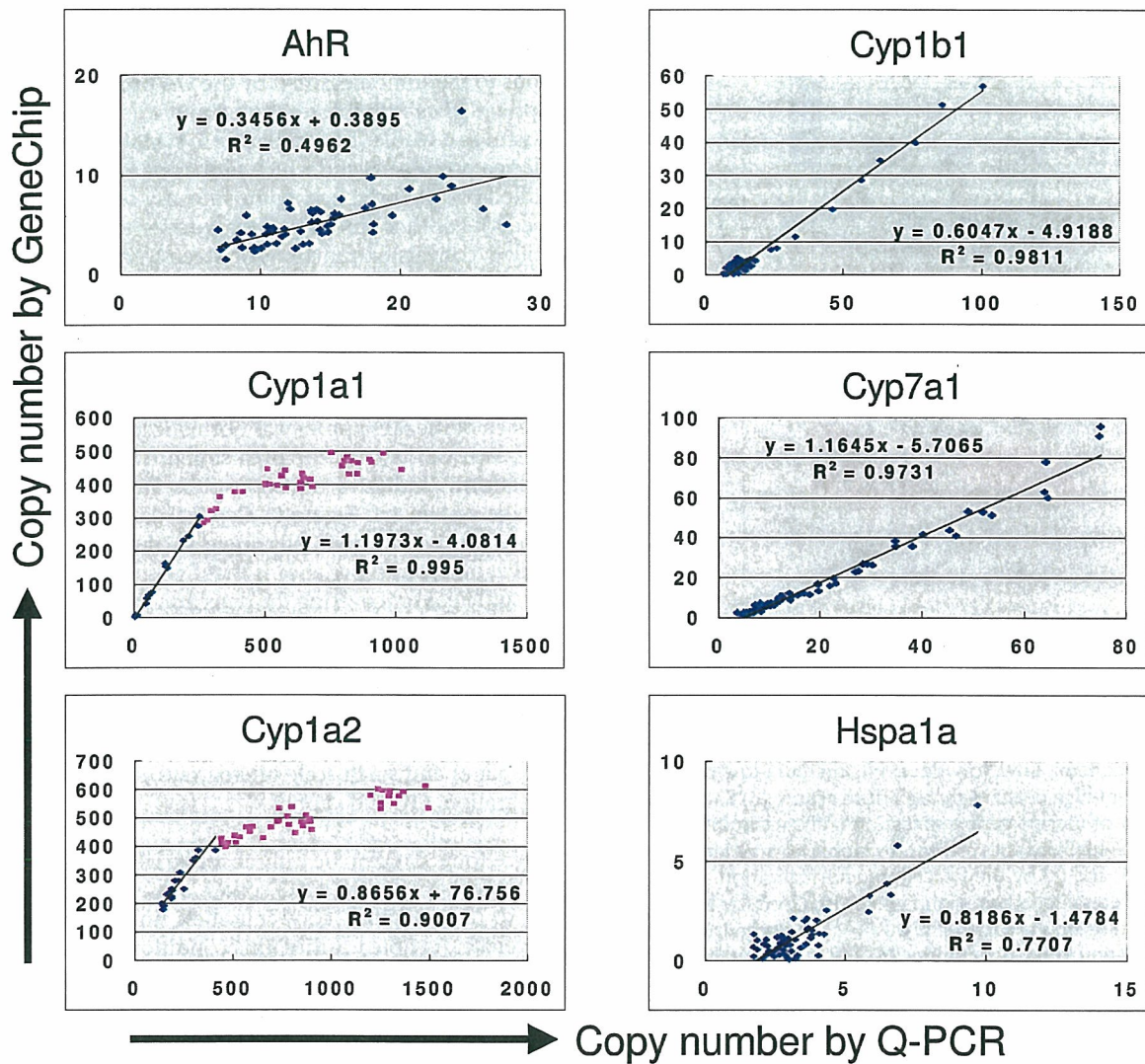


Figure 7

Conversion functions between Q-PCR and GeneChip. The data shown in Figure 5 as 3D surfaces are shown as a scatter plot (60 plots). The regression function can be used to convert Q-PCR to GeneChip and vice versa, with a level of certainty indicated by coefficient of correlation. It is noted that Cyp1a1 and Cyp1a2 became saturated above about 400 copies per cell in GeneChip system (indicated in pink plots). Cyp7a1 showed high linearity, indicating that the variation shown by the split +1sd and -1sd surfaces in Figure 5 reflected biological (animal) variation, not measurement errors.

Another important contribution of Percellome analysis is in the area of archived data in private and public domains. Firstly, Percellome data are the result of a simple linear transformation of the raw microarray data; preserving the distribution and order of the probe set data. Therefore, parametric or non-parametric methods should be able to align the data distribution and generate estimates of mRNA copy number of the non-spiked archival samples.

Any archival samples that are re-measurable by Percellome method will greatly increase the accuracy of estimation. Secondly, percillome can provide appropriate bridging information between old and new versions of Affymetrix GeneChips, such as human HU-95 and HU-133, murine MU-74v2 and MOE430 series. This should also facilitate comparisons between newly generated and archived data.

The Percellome method was developed for a large-scale toxicogenomics project [13] using the Affymetrix GeneChip system. It was intended to compile a very large-scale database of comprehensive gene expression profiles in response to various chemicals from a series of experiments conducted over an extended time period. However, the method also proved to be useful for small-scale platforms such as 96 well plate-based Q-PCRs as shown above, and probably for small-scale targeted microarrays. In both cases, highly inducible or highly transcribed genes are likely to be selected. Therefore, the expression profiles may differ significantly among samples such that profile-dependent normalization (e.g. global normalization) may not be applicable. In such cases, the profile-independent nature of the Percellome method provides a robust normalization.

To demonstrate the profile-independence of the Percellome method, we chose an extreme case – the uterotrophic response assay (cf. Figure 6). The treated uteri were composed of hypertrophic cells with abundant cytoplasm whereas the untreated uteri were composed of hypoplastic cells with scant cytoplasm. This indicates that the uteri of untreated ovariectomized mice were quiescent, and that a majority of the inducible genes were probably transcriptionally inactive. Therefore, the identification of most genes as being induced by 2-fold or greater is reasonable and expected. In most *in vivo* experiments, the gene profiles of the samples are much more similar. However, there is always a set of genes that is found to be "increased" when analyzed on a "per one cell" basis that are declared to be "decreased" by global type normalization, or vice versa. Such increase/decrease calls made by the global type normalization can differ according to the normalization parameters. In both cases, the Percellome method can inform the researcher how much the expression profiles are distorted by the treatment, such as in the case of the uterotrophic assay. We also note that *in vitro* experiments such as cell-based studies tend to generate data similar to that of uterotrophic experiment.

Conclusion

Percellome data can be compared directly among samples and among different studies, and between different platforms, without further normalization. Therefore, "percellome" normalization can serve as a standard method for exchanging and comparing data across different platforms and among different laboratories. We hope that the Percellome method will contribute to transcriptome-based studies by facilitating data exchanges among laboratories.

Methods

Animal experiments

C57BL/6 Cr Slc (SLC, Hamamatsu, Japan) mice maintained in a barrier system with a 12 h photoperiod were

used in this study. For the liver transcriptome experiments, twelve week-old male mice were given a single dose of the test compound by oral gavage, and the liver was sampled at 2, 4, 8 and 24 h post-gavage. For the uterotrophic experiment, 6 week old female mice were ovariectomized 14 days prior to the 7 day repeated subcutaneous injection of a test compound [12]. Animals were euthanized by exsanguination under ether anesthesia and the target organs were excised into ice-cooled plastic dishes. Tissue blocks weighing 30 to 60 mg were placed in an RNase-free 2 ml plastic tube (Eppendorf GmbH., Germany) and soaked in RNAlater (Ambion Inc., TX) within 3 min of the beginning of anesthesia. Three animals per treatment group were used and individually subjected to transcriptome measurement.

Sample homogenate preparation

The tissue blocks soaked in RNAlater were kept overnight at 4°C or until use. RNAlater was replaced in the 2 ml plastic tube with 1.0 ml of RLT buffer (Qiagen GmbH., Germany), and the tissue was homogenized by adding a 5 mm diameter Zirconium bead (Funakoshi, Japan) and shaking with a MixerMill 300 (Qiagen GmbH., Germany) at a speed of 20 Hz for 5 min (only the outermost row of the shaker box was used).

Direct DNA quantitation

Three separate 10 µl aliquots were taken from each sample homogenate to another tube and mixed thoroughly. A final 10 µl aliquot therefrom was treated with DNase-free RNase A (Nippon Gene Inc., Japan) for 30 min at 37°C, followed by Proteinase K (Roche Diagnostics GmbH., Germany) for 3 h at 55°C in 1.5 ml capped tubes. The aliquot was transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes Inc., USA) was added to each well, shaken for 10 seconds four times and then incubated for 2 min at 30°C. The DNA concentration was measured using a 96 well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. λ phage DNA (PicoGreen Kit, Molecular Probes Inc., USA) was used as standard. Measurement by this PicoGreen method and the standard phenol extraction method correlated well (coefficient of correlation = 0.97, data not shown). The smallest sample size for reproducible and reliable DNA quantitation is about 5,000 cells that corresponds to a 6.75 dpc mouse embryo.

The grade-dosed spike cocktail (GSC)

The following five *Bacillus subtilis* RNA sequences were selected from the gene list of Affymetrix GeneChip arrays (AFFX-ThrX-3_at, AFFX-LysX-3_at, AFFX-PheX-3_at, AFFX-DapX-3_at, and AFFX-TrpX-3_at) present in the MG-U74v2, RG-U34, HG-U95, HG-U133, RAE230 and MOE430 arrays: thrC, thrB genes corresponding to nucleotides 248–2229 of X04603; lys gene for diami-

nopimelate decarboxylase corresponding to nucleotides 350–1345 of X17013; pheB, pheA genes corresponding to nucleotides 2017–3334 of M24537, dapB, jojF, jojG genes corresponding to nucleotides 1358–3197 of L38424; TrpE protein, TrpD protein, TrpC protein corresponding to nucleotides 1883–4400 of K01391. The corresponding cDNAs were purchased from ATCC, incorporated into expression vectors, amplified in *E. coli* and transcribed using the MEGAscript kit (Ambion Inc., TX). The mRNA was purified using a MACS mRNA isolation kit (Miltenyi Biotec GmbH., Germany). The concentrations of spike RNAs in the GSC were in threefold steps, from 777.6 pM for AFFX-ThrX-3_at, 259.4 pM for AFFX-LysX-3_at, 86.4 pM for AFFX-PheX-3_at, 28.8 pM for AFFX-DapX-3_at, to 9.6 pM for AFFX-TrpX-3_at. In general, the ratio depends on the linear range of the measurement system and the available number of spikes.

Setting of the "spike factor" and addition of GSC to a sample homogenate according to its DNA concentration

The GSC was added to the sample homogenates in proportion to their DNA concentrations, assuming that all cells contain a fixed amount of genomic DNA (g/cell) across samples. The amount of GSC added to each sample G (l) was given as

$$G = C * v * f \quad (1),$$

where C is the DNA concentration (g/l), v (l) is the volume of homogenate further used for RNA extraction and f (l/g) is the "spike factor", which is an adjustment factor to ensure that the sample is properly spiked by the GSC (cf. Figure 3). Spike factors have been pre-determined for various organs/tissues to reflect differences in their total RNA/genomic DNA ratios (cf. Table 1). In this way, five spike mRNA signals can properly cover the linear dose-response range of the platform. In practice, for the Affymetrix GeneChips, the spike factor is set so that the five GSC spikes cover the range of "Present" calls given by the Affymetrix system, which corresponds to approximately 80 to 7000 in raw readouts given by the Affymetrix MAS5.0 software. A raw readout of 10 by the current Affymetrix GeneChip system corresponds to approximately one copy per cell in mouse liver (spike factor = 0.2), whereas in mouse thymus (spike factor = 0.01) it corresponds to approximately 0.05 copy per cell. For Q-PCR, the same spike factor corresponds to Ct values ranging approximately from 17 to 27, which is well within the linear range of Q-PCR (data not shown).

"Per cell" normalization (Percellome normalization)

Since murine haploid genomic DNA is made of 2.5×10^9 base pairs and one base pair is approximately 600 Daltons (Da), the haploid genomic DNA weighs 1.5×10^{12} Da, corresponding to

$$d = 5 \times 10^{-12} \text{ (g DNA per diploid cell).}$$

Therefore, the cell number per liter of the sample homogenate (N) is given as

$$N = C/d \text{ (cells/l)}$$

where C is the DNA concentration (g/l).

On the other hand, the copy numbers of GSC RNAs in the homogenate are given as follows:

if S_j (mole/l) ($j = 1,2,3,4,5$) is the mole concentration of one of the five spike RNAs in the GSC solution and G (l) is the amount of GSC added to each homogenate, the mole concentrations of the spike RNAs in the homogenate (CS_j) are given as,

$$CS_j = S_j * C * f \text{ (mole/l).}$$

The GSC RNAs in moles per cell (MS_j) are given as

$$MS_j = CS_j / N \\ = S_j * C * f / (C/d)$$

$$= S_j * f * d \text{ (mole/cell)}$$

The copy numbers of the GSC RNAs per cell (NS_j) are given as

$$NS_j = MS_j * A \\ = S_j * f * d * A \text{ (copies per diploid cell)}$$

where A is Avogadro's number.

As a result, the GSC spikes AFFX-TrpX-3_at, AFFX-DapX-3_at, AFFX-PheX-3_at, AFFX-LysX-3_at and AFFX-ThrX-3_at correspond approximately to 5.8, 17.3, 52.0, 156.0 and 468.1 copies per cell (per diploid DNA template) for mouse liver sample homogenates, where the spike factor = 0.2. It is our observation that the RNA/DNA ratios are virtually constant across polyploid hepatocytes (data not shown).

For each Q-PCR plate or GeneChip, the coefficients, α , β , γ and δ of functions {1} or {2} are determined from the GSC values using the least-square method. The signal values or Ct values of all the other mRNAs measured are then converted to copy numbers per cell by {3} or {4}, i.e. the inverses of functions {1} or {2}.

Table 2: Primers for Q-PCR

Gene	Forward	Reverse
AFFX-TrpnX-3_at	TTCTCAGCGTAAAGCAATCCA	GCAAATCCTTTAGTGACCGAATACC
AFFX-DapX-3_at	TCAGCTAACGCTTCCAGACC	GGCCGACAGATTCTGATGACA
AFFX-PheX-3_at	GCCAATGATATGGCAGCTTCTAC	TGCGGCAGCATGACCATTA
AFFX-LysX-3_at	CCGTTTCATGCCACTGAATAC	CCGTTTCGATCCAAATTTCC
AFFX-ThrX-3_at	CCTGCATGAGGATGACGAGA	GGCATCGGCATATGGAAC
Ahr_1450695_at	CAGAGACCACTGACGGATGAA	AGCCTCTCCGGTAGCAAACA
Cyp1a1_1422217_a_at	TGCTCTTGCCACCTGCTGA	GGAGCACCTGTTTGTTCATG
Cyp1a2_1450715_at	CCTCACTGAATGGCTTCCAC	CGATGGCCGAGTTGTTATTG
Cyp1b1_1416612_at	GCCTCAGGTGTGTTTGTATGGA	AGTACAGCCCTGGTGGGAATG
Cyp7a1_1422100_at	TTCTACATGCCCTTTGGATCAG	GGACACTTGGTGTGGCTCTC
Hspa1a_1452388_at	ACCATCGAGGAGGTGGATTAGA	AGGACTTGATTGCAGGACAAAC

The "LBM" ("liver-brain mix") standard sample

A pair of samples having dissimilar gene expression profiles was chosen to evaluate the linearity of the platform. The pairs chosen were brain and liver for mouse and rat, two distinct cancer cell lines for humans, and adult liver and embryo for *Xenopus laevis*. The sample pairs were processed as described above including addition of the GSC. Two final homogenates were then blended at ratios of 100:0, 75:25, 50:50, 25:75 and 0:100 (based on cell numbers) to make five samples. These five samples were measured by Q-PCR and/or GeneChips (MG-U74v2A, MEA430A, MEA430B, MG430 2.0 (shown in Figure 1), RAE230A, HG-U95A, HG-U133, and *Xenopus* array).

Quantitative-PCR

Duplicate homogenate samples were treated with DNaseI (amplification grade, Invitrogen Corp., Carlsbad, CA, USA) for 15 min at room temperature, followed by SuperScript II (Invitrogen) for 50 min at 42°C for reverse transcription. Quantitative real time PCR was performed with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (TAKARA BIO Inc., Japan), with initial denaturation at 95°C for 10 s followed by 45 cycles of 5 s at 95°C and 60 s at 60°C, and Ct values were obtained. Primers for the genes explored in this study were selected from sequences close to the areas of Affymetrix GeneChip probe sets as shown in Table 2.

Affymetrix GeneChip measurement

The sample homogenates with GSC added were processed by the Affymetrix Standard protocol. The GeneChips used were MG-U74v2A for the uterotrophic study and Mouse 430-2 for the TCDD study (singlet measurement). The efficiency of *in vitro* transcription (IVT) was monitored by comparing the values of 5' probe sets and 3' probe sets of the control RNAs (AFFX- probe sets) including the GSC (see Quality Control below). The dose-response linearity of the five GSC spikes was checked and samples showing saturation and/or high background were re-measured

from either backup tissue samples, an aliquot of homogenate, or a hybridization solution, depending on the nature of the anomaly.

Quality control

Any external spiking method, including our Percellome method, is valid for high-quality RNA samples. Therefore, the quality of the sample RNA should be carefully monitored. In addition to a common checkup by RNA electrophoresis (including capillary electrophoresis if necessary), OD ratio, and cRNA yield, we monitor the performance of IVT (*in vitro* translation) or amplification. The 3' and 5' probe set data of the spiked-in RNAs and sample RNAs (actin, GAPD and other AFFX- probe sets) that are prepared in Affymetrix GeneChip are compared to monitor the extension of RNA by the IVT process. When both the spiked-in RNAs and the sample RNAs have similar levels of 5' and 3' signals respectively, it is judged that the IVT extension was normally performed. When both spiked-in and sample RNAs have significantly lower 5' signal than 3' signal, it is judged that the IVT extension was abnormal. When only the sample RNAs showed significantly lower 5' signal than 3' signal, it is judged that the IVT extension was normal but the sample RNAs were degraded. When only the spiked-in RNAs showed significantly lower 5' signal than 3' signal, it is judged that the IVT extension was normal but the spiked-in RNAs were degraded (although we have not encountered this situation). In addition, if the degraded sample was spiked-in by the non-degraded spike RNAs and measured by GeneChip, the position of spiked-in RNAs will be offset toward abnormally higher intensity. Together, this battery of checkups considerably increases the ability to detect abnormal events that will affect the reliability of the Percellome method. When any abnormality was found, each step of sample preparation was reevaluated to regain normal data for Percellome normalization.

The web site for GeneChip data

The GeneChip data are accessible at http://www.nih.gov/tox/TTG_Archive.htm.

Authors' contributions

JK drafted the concept of the Percellome method, led the project at a practical level, and drafted the manuscript. KA developed the algorithm for the Percellome calculation and wrote the calculation/visualization programs. KI developed the laboratory protocols for the Percellome procedures to the level of SOP for technicians. NN developed the Percellome Q-PCR protocol and performed the measurements, and helped in analyzing the Percellome data. AO helped develop the algorithm. YK led the animal studies. TN provided advice and led the toxicogenomics project using the Percellome method, to be approved by the Ministry of Health, Labour and Welfare of Japan.

Additional material**Additional File 1**

Excel spreadsheet file containing 15 Affymetrix Mouse 430-2 GeneChip raw data of five LBM samples in triplicate (cf. Figure 1). The column name LBM-100-0-X_Signal indicates the component percentages, i.e. 100% liver 0% brain, and X = 1,2,3 indicates the triplicates. The LBM-100-0-X_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S1.zip>]

Additional File 2

Excel spreadsheet file containing Percellome data of the same LBM samples, of which raw data is listed in Additional file 1 (cf. Figure 1).

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S2.zip>]

Additional File 3

Excel spreadsheet file containing 2 Affymetrix MG-U74v2 raw data of a blank sample with the GSC (horizontal axis of Figure 2a) and blank with the five spike RNAs at a high dosage (vertical axis of Figure 2a).

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S3.zip>]

Additional File 4

Excel spreadsheet file containing 2 Affymetrix MG-U74v2 raw data of a liver sample with GSC (horizontal axis of Figure 2b) and without GSC (vertical axis of Figure 2b).

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S4.zip>]

Additional File 5

(first quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 2 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 6

(second quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 4 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 7

(third quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 8 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 8

(last quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 24 hr data (15 GeneChip data) of the total of 60 Affymetrix Mouse 430-2 GeneChip raw data of the TCDD study consisting of 20 different treatment groups in triplicate (cf. Figure 5). The column name DoseXXX-TimeYY-Z_Signal indicates the dosage and sampling time after TCDD administration in hours, e.g. XXX = 001 indicates 1 microgram/kg group, YY = 02 indicates two hours after administration, and Z = 1,2,3 indicates animal triplicate. The DoseXXX-TimeYY-Z_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 9

(first quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 2 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 5.

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S9.zip>]

Additional File 10

(second quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 4 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 6.

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[<http://www.biomedcentral.com/content/supplementary/1471-2164-7-64-S10.zip>]

Additional File 11

(third quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 8 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 7.

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Additional File 12

(last quarter of a data set consisting of 2 hr, 4 hr, 8 hr, and 24 hr data, divided because of the upload file size limitation): an Excel spreadsheet file containing 24 hr Percellome data (15 sample data) of the 60 samples of the TCDD study (cf. Figure 5), of which corresponding raw data is listed in Additional file 8.

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Additional File 13

Excel spreadsheet file containing 15 Affymetrix MG-U74v2 A GeneChip raw data of the uterotrophic response study (cf. Figure 6). The column name X-Y_Signal indicates the treatment (V = vehicle, Low = low dose, etc) and animal triplicate (Y = 1,2,3). The X-Y_Detection column indicates P for present, A for absent and M for marginal calls by Affymetrix MAS 5.0 system.

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Additional File 14

Excel spreadsheet file containing Percellome data of the same 15 samples of the uterotrophic response study (cf. Figure 6), of which raw data is listed in Additional file 13.

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Acknowledgements

The authors thank Tomoko Ando, Noriko Moriyama, Yuko Kondo, Yuko Nakamura, Maki Abe, Nae Matsuda, Kenta Yoshiki, Ayako Imai, Koichi Morita, Hisako Aihara and Chiyuri Aoyagi for technical support, and Dr. Bruce Blumberg and Dr. Thomas Knudson for critical reading of the manuscript. This study was supported by Health Sciences Research Grants H13-Seikatsu-012, H13-Seikatsu-013, H14-Toxico-001 and H15-Kagaku-002 from the Ministry of Health, Labour and Welfare, Japan.

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Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates

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Dietary and xenobiotic compounds can disrupt endocrine signaling, particularly of steroid receptors and sexual differentiation. Evidence is also mounting that implicates environmental agents in the growing epidemic of obesity. Despite a long-standing interest in such compounds, their identity has remained elusive. Here we show that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo*. TBT is a dual, nanomolar affinity ligand for both the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor γ (PPAR γ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipo-

genesis and lipogenic pathways *in vivo*. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues after organotin, RXR, or PPAR γ ligand exposure. TBT represents, to our knowledge, the first example of an environmental endocrine disrupter that promotes adipogenesis through RXR and PPAR γ activation. Developmental or chronic lifetime exposure to organotins may therefore act as a chemical stressor for obesity and related disorders. (*Molecular Endocrinology* 20: 2141-2155, 2006)

ORGANOTINS ARE A diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the 1960s as antifouling agents in marine shipping paints, although such use has been restricted in recent years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high-value food crops (1, 2). Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems, and tex-

tiles. Mono- and diorganotins are prevalently used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPTO results in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks (3, 4). Bioaccumulation of organotins decreases aromatase activity leading to a rise in testosterone levels that promotes development of male characteristics (5). Imposex results in impaired reproductive fitness or sterility in the affected animals and is one of the clearest examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species (6, 7), but TBT is only reported to have modest adverse effects on mammalian male and female reproductive tracts and does not alter sex ratios (8, 9). Instead, hepatic-, neuro-, and immunotoxicity appear to be the predominant effects of organotin exposure (10). Hence, the current mechanistic understanding of the endocrine-disrupting potential of organotins is based on their direct actions on the levels or activity of key steroid-regulatory enzymes such as aromatase and more general toxicity mediated via damage to mitochondrial functions and subsequent cellular stress responses (11-15).

However, it remains an open question whether *in vivo* organotins act primarily as protein and enzyme

First Published Online April 13, 2006

Abbreviations: Acac, Acetyl-coenzyme A carboxylase; b.w., body weight; C/EBP, CCAAT/enhancer binding protein; 9-*cis* RA, 9-*cis* retinoic acid; DMSO, dimethylsulfoxide; F, forward; Fatp, fatty acid transport protein; LBD, ligand-binding domain; LXR, liver X receptor; MDIT, 3-isobutyl-1-methylxanthine, dexamethasone, insulin and T₃ adipocyte differentiation mix; PPAR, peroxisome proliferator-activated receptor; R, reverse; RAR, retinoic acid receptor; RXR, retinoid X receptor; Srebf1, sterol-regulatory element binding factor 1; TBT, tributyltin chloride; TPTO, triphenyltin oxide; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl-1-propenyl) benzoic acid; VDR, vitamin D receptor.

Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

inhibitors, or rather mediate their endocrine-disrupting effects at the transcriptional level. Recent work has shown that aromatase mRNA levels can be down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, retinoid X receptor (RXRs) or peroxisome proliferator-activated receptor γ (PPAR γ) (16–18). Furthermore, Nishikawa *et al.* (19) have demonstrated that the gastropod *Thais clavigera* RXR homolog is responsive to 9-*cis*-retinoic acid (9-*cis*-RA) and TBT, and 9-*cis* RA can also induce imposex, suggesting a conserved transcriptional mechanism for TBT action across phyla. These ligand-dependent transcription factors belong to the nuclear hormone receptor superfamily—a group of approximately 150 members (48 human genes) that includes the estrogen receptor, androgen receptor, glucocorticoid receptor, thyroid hormone receptor, vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), PPARs, and numerous orphan receptors. We were therefore intrigued by the similar effects of TBT and RXR/PPAR γ ligands on mammalian aromatase mRNA expression and hypothesized that TBT might be exerting some of its biological effects via transcriptional regulation of gene expression through activation of one or more nuclear hormone receptors.

Our results show that organotins such as TBT are indeed potent and efficacious agonistic ligands of the vertebrate nuclear receptors, retinoid X receptors (RXRs) and PPAR γ . The physiological consequences of receptor activation predict that permissive RXR heterodimer target genes and downstream signaling cascades are sensitive to organotin misregulation. Consistent with this prediction we observe that organotins phenocopy the effects of RXR and PPAR γ ligands using *in vitro* and *in vivo* models of adipogenesis. Therefore, TBT and related organotin compounds are the first of a potentially new class of environmental endocrine disrupters that targets adipogenesis by modulating the activity of key regulatory transcription factors in the adipogenic pathway, RXR α and PPAR γ . The existence of such xenobiotic compounds was previously hypothesized (20, 21). Our results suggest that developmental exposure to TBT and its congeners that activate RXR/PPAR γ might be expected to increase the incidence of obesity in exposed individuals and that chronic lifetime exposure could act as a potential chemical stressor for obesity and obesity-related disorders.

RESULTS

Organotins Are Agonists of Vertebrate RXR and RXR-Permissive Heterodimers

Many known or suspected environmental endocrine-disrupting chemicals mimic natural lipophilic hormones that act through members of the superfamily of nuclear receptor transcription factors (22, 23). In a

screen of high-priority endocrine-disrupting chemicals against a bank of vertebrate nuclear receptor ligand-binding domains (LBDs), we observed that organotins, specifically tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), could fully activate an RXR α LBD construct (GAL4-RXR α) in transient transfection assays. Both TBT and TPTO were as potent (EC_{50} ~3–10 nM) as 9-*cis* retinoic acid, an endogenous RXR ligand and approximately 2- to 5-fold less potent than the synthetic RXR-specific ligands LG100268 (EC_{50} ~ 2 nM) or AGN195203 (EC_{50} ~ 0.5 nM) (Fig. 1A and see Table 2). Maximal activation for TBT reached the same levels as LG100268 or AGN195203.

We next tested whether activation by TBT was unique to RXR α only, restricted to RXR heterodimer complexes, or a general nuclear receptor transcriptional response (Fig. 1, B–D, and Table 1). TBT activated RXR α and RXR γ from the amphibian *Xenopus laevis* in addition to human RXRs (Table 1). Our results are consistent with recent findings by Nishikawa *et al.* (19, 24) that organotins promote activation of all three human RXR subtypes in a yeast two-hybrid screen. We also observed significant activation of receptors typically considered to be permissive heterodimeric partners of RXR including human PPAR γ (Fig. 1B, ~30% maximal activation of 10 μ M troglitazone, but note that activation is compromised by cellular toxicity above 100 nM), PPAR δ , liver X receptor (LXR), and the orphan receptor NURR1. In contrast, typical nonpermissive partners such as RARs, thyroid hormone receptor, and VDR failed to show activation by organotins (Fig. 1C and Table 1). Murine PPAR α was also not activated by TBT although it was fully activated by its specific synthetic agonist WY-14643 (Fig. 1D). The steroid and xenobiotic receptor was likewise unresponsive. The orphan receptor NURR1, which has no discernable ligand pocket and is believed to be ligand independent (25), was nevertheless activated 7- to 10-fold at 100 nM TBT. Similarly, other RXR-specific ligands, *e.g.* LG100268, activated NURR1 to the same degree, suggesting that this response occurred through NURR1's heterodimeric partner RXR as has been previously described (25, 26). Like other RXR-specific ligands, tributyltin was also able to promote the ligand-dependent recruitment of nuclear receptor cofactors such as receptor-associated coactivator 3 (ACTR), steroid receptor coactivator-1, and PPAR-binding protein in mammalian two-hybrid interaction assays (data not shown). We infer from these results that nuclear receptor activation by TBT activation is specific to a small subset of receptors and not a consequence of a general effect on the cellular transcriptional machinery.

We next investigated the relationship between the structure of the tin compounds and RXR activation by testing the response of GAL4-RXR α to mono-, di-, tri-, and tetra-substituted butyltin, branched side chains, variations in the alkyl chain length, and changes in the halide component (Fig. 1A and Table 2). Overall, trialkyltin compounds were the most effective with nano-

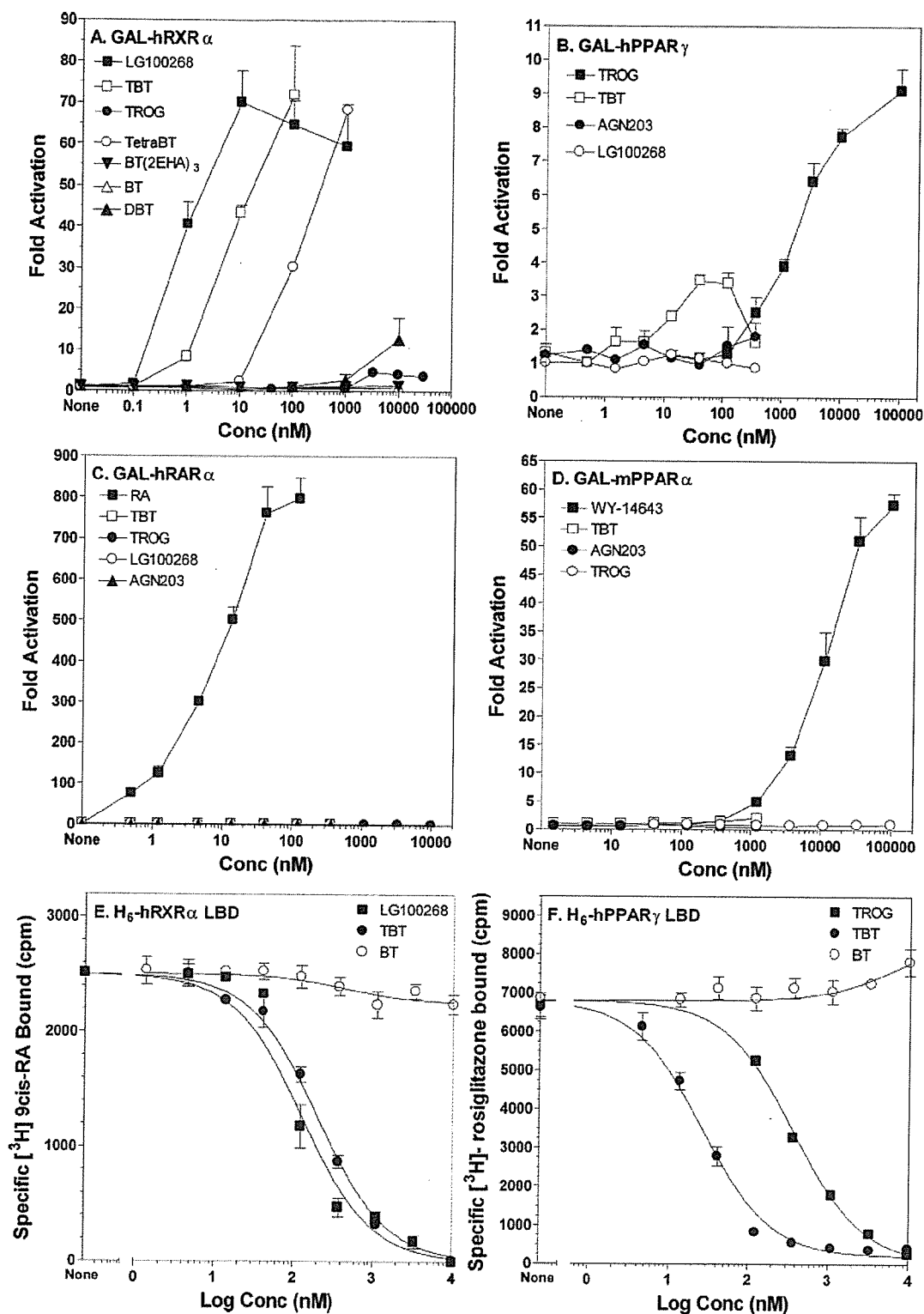


Fig. 1. Organotin Are Agonist Ligands of RXR α and PPAR γ

Organotin are high-affinity ligand agonists of RXR α and PPAR γ . A–D, Activation of GAL4-hRXR α , -hPPAR γ , -hRAR α , or -hPPAR α in transiently transfected Cos7 cells by organotin and receptor-specific ligands. Data represent reporter luciferase activity normalized to β -galactosidase and plotted as the average fold activation \pm SEM ($n = 3$) relative to solvent-only controls from representative experiments. E and F, Competition binding curves of histidine-tagged RXR α or PPAR γ 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
RXR α (<i>Homo sapiens</i>)	60	Yes
RXR α (<i>X. laevis</i>)	25	Yes
RXR γ (<i>X. laevis</i>)	7.0	Yes
NURR1 (<i>H. sapiens</i>)	7.0	Yes
LXR (<i>H. sapiens</i>)	2.1	Yes
PPAR α (<i>Mus musculus</i>)	0.7	Yes
PPAR γ (<i>H. sapiens</i>)	5.3	Yes
PPAR δ (<i>H. sapiens</i>)	1.7	Yes
RAR α (<i>H. sapiens</i>)	0.7	No
TR β (<i>H. sapiens</i>)	0.4	No
VDR (<i>H. sapiens</i>)	0.5	No
SXR (<i>H. sapiens</i>)	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 EC₅₀ 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-activ-

Table 2. Organotin EC₅₀ Values for Nuclear Receptor LBDs

Ligand	GAL4-NR LBD Transactivation (EC ₅₀ Values, nM)		
	hRXR α	hRAR α	hPPAR γ
LGD268	2–5	na	na
AGN195203	0.5–2	na	na
9- <i>cis</i> RA	15	na	na
all- <i>trans</i> 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. EC₅₀ values were determined from dose-response curves of GAL4-NR LBD construct activation in transiently transfected Cos7 cells after 24-h ligand exposure.

ity relationship. A reduction in hydrophobicity from butyl to ethyl side chains raised the EC₅₀ value by almost 1000-fold into the micromolar range. Trimethyltin was weakly active only above 100 μ M (Table 2). Substitution of the halide component had no significant effect on the EC₅₀ values for TBT, probably due to the lability of the halide atom through exchange in aqueous tissue culture media where chloride ions are prevalent.

TBT Is a Potent Ligand of Both RXR α and PPAR γ

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 RXR α (H₆-RXR α) and PPAR γ (H₆-PPAR γ) 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 RXR α . 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 \pm 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 PPAR γ LBD (Fig. 2B). The deduced K_i of 20 nM (17–40 nM; 95% confidence interval) was slightly higher than for RXR α but significantly better than the K_i for the PPAR γ agonist troglitazone, which

Table 3. TBT Binding Constants (K_d) for hRXR α and hPPAR γ LBDs

Ligand	Receptor Competitive Inhibition Binding Constants K _i (nM \pm 95% CI)		
	H ₆ -RXR α	H ₆ -PPAR γ	Published
TBT	12.5 (10–15)	20 (17–40)	
LG100268	7.5 (6–10)	ND	3 \pm 1 ^a
Troglitazone	ND	300 (270–335)	300 \pm 30 ^b

Competition binding curves were determined at constant ³H-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 \pm 95% confidence intervals (n = 3). CI, Confidence interval; ND, not determined.

^a RXR α :LG100268 K_d = 3 \pm 1 nM (27).

^b PPAR γ :troglitazone K_d = 300 \pm 30 nM (28).

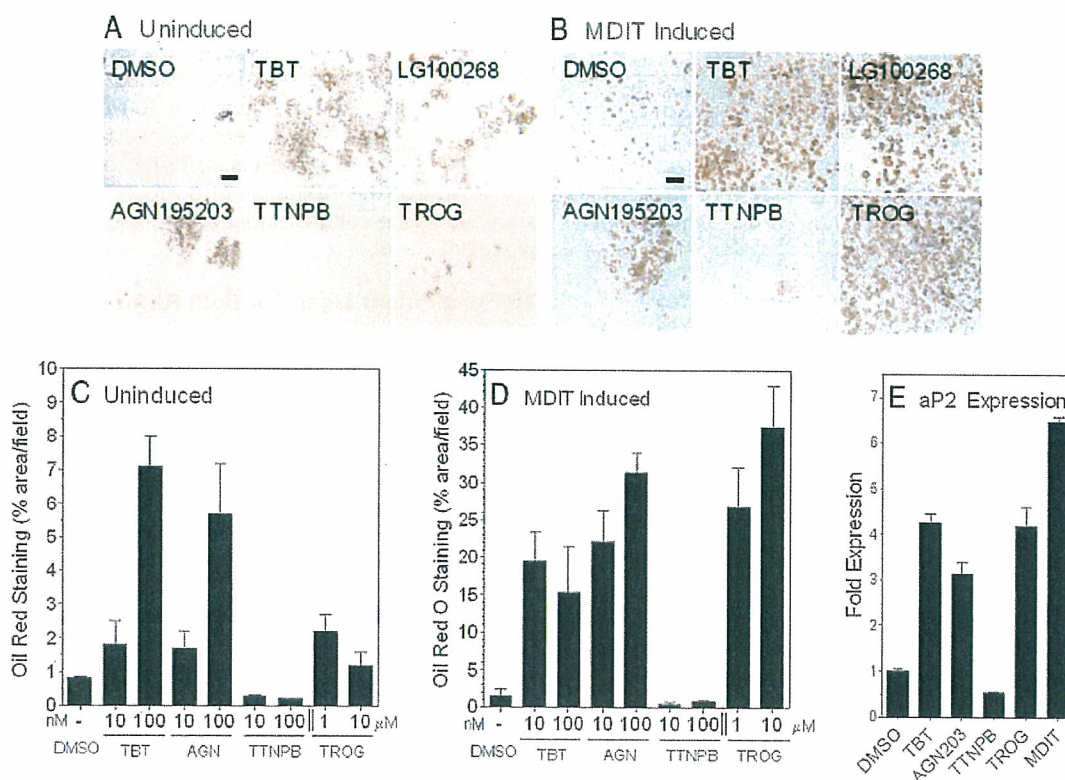


Fig. 2. Tributyltin induces adipogenesis in 3T3-L1 cells

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 \pm 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 RXR α (12.5 nM) and PPAR γ (20 nM) are also in close agreement with EC_{50} values obtained from transient transfection assays using GAL4-RXR α and GAL4-PPAR γ 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 RXR α and PPAR γ , 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 PPAR γ 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 RXR α :PPAR γ 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/EBP α and PPAR γ (32–34). The combination of C/EBP α expression together with PPAR γ signaling efficiently drives terminal adipocyte differentiation and lipid accumulation. We therefore tested whether TBT signaling through RXR:PPAR γ could promote adipogenesis in the murine 3T3-L1 differentiation assay and compared its effect to other RXR-specific or PPAR γ 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-

differentiation 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 PPAR γ 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 PPAR γ 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:PPAR γ targets such as adipocyte-specific fatty acid-binding protein (aP2) mRNA. The aP2 promoter contains response elements sensitive to C/EBP factors and RXR α :PPAR γ 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 RXR α :PPAR γ Signaling *in Vivo*

The ability of organotins to regulate RXR α :PPAR γ 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 RXR α , PPAR γ , C/EBP $\alpha/\beta/\delta$, and sterol regulatory element binding factor 1 (Srebf1) as well as known target genes of RXR α :PPAR γ 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 RXR α and PPAR γ transcription in liver (Fig. 3, A and B). A more pronounced decrease was observed for RXR α , PPAR γ , C/EBP α , and C/EBP δ 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/EBP β 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/EBP β , 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:PPAR γ 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:PPAR γ 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 PPAR γ 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:PPAR γ , 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 PPAR γ signaling (49, 54).

Taken together, these data show that TBT exposure induces lipogenic RXR:PPAR γ target gene expression, in adipose tissue and liver, and modulates associated early adipocyte differentiation factors such as C/EBP β 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

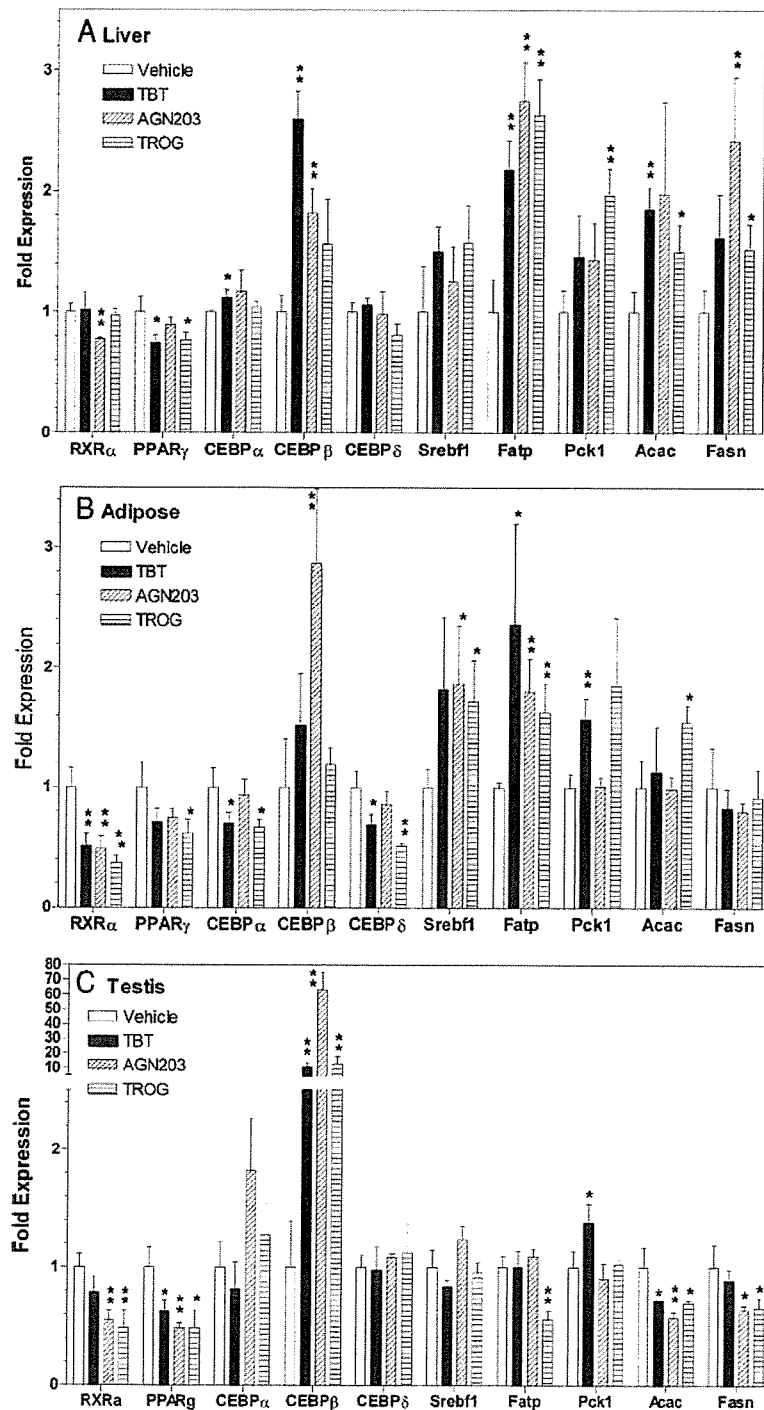


Fig. 3. *In Vivo* Induction of Adipogenic Modulators and RXR:PPAR γ Target Genes

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 \pm 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-

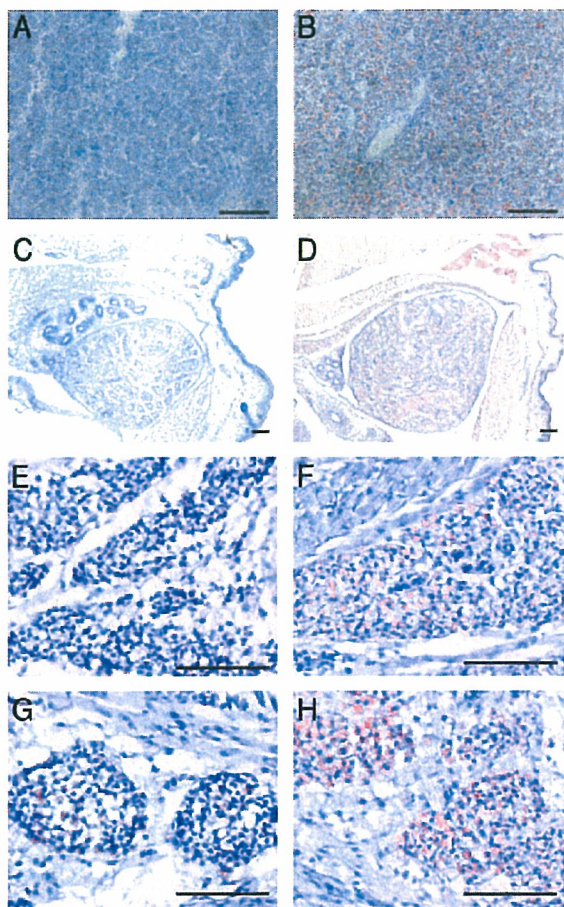


Fig. 4. *In Utero* Exposure to TBT Increases Adiposity in Mouse Liver, Testis, and Adipose Depots

Histological sections (12 μ m) 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 μ m.

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 \pm 0.70 (n = 9) vs. 25.53 g \pm 0.39 (n = 10), P = 0.583; female, 21.22 g \pm 0.41 (n = 10), vs. 20.24 g \pm 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 \pm 0.020 (n = 9) vs. 0.36 g \pm 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/PPAR γ 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 μ M), and estradiol (1–10 nM) on developing *X. laevis* tadpoles from stage 48 to metamorphosis. 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/PPAR γ ligands in mammals (17, 18). *Xenopus* aromatase expression was similarly repressed 2- to 3-fold by 10 nM TBT, AGN195203, LG100268, or 1 μ M 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/PPAR γ 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 μ M 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 PPAR γ ligands are therefore consistent with a common mechanism of action through RXR:PPAR γ 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,

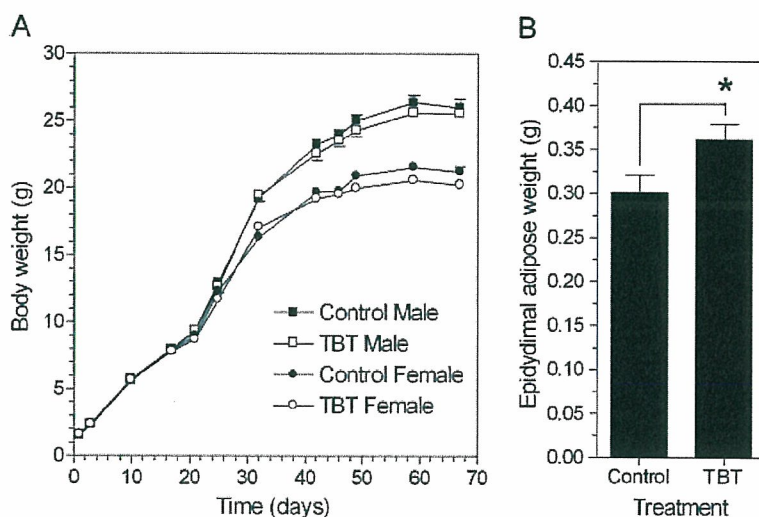


Fig. 5. *In Utero* Exposure to TBT Increases Adipose Mass But Not Body Weight in Adult Mice

A, Growth curves of C57BL/6 male and female pups exposed to control (sesame oil) or TBT *in utero* (E12–18). Data are mean \pm 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: $0.30 \text{ g} \pm 0.020$ ($n = 9$) vs. $0.36 \text{ g} \pm 0.018$ ($n = 10$); *, $P = 0.0374$]. Data represent mean \pm SEM ($n = 9$ – 10).

high-affinity xenobiotic ligand for RXR α and PPAR γ . 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/PPAR γ 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 PPAR γ 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 PPAR γ above $10 \mu\text{M}$ *in vitro*. However, in accord with our findings, they show that TBT activated PPAR γ 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 PPAR γ in cells ($\sim 30\%$ at 100 nM TBT cf troglitazone) is consistent with one of two possibilities: either non-specific 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 RXR α :PPAR γ , 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:PPAR γ signaling pathway are predictable and should follow known aspects of RXR/PPAR γ biology. The RXR:PPAR γ pathway plays a key role in adipocyte differentiation and energy storage, and is central to the control of whole-body metabolism (58). PPAR γ activation increases the expression of genes that promote fatty acid storage and represses genes that induce lipolysis in adipocytes in white adipose tissue (59). PPAR γ 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, PPAR γ agonists comprise a class of phar-