is initially highly effective, within 2–3 years of therapy most patients develop ADT-resistant tumors, for which no effective treatment is available [127,128].

It was shown by Wetherill and colleagues that low concentrations of BPA (1–10 nM) are capable of activating the mutant AR (AR-T877A), that is selected for during ADT therapy in prostate cancer cells. Under conditions of androgen ablation, BPA activation of the mutant AR induced cellular proliferation [26]. In contrast, BPA has no agonistic activities on the wild-type AR [23,24,29]. Wetherill et al. also demonstrated that BPA competes with DHT for AR-T877A binding by a non-competitive mechanism, and enhances mutant AR activity in both androgen-dependent (mutant AR-T877A) and androgen-independent (mutant AR-H874Y) prostate cancer cell lines in the presence of DHT [28]. In contrast, in an androgen-dependent prostate cancer cell line LAPC-4 that expresses a wild-type AR, low doses of BPA only marginally activate the receptor and fail to show additive effects with DHT on AR transactivation [28]. In addition, this group showed that the ability of BPA to activate tumor-derived AR is conserved across multiple mutant ARs (AR-V715M, AR-T877S, and AR-H874Y), as demonstrated by both yeast-based and mammalian cell assays. These mutations in the AR were identified in patients with metastatic androgen-independent prostate tumors following ADT [129,130]. At higher doses of 1–10 μM BPA, growth inhibition of androgen-dependent prostate cancer cell lines (LNCaP and LAPC-4) was observed. Thus, the differential effects of lower and higher doses of BPA were demonstrated, as the growth inhibitory effect at higher doses is seen only in androgen-dependent prostate cancer cells [28]. These results are in agreement with previous studies that showed inhibition of AR activity at high dose exposure to other endocrine disruptors [131,132] and BPA [22–24,27,29,35]. Collectively, the above *in vitro* studies, offer a mechanism by which environmentally relevant BPA exposures in prostate cancer might lead to tumor cell proliferation and suggest that BPA can interfere with therapy in prostate cancer patients by activating mutant ARs that are frequently selected for during ADT; these effects of BPA could contribute to the disease relapse. In fact, a recent study by Wetherill and Hess-Wilson et al. suggests that exposure of advanced prostate tumors expressing specific somatic mutations (AR-*T877A*) in the androgen receptor gene to environmentally relevant doses of BPA may facilitate ADT bypass and advance tumor recurrence [133].

12. Effects of BPA on cells and tissues of the male reproductive system

A study in adult Leydig cells by Akingbemi et al. demonstrated that low-dose BPA (0.01 nM) decreases testosterone biosynthesis by 25% as a result of decreased expression of the steroidogenic enzyme 17α-monooxygenase (17alpha-hydroxylase/17–20 lyase) [134]. Further, post-natal exposure to BPA decreased serum 17β-estradiol levels in Long-Evans rats exposed to 2.4, 10, or 100 μg/kg/day of BPA by inhibiting Leydig cell aromatase activity and exposure to lowest and environmentally relevant BPA dose (2.4 μg/kg/day) decreased the mRNA levels of lutenizing hormone (LH)-β and increased ERβ expression in the pituitary [134]. These findings demonstrate that the perinatal period is a sensitive window of exposure to BPA in this animal system. The effects of BPA on steriodogenesis in Leydig cells was also analyzed by measuring the LH receptor-mediated cAMP and progesterone production in cultured mouse Leydig tumor cells [135]. This study found that BPA inhibited cAMP production and progesterone synthesis by preventing coupling between LH receptor and adenylate cyclase. However, estradiol did not inhibit cAMP production, thus further emphasizing the complexity of endocrine disruption.

In the testis, Sertoli cells establish intercellular junctions that are essential for spermatogenesis. The SerW3 Sertoli cell line displays some features of native Sertoli cells. Fiorini et al. studied the mechanism of BPA action on Sertoli cells [136]. Using Western blot and immunofluorescence analyses it was found that SerW3 Sertoli cells express typical components of tight (occludin and zonula occludens-1), anchoring (N-cadherin) and gap (connexin 43) junctions. Similar to other endocrine disruptors, BPA affected intercellular junctions by either reducing their amount, or by inducing aberrant intracellular localization of these membrane proteins [136].

Song et al. investigated the action of BPA on orphan nuclear receptor Nur77 gene expression and steroidogenesis, as it plays an important role in the regulation of LH-mediated steroidogenesis in testicular mouse K28 cells [137]. The K28 mouse Leydig tumor cell line has been characterized as a suitable cell culture model for steroidogenesis [138,139]. It was found that exposure to 10 nM BPA induces Nur77 gene expression in K28 cells after 30 min treatment and BPA-mediated induction of Nur77 gene expression is ERK mitogen-activated protein kinase (MAPK) and PKA dependent. At high BPA concentrations (1 μM), progesterone biosynthesis was increased in K28 cells; this effect was suppressed by overexpression of dominant negative Nur77. Moreover, high dose BPA effects on steroidogenesis were confirmed *in vivo* where BPA injection into prepubertal mice (25 mg/kg; 18-day old) resulted in induction of Nur77 mRNA and increased concentrations of testicular testosterone *in vivo* [137]. Taken together, these results demonstrate that BPA can induce Nur77 gene expression, which subsequently alters the steroidogenesis in testicular Leydig cells. Post-natal exposure to a low dose of BPA (2.4 μg/kg/day) was also found to reduce expression of cortactin, an actin-binding protein, in mouse testes [140]. Thus, the studies described above implicate adverse effects of low dose environmental BPA exposure on male fertility.

13. Thyroid hormone action, thyroid receptor and BPA

Thyroid hormone (TH) is essential for normal brain development, and recent studies indicate that the developing brain is very sensitive to small perturbations in thyroid hormone status. As a result, understanding the action of environmental chemicals that can interfere with TH signaling mechanisms is highly important to human health. The first environmental chemical shown to bind to the thyroid hormone receptor (TR) was BPA and its brominated and chlorinated derivatives. It was demonstrated by Meerts et al. in 2001 that polybrominated BPA compounds have estro-

genic activities and are able to bind to the ERα [141]. A study in 2002 by Kitamura et al. showed that BPA, tetrabromo- and tetrachloro-BPA (TBBPA ad TCBPA, respectively) could displace thyroid hormone ^{125}I-T_3 from cell nuclei isolated from the estrogen-responsive pituitary cell line MTt/E-2 [33]. Although these data indicate TR binding, it is not clear whether this binding is specific to the TRβ, TRα or both. In the thyroid hormone-responsive pituitary cell line GH3 (in which the growth hormone secretion depends markedly on thyroid hormones, but little on estrogen), TCBPA and TBBPA increase cell proliferation at the dose of 100 μM and 1–100 μM, respectively [33]. These results indicate that brominated BPA derivatives act as TR agonists in pituitary GH3 cells. Further support for this interpretation was the observation that these two halogenated BPA compounds increase GH3 cell proliferation, and produce growth hormone (GH) production in the presence of thyroid hormone T3 in an additive way. In contrast, Moriyama et al., who utilized rat liver nuclei that express both the TRα and TRβ, showed that BPA binds to the TR and acts as an antagonist on this receptor [36]. This conclusion was based on two types of experiments. In the first approach, they fused the ligand binding domain (LBD) of either TRα or TRβ to the Gal4 DNA binding domain and performed *in vitro* expression assays using a Gal4 response element. It was found that BPA at 1.0 μM inhibited both the TRα- and TRβ-stimulated response induced by thyroid hormone T_3. In the second approach, investigators co-transfected full length TRα and TRβ into TSA-201 cell (a clone of human embryonic kidney 293 cells) with one of three types of thyroid hormone response elements (TRE): a palindrome, the malic enzyme TRE, and the TSHα TRE. They found that in the presence of both TRα and TRβ, BPA produced a dose-dependent inhibition in the T_3 response (10 nM T_3) on both the palindromic and the malic enzyme TRE, but produced a dose-dependent induction in the T_3 response on the TSHα TRE. Because the TSHα TRE is a negative response element, the authors speculated that BPA stabilized the interaction between TRα1 or TRβ1 and a nuclear repressor N-CoR. To test this hypothesis, Moriyama et al. performed a mammalian two-hybrid experiment using GAL4-NCoR and TR-VP16 with their GAL4 reporter construct in TSA-201 cells. These experiments fully supported the concept that BPA binding to TR stabilized the interaction between the TR and N-CoR. This hypothesis that is consistent with the observations that BPA suppresses T_3 action on positive TREs (malic enzyme and a DR4), and enhances T_3 action on a negative TSHα TRE.

The above studies indicate that BPA is an antagonist on the TR, but that halogenated BPA derivatives (commonly used as flame retardants) are TR agonists. Moreover, the affinity of the TR for brominated TBBPA or TCBPA appears to be higher than for BPA. Finally, these data indicate that BPA and its halogenated derivatives can bind two classes of TRs equally well. However, the latter concept is inconsistent with the work of Ghisari and Bonefeld-Jorgensen where BPA and TB-BPA were found to exert thyroid hormone-like activity in GH3 cells by increasing cell proliferation [30]. Interestingly, BPA, but not TBBPA, increased GH3 cell proliferation in the presence of a pure estrogen receptor antagonist ICI 182,780, suggesting that ER-mediated transactivation is not involved and that BPA may exert effects directly on the TR [30]. Other studies have found that TBBPA has a lower affinity for the ER than does BPA [142]. It is unclear why BPA appeared to have a stronger action on the TR than TB-BPA in this study. Moreover, BPA appeared to act as an agonist, which is not consistent with previous *in vitro* studies.

The contribution of rapid intracellular signaling effects are well described for T_3, estradiol, and BPA and cannot be ruled out as contributing to the actions of BPA in this cell model system (reviewed in [143,144]). A potential contribution of rapid signaling effects in TH/TR-mediated effects of BPA and/or TB-BPA are supported by the work of Canesi et al. who showed that BPA (25 μM) and TBBPA (5 μM) stimulate rapid and transient activation of ERK-MAPK and PKC in hemocytes isolated from the marine mussel *Mytilus galloprovincialis* [75]. While these studies reinforce the potential contribution of rapid signaling to BPA-mediated actions at the TRs, the high concentrations of BPA required to activate signaling effects limit their significance.

Other halogenated BPA derivatives were shown to act as TR antagonists in the study by Kitamura et al. [34]. The investigators used CHO cells in a transient transfection assay in which human TRα or TRβ were co-transfected with a palindromic TRE. In these experiments, TBBPA and TCBPA were potent antagonists of T_3, producing significant inhibition of T_3 action at concentrations as low as 3 μM, but this action depended upon the compound and the TR isoform. In general, TBBPA was more potent than TCBPA, and TRα was more sensitive to each of these compounds. These findings are not consistent with the initial studies reviewed above in which the halogenated BPA derivatives acted as agonists and parent BPA acted as an antagonist. Differences in the cellular context (i.e., use of different cell lines), and/or differences in the reporter construct (i.e., the use of a DR4 versus a palindrome) may account for the reported experimental differences.

Kitamura et al. followed these observations with a comparison of effects of BPA, TBBPA and TC-BPA on ER, AR and TR signaling [35]. This study supported their previous observations that halogenated derivatives of BPA act as TR agonists in pituitary GH3 cells [35]. Interestingly, the biologically active dose in these experiments was one or two orders of magnitude higher than doses producing estrogenic or androgenic effects, concentrations ranged from 1 to 100 μM. In contrast to previous studies by Moriyama et al. [36], an agonistic or antagonistic action of BPA on the TR in this system was not observed.

Hamers et al. have profiled a number of polyhalogenated aryl hydrocarbons for their ability to signal through the AhR receptor, ER, AR, and TR [31]. In addition, they characterized the ability of these compounds to displace T_4 from transthyretin, or to affect estrogen sulfotransferases. In these studies, TB-BPA was a very potent T_4 competitor in the TTR-binding assay ($IC_{50} < 0.1$ μM) with a 1.6 times higher TTR-binding potency than the natural ligand T_4. In their GH3 cell-based assays, BPA, TB-BPA and TC-BPA were nearly inactive, yet TB-BPA was found to potentiate the effects of T_3 in the T-screen assay.

Finally, Schricks et al. modified the T-screen to produce a more quantifiable assay [145]. This assay utilized a GH3 rat pituitary cell line that specifically proliferates when exposed to thyroid hormone T_3. The growth stimulatory effect is mediated

via T_3-receptors. In this proliferation assay, resozurine is used to measure cell proliferation. It was found that neither BPA nor the halogenated derivatives were active as agonists or antagonists in this system. However, it was shown that BPA significantly potentiated the effect of T_3.

Taken together, *in vitro* studies demonstrate that BPA binds to both the TRα and TRβ with relatively low affinity. In addition, the halogenated flame retardants, TB-BPA and TC-BPA bind with higher affinity. However, some studies indicate that BPA acts as an antagonist on the TR [32,34–36], whereas other studies conclude that BPA acts as an agonist [30,33], and some studies indicated that BPA and its halogenated derivatives are not active on thyroid hormone signaling. It is unclear why such differences in experimental results were obtained. Several investigators have employed GH3 cells in their studies, and have still obtained different results. This could be partially due to experimental design differences: initial reports utilized the MTT assay to evaluate relative growth, as well as growth hormone production (GH is controlled by thyroid hormone) [33], whereas in later studies the authors used a modified version of a cell proliferation assay (the use of resazurine), which may differ in its sensitivity [145]. It is also important to consider the possibility that differences in cell lines may have developed and that the differing results might reflect differences in transcriptional machinery that impact the ability of the TR to drive gene expression. If these compounds bind to the TR but produce a slightly different conformational response in the protein, it is theoretically possible that the TR will interact differentially with various cofactors. The initial observation of Moriyama et al. indicating that BPA can alter the strength of the interaction between TR and NCoR supports this interpretation [36].

If BPA and its halogenated derivatives interfere with thyroid hormone signaling, *in vivo* effects that are consistent with this concept might be observed. In this regard, Iwamuro et al. found that BPA reduces the rate of metamorphosis in *Xenopus* and inhibites T_4-accelerated metamorphosis [146]. These investigators also showed *in vitro* that T_4-induced tail resorption inhibited by BPA in a dose-dependent manner. These findings are fully consistent with the hypothesis that BPA acts as an antagonist of the *Xenopus* thyroid receptorthyroid receptor. This finding is consistent with those of Kitamura et al. where T_3-induced tail shortening is inhibited by TB-BPA in a Ranid [34]. Further, Kudo et al. found that 3,3′,5-tribromobisphenol A and TB-BPA both exhibited T_3 agonist and antagonist activities at submicromolar concentrations in a *Xenopus laevis* cell line and in tadpoles [147]. These compounds competed with T_3 binding to xTTR and xTR *in vitro*, suggesting these proteins are possible target sites for BPA action *in vivo*. In addition, the dose at which TR signaling was influenced was lower than the IC_{50} values for the receptor. Therefore, the authors suggest that novel molecular mechanisms of interaction with the TR may be at play. Interestingly, Sugiyama et al. reported that TCBPA could inhibit the effect of T_3 on a luciferase construct driven by a *Xenopus* TRE (the TH/bZIP TRE), but that TB-BPA acts as a TR agonist in the absence of T_3 [148].

Iwamuro et al. investigated the effects of BPA on the expression of TR-α and TR-β, and retinoid X receptor (RXR) gamma mRNA in tails of stage 52–54 *Xenopus* tadpoles in organ culture in the presence or absence of different concentrations of T_3 [32]. In the absence of T_3, BPA at all concentrations examined did not show remarkable effects on tail length, but blocked T_3-induced tail resorption in a concentration-dependent manner. Changes in expression of TRα and TRβ mRNAs from the tail specimens (measured by RT-PCR) indicated that low BPA dose (0.1 μM) showed an apparent antagonistic effect towards the receptors and reduced mRNA levels. When administered together with T_3, the antagonistic effects of BPA were more pronounced. Autoinduction of both TRα and TRβ genes by T_3 was inhibited by BPA, but the effect was less marked on TRα than on TRβ. Gene expression of RXRγ, a partner for heterodimer formation of TRs, was moderately suppressed either by T_3 or by BPA, but no additive effects were observed.

Those studies show that somewhat different findings are reported using amphibian models as well as those reviewed above for other *in vitro* models. Although some of these data appear conflicting, it is clear that BPA and its halogenated derivatives can interfere with TH signaling in the amphibian model. The differences among studies characterizing these compounds as agonists or antagonists likely reflect the complexity of mechanisms underlying BPA actions on the TR.

Findings from studies in mammals are also complex. For example, Zoeller et al. [37] reported that BPA could increase serum total T_4 in rat pups, but also increased RC3 expression in the dentate gyrus. Their interpretation was that BPA acted as a selective TRβ antagonist, causing an increase in serum T_4. However, because RC3 expression is driven by the TRα in the dentate gyrus, these authors speculated that BPA was not exerting the same action on the TRα. In contrast, Nakamura et al., who studied whether prenatal exposure to low-doses of BPA by injection of pregnant mice with 20 μg/kg/day BPA affected the brain morphology and expression of genes related to brain development, reported that BPA exposure causes changes in the histogenesis of the cerebral cortex and increases the expression of a number of genes, including the TRα gene. These results were interpreted as reflecting a thyroid hormone-like effect of BPA in the cortex [149]. A similar observation was also made in *Xenopus* where TB-BPA increased TRα, but not TRβ, expression [150].

These studies suggest that BPA and a variety of its derivatives interfere with thyroid hormone signaling during development. The mechanism(s) underlying these effects are likely to be complex given that the dose of BPA required to produce effects on molecular events may be lower than the IC_{50} for binding to the thyroid receptor. It is also likely that BPA and its derivatives alter the relationship of the TR to various co-modulatory molecules. Moreover, BPA and its derivatives may also influence the abundance of the receptor. Thus, the mechanisms associated with BPA endocrine disrupting effects on the TH system are currently ill-defined and warrant further detailed investigation.

14. BPA and rapid signaling systems

BPA also exerts cell- and tissue-specific effects that act through mechanisms which modulate a variety of cell signaling pathways. The impact of BPA exposure is observed within

seconds to minutes, and is initially independent of the nuclear hormone receptor mediated transactivation activity that induces later effects on gene expression. However, these rapid signaling pathways interact with the traditional nuclear hormone receptor pathways [144,151].

For specific cell types, the features of the mechanisms associated with rapid signaling for estradiol, thyroid hormone and some endocrine disruptors, including BPA, are becoming more understood with recent research findings showing the presence of extracellularly accessible binding sites that act to modulate intracellular signaling [12,143,144,152]. Although the properties of the receptors involved in the initiation of rapid signaling effects in different tissues/cells are often related to those of a nuclear hormone receptor-like protein [20], some receptor/signaling pathways can be explained by an alternative transmembrane heptahelical G-protein coupled receptor [153]. A candidate G-protein coupled membrane estrogen receptor (GPR30) that binds estradiol and the endocrine disruptor *ortho,para*-dichlorodiphenyldichloroethylene (*o,p'*-DDE) with relatively low affinities has been described [71]. At GPR30, an IC_{50} for BPA was found to be 630 nM and its RBA (when compared to 17β-estradiol) equals 2.83 nM [79]. It is notable that the RBA for BPA at GPR30 is higher than that observed for ERα or ERβ. In addition, this study demonstrated that following a 30 min treatment, both estradiol and 200 nM BPA increase cAMP activity, a GPR30-dependent signaling pathway activated by estrogens, in stably transfected ER-negative HEK 293 cells [79]. Thus, it is likely that BPA exerts some effects through this novel seven-transmembrane estrogen receptor GPR30, although the consequences of inappropriate activation of this signaling pathway are currently unknown. It is important to caution that even in controlled recombinant over-expression systems, the role of GPR30 receptor in rapid estradiol-mediated signaling is still controversial [154,155].

14.1. Rapid signaling effects in pituitary cells

Investigations of potential impact of BPA on pituitary physiology have been largely focused on examining pituitary tumor cell growth and disruption of prolactin release. Established GH3 rat pituitary tumor cell line has been a valuable model in characterizing the mechanisms involved in the estrogenic release of prolactin [156]. Steinmetz et al. demonstrated that 1 μM BPA stimulates prolactin release in GH3 cells and induces hyperprolactinemia in F344 rats [157]. Watson et al. identified an extracellularly accessible plasma membrane ER on GH3/B6 rat pituitary tumor cell line [158,159], and have concluded that concentrations of BPA as low as 1 fM can cause an influx of calcium from extracellular sources via nifedapine-sensitive L-type calcium channels within 1 min of exposure [20,72,77]. In addition, these low concentrations of BPA could induce rapid release of prolactin from pituitary cells; effects that are mediated by the elevation of intracellular calcium and similar to those observed for estradiol [77]. Because immunoselected or limiting dilution selected GH3/B6 cells with very low expression levels of a cell surface ERα epitope were non-responsive, these rapid signaling effects of BPA were associated with expression of an extracellular accessible ERα-like protein. In this GH3/B6 cell model, several other potential endocrine disrupting compounds (DDE, coumestrol, endosulfan, and nonylphenol) induced small increases in ERK1/2 phosphorylation; however, rapid increases in ERK-phosphorylation were not observable in response to 1 nM BPA [68,69].

In contrast to the results from the GH3 cell model, rapid modulation of ERK-signaling in response to BPA has been observed in neurons and immune cells; observations lending further support for the cell/tissue specific effects of BPA. For example, Canesi et al. showed that concentrations of BPA on *Mytilus* hemocytes induced small, but significant, and transient increases in ERK1/2 and PKC pathways with later increases in ERK2 and STAT3 phosphorylation [75]. There results are in agreement with a study of tetrabromobisphenol A (TB-BPA) toxicity, where Reistad et al. found that TBBPA can rapidly (2–4 min) activate the ERK1/2 pathway in human neurophil granulocytes and that in high concentrations TBBPA or BPA (2–12 μM) stimulate production of reactive oxygen species. Both TBBPA and high concentrations of BPA (20 μM) were shown to induce a concentration dependent increase in intracellular free calcium levels [160].

14.2. Rapid and low dose actions of BPA related to the developing CNS

Rapid low dose BPA-mediated effects on ERK signaling were characterized *in vivo* and *in vitro* in isolated primary cultures of cerebellar granule cell neurons [46,153,161]. An analysis of BPA concentration dose response by Zsarnovszky et al. revealed that BPA stimulates an inverted U-shaped curve with efficacy and potency equal to estradiol in the low dose range (10 fM to 10 nM) [46]. In the presence of pharmacological concentrations (micromolar range) of BPA and estradiol, ERK-phosphorylation was also observed, demonstrating a clear non-monotonic and biphasic dose response. Developmental and pharmacological studies of the rapid actions of estrogen and BPA on cerebellar signaling by Belcher et al. revealed a mechanism that induces cell specific activation of ERK1/2-phosphorylation that involves G-proteins, PKA and the Src-family tyrosine kinase, but not EGF receptor or the PI3kinase/AKT pathway [161]. Further, the results of experiments assessing the effects of binary mixtures of estradiol and BPA highlighted the complexity of the rapid signaling mechanism in these developing neurons [46]. These results demonstrate the ability of BPA to act as a highly potent EDC with a potential to disrupt the rapid signaling of estradiol at very low concentrations during brain development.

Numerous *in vivo* studies have shown that prenatal and neonatal exposure to low doses of BPA can disrupt normal sexual differentiation in the rodent brain [162,163]. BPA at 40 μg/kg dose can antagonize the action of estradiol in the adult rat hippocampus by blocking the stimulatory effect of estradiol on synaptogenesis [43]. Additional studies have also implicated BPA in modifying the activity of the mesolimbic dopamine system through upregulation of dopaminergic neurotransmission, an effect that in part involves increased dopamine D1 receptor expression. These endocrine disrupting activities of BPA could

potentially influence reward-seeking behaviors associated with drugs of abuse. In the pheochromocytoma PC12 cell model, high concentrations of BPA (25–150 μM) stimulate rapid release of dopamine through guanine nucleotide-binding protein and N-type calcium channels, illustrating that exposure to BPA may influence the function of dopaminergic neurons [76]. While high concentrations of BPA were used in this study, it is possible that rapid signaling mechanisms involving mobilization of intra- and extracellular calcium contribute to the impact of BPA through presynaptic influences on dopaminergic neurotransmission.

In an effort to discriminate rapid signaling actions of BPA from BPA-mediated gene expression, Yamaguchi et al. examined the effects of low dose BPA (1 pg/mL to 1 μg/mL) on the differentiation of serum-free mouse embryo (SFME) cells and astrocyte progenitor cells [40]. These investigators monitored glial fibrillary acidic protein (GFAP) expression as a marker of differentiation and found that GFAP expression was significantly increased in SFME cells in the presence of 1–100 pg/mL BPA. The BPA-induced increases in GFAP were due to activation of signal transducer and activator of transcription 3 (STAT3) and Smad1. In isolated murine midbrain astrocytes or in astrocyte/neurons co-culture Miyatake et al. demonstrated increased GFAP immunoreactivity and increased intracellular calcium in response to dopamine at BPA concentrations as low as 100 fM and 1 pM, respectively [44]. An inverted U-shaped low dose response was observed between 100 fM and 10 pM, with a second phase of effects observed at concentrations of 10 nM to 1 μM. Similar effects were not observed in response to estradiol. The investigators suggest that these results provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes, which may contribute to potentiate the development of psychological dependence on drugs of abuse.

Together, the results from these CNS-related models highlight the complexity of BPA endocrine disruption effects at low physiological concentration, and the extreme importance of appropriate experimental design, such as including positive and negative controls for each experiment and doing complete dose response analysis for each end-point.

14.3. Rapid BPA effects on the endocrine pancreas

A "non-classical membrane ER" (ncmER) has been described in the endocrine pancreas that mediates the actions of BPA at concentrations as low as 0.1 nM [70]. This action is also involved in the activation of the transcription factor calcium-dependent cAMP-responsive element binding protein (CREB) where 1 nM BPA induces activation [73]. This study showed that BPA and estradiol activate CREB with the same potency as estradiol. In another study, 1 nM BPA potentiated glucose-induced calcium ion oscillations in freshly isolated islets of Langerhans [70], also implicating ncmER.

Involvement of the ncmER in modulation of glucose induced calcium signals in glucagon releasing alpha-cells of the pancreas has also been demonstrated [66]. It was shown that 1 nM BPA can suppress low-glucose induced calcium ion oscillations in alpha cells, the signal that triggers glucagon secretion, through the action of ncmER, G-proteins, and PKG. Moreover, it was demonstrated that BPA exposure rapidly increases insulin release in adult mice following a single dose of 10 μg/kg/day [67]. This effect was insensitive to an antiestrogen ICI 182,780, indicating that classic ERs may not be involved. In contrast, long-term exposure alters pancreatic insulin content and induces insulin resistance in a classic ER-dependent manner [67].

In agreement with previous findings, Adachi et al. reported that long-term exposure to 10 μg/L BPA induces insulin secretion in rat pancreatic islets following stimulation with 16.7 mM glucose. This effect is significantly suppressed by 1 μM ICI 182,780, suggesting that long-term exposure to BPA potentiates glucose-induced insulin secretion via a classic ER-mediated pathway [21]. In the same study, the maximal effective dose of estradiol for insulin secretion was also10 μg/L. However, in contrast to estradiol, BPA exhibited only long-term effects and did not induce acute insulin secretion from islets via plasma membrane ERs.

The rapid effects of BPA on intracellular calcium ion concentration have also been measured and compared to that of estradiol in breast cancer cell models (MCF-7 and MDA-MB-231). A rapid (within 1.5 min) influx of calcium was observed in response to BPA that was significant at the lowest dose tested (0.1 nM) [74]. This response was not blocked by anti-estrogens, demonstrating ER-transactivation independence of these rapid signaling effects of BPA at nanomolar concentrations. In sum, it has been shown that BPA can activate classic ERs in organelles other than the nucleus in target cells. In addition, BPA can bind and activate other estrogen binding proteins, for example ncmER and GPR30. BPA activates these receptor-mediated pathways at concentrations similar to those described for estradiol. However, further studies are needed to delineate BPA's mode of action in the pancreas, CNS and other tissues. Clearly, the studies described above are just starting to unveil the possible link between environmental estrogens, insulin resistance and glucose metabolism.

15. Immune system, allergic response and BPA exposure

It is well established that estrogens play a role in the immune system and recent research shows that BPA is capable of influencing the immune system functions [164,165]. It was demonstrated that at concentrations as low as 10 nM, BPA decreases the adherence index of rat peritoneal macrophages *in vitro* [166]. Based on the understanding that adhesion is the first step in the phagocytic process of macrophages and in antigen presentation, the authors of this study suggested that BPA may modulate immune and inflammatory responses.

To examine the effects of estradiol and BPA on chemokine production, expression of monocyte chemoattractant protein-1 (MCP-1, a member of the chemokine family) in human breast cancer cell line MCF-7 was studied [60]. MCF-7 cells produce a large quantity of MCP-1 in response to interleukin-1 alpha (IL-1α). Addition of 0.1 μM BPA to MCF-7 cells inhibited MCP-1 mRNA and protein expression and electrophoretic mobility shift assay and supershift analysis revealed that treatment with 0.1–1 μM BPA diminished the IL-1α-induced complex formation, although to a lesser degree than estradiol. These results

suggest that BPA represses MCP-1 expression in a different manner than estradiol, possibly depending on the presence of cell-type specific cooperating factors and/or signaling pathways. Further studies are needed to decipher the molecular mechanism of cell type- and stimulus-specific responses.

Regulation of immunoglobulin (Ig) production by BPA and estradiol was examined by Han et al. [63]. Mouse splenocytes (BALB/c mice) were treated with 10 nM to 1 uM BPA or estradiol and the levels of IgM, IgE, IgA and IgG were measured. While IgA and IgG levels were not affected by either treatment, estradiol induced decreases in IgM and increased IgE levels. In contrast, 1 μM BPA treatment resulted in enhanced IgM production and decreased IgE levels [63].

Youn et al. examined the immune response following BPA exposure in the mouse spleen cells [58]. BPA was administrated to mice in drinking water for 4 weeks at 0.015, 1.5 and 30 mg/mL. It was found that BPA induced prolactin production in the spleen, and exposure of BPA increased the activity of splenocyte proliferation. Interestingly, the production of a strong Th-1 type cytokine (IFN-gamma) was induced, while Th-2 type (IL-4) was suppressed by BPA treatment. Based on those findings, the authors speculated that stimulation of prolactin production by estrogenic effects of BPA can affect cytokine production, thus leading to imbalanced cellular immune response. A follow-up study by Yoshino et al. demonstrated that prenatal exposure to BPA results in up-regulation of immune responses [56]. This group measured proliferative responses of spleen cells to antigen, anti-HEL IgG2a and interferon-gamma (IFN-gamma) secreted from splenic lymphocytes were also measured as indicators of T helper 1 (Th1) immune responses, while anti-HEL IgG1 and interleukin-4 (IL-4) were measured as indicators of Th2 responses. The results showed that fetal exposure to BPA was followed by significant increases in anti-HEL IgG as well as antigen-specific cell proliferation, suggesting that prenatal exposure to BPA may result in the up-regulation of immune responses in adulthood.

Goto et al. examined the effects of BPA on mouse splenocyte (BALB/c female mice) proliferation and found that at high concentrations of BPA (20 μM) enhanced Mac1+ splenocyte proliferation [64]. However, the dose used in this study is clearly outside of the physiologic range. To evaluate the effects of estradiol and BPA on the immune function, Sakazaki et al. investigated whether ERα is expressed in splenic T and B cells, and what effects these compounds have on mouse lymphocyte mitogenesis [65]. This group showed that ERα is expressed in both male and female mouse splenic lymphocytes. Next, lymphocytes from mouse spleen were exposed to estradiol or BPA under growth stimulation by lipopolysaccharide or concanavalin A as a mitogen specific for either B or T cells, respectively. It was found that exposure to estradiol (10 nM to 10 μM) or BPA at 1 μM inhibits lymphocytes mitogenesis, especially B cells. However, this group did not determine whether BPA-mediated suppression of lymphocyte proliferation was mediated by the ER-α in these cells.

Another study examined the effects of BPA at low concentrations (0.1–10 nM) on leukocyte differentiation by measuring superoxide production by differentiated HL-670 cells, a human promyelocytic cell line, in the presence of absence of tamoxifen [53]. It was found that that BPA enhances leukocyte differentiation through an ER-independent pathway. Thus, it is possible that long-term exposure to low dose BPA might significantly affect the innate immunity in humans.

The effects of BPA on the production of interleukin-4 (IL-4), a pro-inflammatory cytokine closely associated with allergic immune responses, were examined by several investigators [52,61]. Using different experimental systems, these investigations are in agreement: treatment with BPA results in enhanced production of various cytokines, including IL-4. Yamashita et al. demonstrated BPA effects on murine thymocytes *in vitro* [55]. They showed that precultured thymocytes with 10 μM BPA exhibited enhanced production of IL-3, IL-4 and interferon-gamma. Tian et al. studied the effects of BPA on the *in vitro* production of Th1 and Th2 cytokines in mesenteric lymph node cells from Trichinella spiralis (Ts)-infected mice inoculated orally with BPA [52]. They found that IL-4 production by Th2-dominant mesenteric lymph node cells from Ts-infected mice increased significantly by addition of 3 μM BPA. However, IL-5 production was not affected. This group demonstrated that the IL-4 production was increased both *in vitro* and *in vivo* by treatment with BPA, and this study suggests that BPA might cause allergic diseases by stimulating the IL-4 production by Th2 cells. It was also shown that BPA significantly enhanced IL-4 production in keyhole limpet haemocyanin (KLH)-primed CD4+ T cells in a concentration-dependent manner [61]. The lowest dose of BPA that had an effect on IL-4 production in this study was 10 μM. Furthermore, BPA enhanced the activation of IL-4 gene promoter in hybridoma EL4 T cells transiently transfected with IL-4 promoter/reporter constructs. The authors also assessed the contribution of intracellular calcium-mediated signaling to BPA-enhanced IL-4 production by pre-treating lymph node cells from the immunized mice with various inhibitors known to interfere with calcium homeostasis. BPA increased intracellular calcium ion levels by inhibiting intracellular calcium pumps. The enhancement of IL-4 production by BPA was significantly reduced by nitrendipine, which blocks calcium ion influx and by FK506, a calcineurin inhibitor, demonstrating that the enhancement of IL-4 by is mediated by a calcium/calcineurin/NF-AT signaling pathway [61].

A study by Kim et al. demonstrated that BPA may affect the regulation of the immune system by reducing nitric oxide (NO) and tumor-necrosis factor-alpha (TNF-alpha) via the inhibition of nuclear factor (NF)-kappaB transactivation. These effects of BPA are mediated through the ER, as shown in this study [48]. Peritoneal macrophages isolated from specific pathogen free-BALB/C mice and RAW 264.7 cells, a mouse macrophage cell line, were used in the study. Ten to 50 μM BPA was used. In agreement with this study, a follow-up study by Hong et al. found that BPA treatment enhanced NO production in mouse macrophages *in vitro* [62].

The involvement of endocrine disruptors in autoimmune disease was examined in the study by Yurino and colleagues. In those studies it was found that demonstrated that endocrine disruptors including DES and BPA enhance autoantibody production by B1 cells both *in vitro* and *in vivo* [59].

The production of IL-2, a cytokine that plays an important role in adaptive immune response, was examined in CD4+ Jurkat T cells in response to BPA treatment [49]. Interestingly, other endocrine-disrupting compounds (coumestrol, DDT and TCDD), but not BPA, significantly suppressed IL-2 production at the transcriptional and translational levels. These results demonstrate that BPA elicits differential responses on cytokine production and may be cell-type and stimulus specific. Recently, Yamashita et al. examined BPA effects on murine spleen cells and thymus cells *in vitro* [54]. They reported that at 0.01–1 μM BPA concentrations BPA stimulates production of IL-1, IL-6, IL-12, TNF and MCP-1. The maximum response was observed at 0.1 μM BPA. Alizadeh et al. demonstrated that low dose BPA exposure (0.1 mg/g/body weight) results in augmentation of Th1 immune response [47], thus further implicating BPA involvement in the immune system functions.

16. Conclusions and levels of confidence from the results of mechanistic *in vitro* studies

16.1. Based on existing evidence, we are confident of the following

The criterion for achieving this confidence level is that multiple independent studies had been conducted that showed the same or similar outcome.

a. BPA can act as an estrogen. Its effects are, however, cell type specific.
b. Timing (developmental stage) of exposure and exposure dose/concentration are critical.
c. When BPA binds to classic nuclear estrogen receptors and induce specific ERE binding, BPA is usually less potent than estradiol.
d. When BPA action is mediated by estrogen receptors outside the nucleus, its potency is as high as that of estradiol, ranging within the pico- and nano-molar concentrations.
e. Because of cell-type specific expression patterns and the role of varied specific co-regulatory factors, the effects of BPA might be different in individual cell types and these effects can vary depending on intrinsic and extrinsic influences.
f. BPA is not simply a SERM (selective estrogen receptor modulator).
g. BPA exerts pleiotropic cellular and tissue-type specific effects and non-monotonic dose–response at the cellular and intracellular levels at low physiologically relevant concentrations.

16.2. We consider the following to be likely but requiring confirmation

The criterion for achieving this confidence level is that significant effects have been reported, but the number of independent replications is limited. However, confidence in the findings is increased by the plausibility of the results, based on mechanistic information available from other related studies.

a. BPA exerts some of its effects by binding to the nuclear steroid receptors for estrogen (ERα and ERβ) to subsequently impact expression of estrogen-responsive gene products via the EREs. Thus, cell types that express specific ERs and co-modulatory elements can be especially sensitive to the effects of BPA.
b. BPA exerts some of its effects by binding to nuclear steroid receptors for androgens—the (AR). BPA can act as an antagonist for the wild type AR in some tissues.
c. Ligand-binding domain somatic mutants of ARs are important in prostate cancer: they can be activated by low dose (chronic exposure) of BPA.
d. In cells from the male reproductive system BPA impacts the biosynthesis of steroids which results in altered steroid hormone concentrations.
e. Compared to the impact of brominated BPA derivatives, these studies point toward BPA at physiological concentrations ($\leq 1 \times 10^{-7}$ M) has a relatively minor influence on the TH system.

16.3. Research on BPA suggests several broad themes that should be pursued in the future

a. Because of the apparently complex nature of BPA action on the TH-signaling systems studied to date, additional studies are required in order to develop a mechanistic understanding of BPA's influence of TH-signaling, which has an important impact on the development of wildlife and humans.
b. Increase our knowledge of the physiological/biological relevance of BPA binding to the ER, AR and TR.
c. Investigate the mechanisms responsible for inverted U dose response.
d. We need to develop sensitive cell model systems that detect altered patterns of differentiation and physiologically relevant responses, avoiding systems in which regulators are over-expressed.
e. Identify other BPA-binding proteins and investigate GPR-30 actions in specific cell types upon exposure to endocrine disrupting compounds.
f. Define additive and synergistic effects of BPA and endogenous hormones and other endocrine disruptors.
g. While there are numerous *in vivo* studies demonstrating impact on the nervous system upon early exposures to BPA, much additional research is necessary in order to assign specific molecular mechanism through which BPA acts to impact specific neuronal and glial populations in the developing and mature CNS.
h. Explore low dose BPA exposure on the immune system.
i. Molecular mechanism(s) of acute versus chronic exposure to BPA need to be distinguished and deciphered.
j. Define cell and tissue-type sensitivity and windows of susceptibility to BPA across the life span of experimental animals and humans.

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特集　環境化学物質の作用メカニズムを解き明かす

トキシコゲノミクスの新展開
Percellomeプロジェクトによる2,3,7,8-TCDD－2,3,7,8-TCDF比較

An Attempt for Adding a New Dimension to Toxicogenomics Research : 2,3,7,8-TCDD–2,3,7,8-TCDF Comparison Trial in The Percellome Project

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Percellomeトキシコゲノミクスはマイクロアレイという数万遺伝子の発現レベルを一気に測定するハイスループット技術を利用し，全遺伝子のカスケード解明を最終目標としつつ，従来に比べてより早く安くかつ正確な毒性評価系の確立を目指すものである．筆者らはこのような次世代の毒性評価・予測技術を開発するために，細胞1個当たりのmRNAコピー数を測定するPercellome法を開発した．今までに90以上の化学物質についての網羅的遺伝子発現情報を得て，なお追加中である．本稿では環境化学物質の一例としてダイオキシンの分子毒性に関わる知見を紹介する．

key words
Percellome Project，遺伝子発現カスケード，分子毒性学

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1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌攪乱関連などの分子毒性学研究，Percellomeトキシコゲノミクスプロジェクトなどを厚生労働所掌業務の一環として有機的に推進．

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はじめに

毒性学は生体（Biosphere）と外来性物質（Chemosphere）との相互作用を研究する分野であり，目的は“ヒトの安全”である．日常遭遇する化学物質（生活化学物質，環境化学物質，医薬品や食品を含む）が摂取された際の安全性を担保するため（毒性評価）に，人体実験が困難な場合，身代わりとしての実験動物の毒性所見をヒトに外挿することが行われてきた．これは両者が基本的に同等の生体反応を示すという前提に基づいている．そして，酵素，膜，DNAなど比較的普遍的かつ基本的な標的が主な検討対象となってきた．現在の分子毒性学は，生体反応メカニズムに踏み込み，受容体，転写因子などとの選択的結合によるシグナル伝達障害などの標的特異性の高いものや，エピジェネティックな遅発影響なども直接的な対象とするようになり，基礎分子生物学と直結する時代に入っている．古い話ではあるもののいまだに分子機構の解明が完結していないサリドマイドの催奇形性問題，あるいは，最近の健常人ボランティアに対するバイオ医薬品（治験薬）の微量投与がその全員を集中治療室送りにした事件は，種差問題の解決を含む分子毒性評価法の確立の重要性と，その現状を示していると考えられる．

Ⅰ．トキシコゲノミクス

分子毒性メカニズム解析のためのツールの1つにmRNAを対象とするトキシコゲノミクスがあり，見落としのない網羅性が要求される毒性学では全遺伝子のカスケード解明がその最終目標となる．これにより従来に比べて早く安く正確な毒性評価を目指すことができる．そして，種差・個体差，一生涯の反応性を修飾する胎生期・周産期影響，あるいは複合作用などを包括的に扱う際には，生命科学の各分野との緊密な連携が必須となる．また，従来の毒性学に対してのトキシコゲノミクスは，例えとしては光学顕微鏡に対しての電子顕微鏡のような立場にあると考えられる．電子顕微鏡が広く用いられるようになるには，教科書や図譜が必要であったように，トキシコゲノミクスの実用化にはある程度の量のデータの蓄積と解析のための基礎研究（関連分野との連携を含む）が必要である．そこで，筆者らは，情報の互換性を確保するために細胞1個当たりのmRNA発現コピー数を得るPercellome手法を開発した．これを基盤としたプロジェクトを展開中であり，今までに90以上の化合物についてのデータを蓄積し，その解析ツールを開発している．

Ⅱ．Percellome法

原理は単純で，サンプルの細胞数を測る代わりにDNA濃度を精密に計測し，それをもとに外部標準mRNA（スパイクRNA）を細胞1個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出・測定に移る．スパイクRNAの測定値を基準に，サンプルの各RNAの測定値を細胞1個当たりのコピー数に換算する[1)~3)]．これにより，実験操作，試薬やマイクロアレイのロット差などによる系統誤差を相殺するという本来の目的が果されるほか，測定過程における各種の異常が高感度に検出されることから，品質管理精

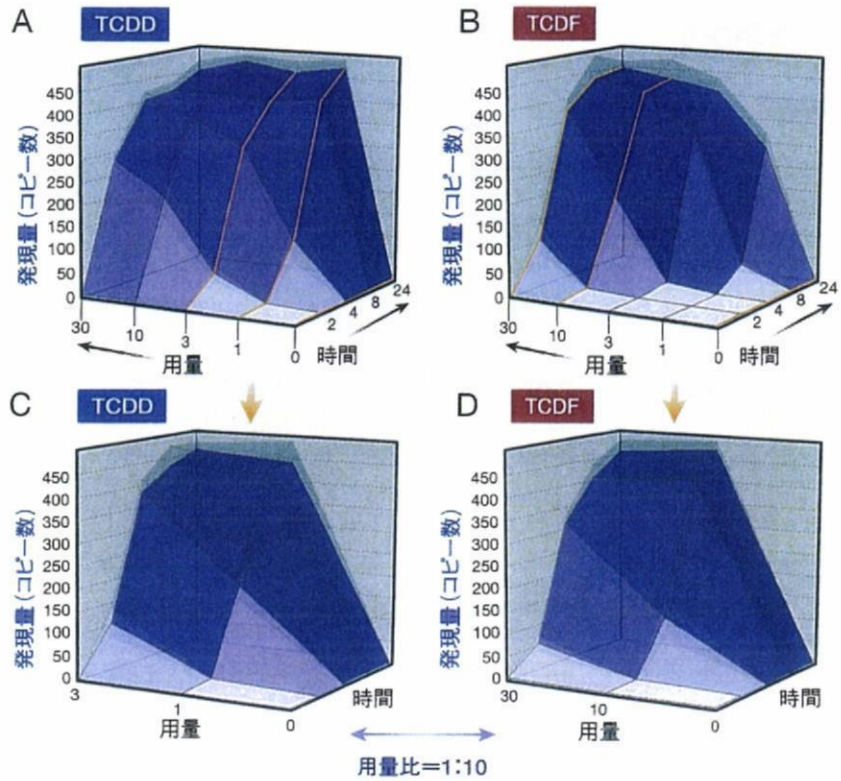

図1. TCDDとTCDFのPercellomeデータ：TEF依存遺伝子の抽出法（1）

TCDDおよびTCDFの単回経口投与をC57BL/6雄マウスに行った．用量は両実験とも0（溶媒対照），1，3，10，および30 μg/kgとし，投与後2，4，8，および24時間後に肝を採取しマイクロアレイ解析を行った（両動物実験は1カ月を隔てて，国立医薬品食品衛生研究所，環境保全型動物実験施設内にて厳重管理の下に実施された）．代表例としてcytochrome P450, family 1, subfamily a, polypeptide 1（Cyp1a1; Affymetrix probe ID 1422217_a_at）を示す．
A：TCDDによるCyp1a1の発現変動のSurface（反応曲面）表示．
B：TCDFによるCyp1a1の発現変動のSurface表示．丁度，用量について10倍ずれた反応を示している．
C：TCDDの3，1，0から作製したSurfaceとD：TCDFの30，10，0から作製したSurfaceが形状および発現値ともにほぼ完全に一致している．このような遺伝子をTEF依存性とした．

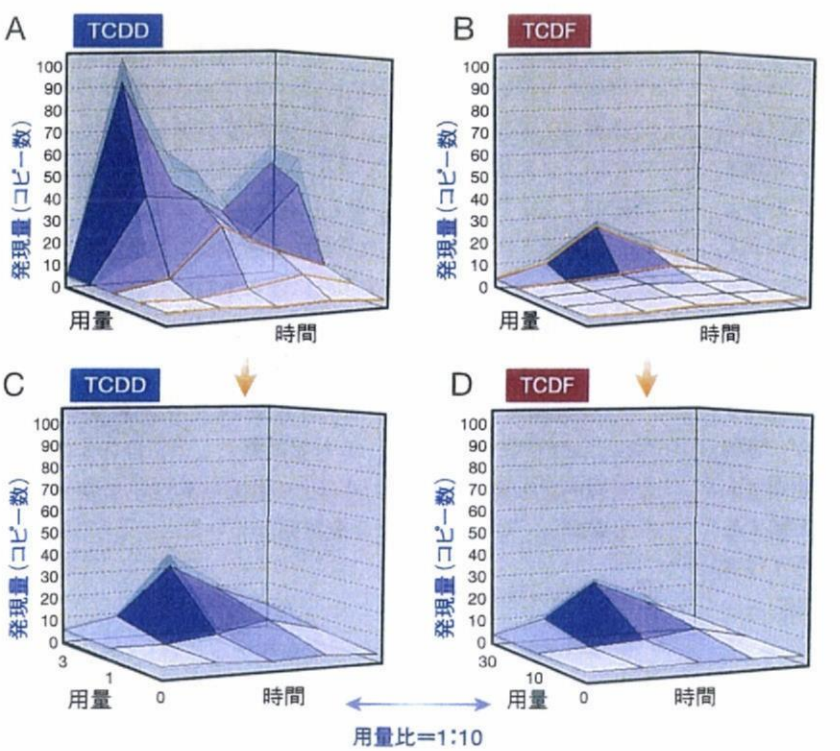

図2. TCDDとTCDFのPercellomeデータ：TEF依存遺伝子の抽出法（2）

TEFに従うもう1つの例としてTCDD-inducible poly (ADP-ribose) polymerase（Tiparp；1452160_at）を示す．A，B，C，Dは図1と同様の表示．AとBを比較すると一見違った反応をしているようだが，CとDを比較するとTEF依存性であることがわかる．

度の向上が図られている．例えば，高密度マイクロアレイで問題となるプローブの飽和によるダイナミックレンジの狭小化の検証・回避に役立っている．新世代Affymetrix GeneChipにおいて高発現遺伝子プローブが容易に飽和し高用量域で定量性を失う現象は，一般的なデータ標準化手法では検出困難であり，Percellome法を用いて初めて直接的に感知することができる．現在，筆者らはサンプルRNA量をメーカー推奨プロトコールの半量にすることなどにより効率的にこれを回避している．

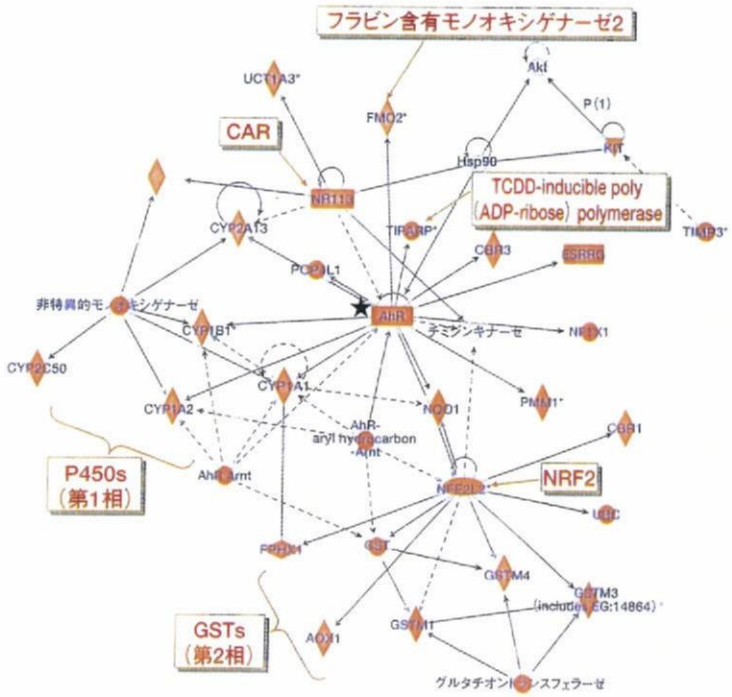

図3. TEF依存遺伝子の描くPathway

図1，2の方法で抽出されたTEF依存遺伝子約140をIngenuity Pathways Analysis 5.5（Ingenuity Systems, Inc.）に投入し，得られるPathwayの代表的なものを示す．AhR（★）を中心にNRF2を含む転写因子群を介して，第1相および第2相の代謝酵素を含むAhR依存的な遺伝子を認める．
赤色；計算に投入したTEF依存遺伝子のうち，このPathwayに含まれるもの．
灰色；Pathwayのメンバーとしてソフトウェアが拾ったもの．

Ⅲ. Percellome Project

マウス肝を中心とした約90化合物（毒性学的情報の豊富なもの）の単回暴露による初期応答遺伝子データベースを構築するとともに，シックハウス症候群[注1]を考慮した低用量域での吸入トキシコゲノミクス，発生毒性についての胎児トキシコゲノミクス，また*in situ*ハイブリダイゼーションによる局在確認系を立ち上げた．現在，これらに加えて反復暴露による慢性毒性，および多臓器間の関連性を検討する研究を展開中である．データの一部はホームページ（http://toxicomics.nihs.go.jp/db/）にて公開中であり，今後，内容を拡充する予定である．

ここで，本プロジェクトにより明らかとなった一般的な注意点について簡単に触れる．それは，再現性の高いデータを得るためには実験管理を厳重にする必要があるという点である．マウス肝で有意に発現する12,000（いずれかの時点で3コピー/細胞以上）の遺伝子のうち，概日変動を示す遺伝子が3,600以上あり，最大で数十倍以上の発現差が見られる．この変動が実験の邪魔をしないようにするためには，動物飼育施設の明暗サイクルを2週間以上一定に保ちマウスの概日リズムを安定化し，そのうえで，マウスに検体を投与する時刻，およびサンプルを採取する時刻を±30分以内に限定する必要がある．概日リズムは肺，腎，脳，心，胸腺などにも明瞭に存在するので，同様の注意が必要である．培養細胞実験においても細胞密度，培地交換，シャーレのインキュベーター内の位置，倒立顕微鏡での観察の影響（操作による培地攪拌による刺激），多穴プレートでは辺縁と中央の違いなどが，遺伝子発現データに大きく影響することを観察している．

注1　住宅の高気密化や化学物質を放散する建材・内装材の使用などにより，新築・改築後の住宅やビルにおいて，化学物質による室内空気汚染などにより，居住者に生じる様々な健康障害の総称．

Ⅳ. Percellomeを活かしたデータ解析

Percellome法の絶対量化データの最大の特徴は，原点（zero）から表示可能なコピー数という“名数”である点にある．基底発現量がわかる，データの四則演算が可能であり，例えば処置群から溶媒対照群の値を引くことで概日リズムや溶媒による影響を容易に取り除くことができる，などの利点が多い．これらは発現比を用いた解析では問題となる操作であるが，Percellome法であれば自然な処理が可能である．さらに，この性質を利用し，用量と時間が発現変動を規定するとの前提に基づき，用量・時間・発現値を三次元直行座標に置いた1つの三次元グラフに反応曲面（Surfaceと呼称）として可視化して，直感的なデータの把握を実現している．1つの化合物についてGeneChipのプローブセット数に対応する約45,000枚のSurfaceが描出される．この中から生物学的に有意であると考えられるパターンを含む特徴的な発現パターンを効率的に自動的抽出するRSort（Surfaceの凹凸の特徴を解析し，その明瞭さの順にソートする）アルゴリズムを考案，活用している．また，別途に独自開発した教師無しクラスタリングアルゴリズム（MADIC[4]など）を活用して，発現パターンが類似しているものを網羅的にクラスター化している．2枚のSurfaceの形の類似性を数値化するアルゴリズム（tmf）も開発済みであり，注目すべき遺伝子の発現パターンを鋳型として同様の発現誘導パターンを呈する遺伝子を自動抽出できるようになっている[5]．

Ⅴ. TCDDとTCDFのPercellome比較によるTEF依存遺伝子および非依存遺伝子の解析アプローチ

ここでは解析例として，TCDD（2,3,7,8-tetrachlorodibenzo-*p*-dioxin）とTCDF（2,3,7,8-tetrachlorodibenzofuran）の遺伝子発現応答データを比較しTEF（toxicity equivalence

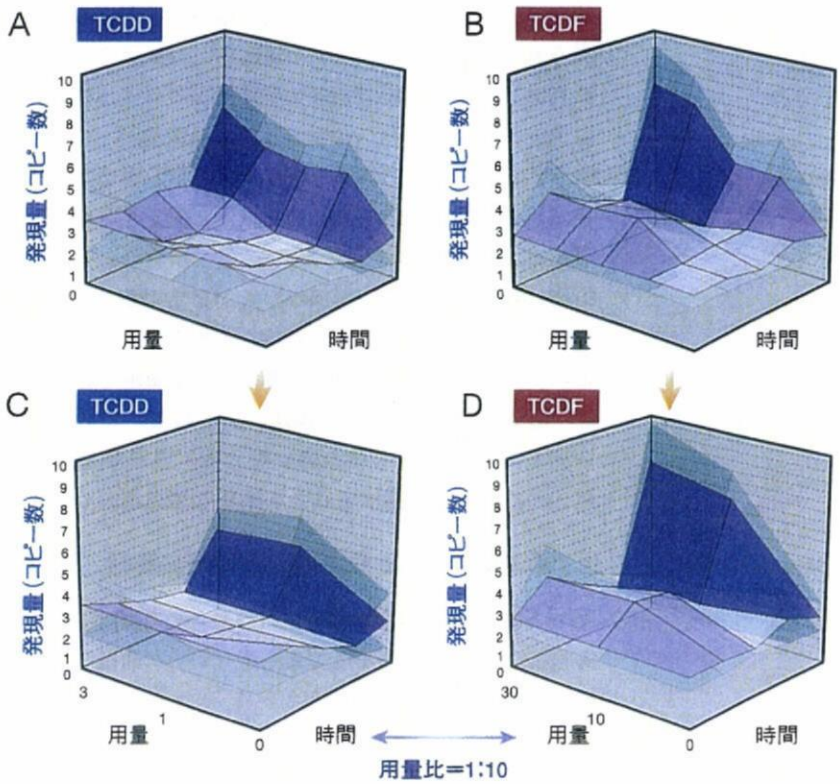

図4. TEF非依存遺伝子

TEFに従わない遺伝子の一例として，Hectd2 (HECT domain containing 2, 1433944_at)のSurfaceを示す．A, B, C, Dは図1と同様の表示．2時間目の応答の違いのほか，24時間目の応答がTCDF＞TCDDである．

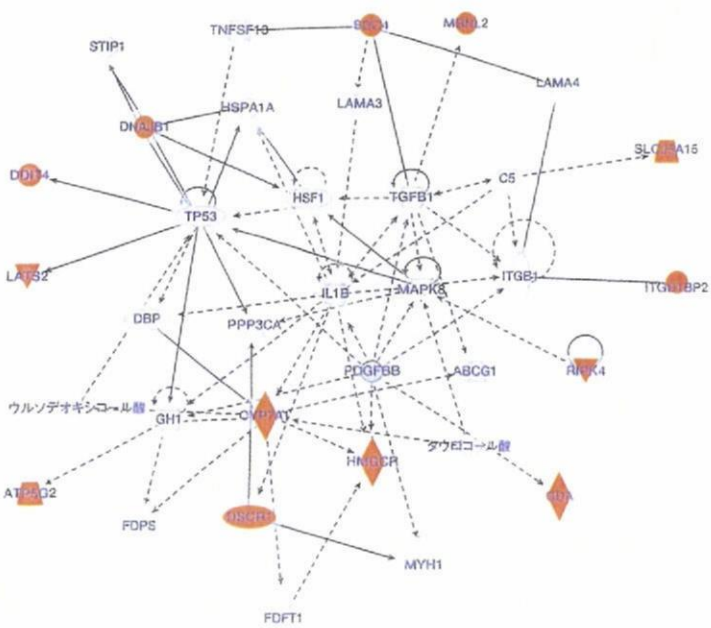

図5：TEF非依存遺伝子の描くPathway

図1の方法を利用し抽出されたTEF非依存遺伝子約20をIngenuity Pathways Analysis 5.5 (Ingenuity Systems, Inc.) に投入し，得られるPathwayの代表的なものを示す．AhRは含まれず，p53，TGF-β，MAPK8などが見られる．赤色；計算に投入したTEF非依存遺伝子のうち，このPathwayに含まれるもの．灰色；Pathwayのメンバーとしてソフトウェアが拾ったもの．

factor) [6]に従う遺伝子と従わない遺伝子を検討した事例を紹介する．

ダイオキシン類，すなわちダイオキシン，ジベンゾフラン，およびコプラナーPCBは，そのいずれにもベンゼン環に結合する塩素の数の違う異性体や同族体が多数あり，個々はそれぞれダイオキシンとしての生物活性の強さ，例えば*in vitro*実験系でCyp1a1の発現を誘導する能力に違いがある．他方，環境中では，これらダイオキシン類の同族体などを様々な比率で含む混合物として検出されることから，その生物影響の総体強度を推定するために，個々の同族体の活性を合計して評価することが行われている．その際の強度の単位にTEFが用いられる．TEFは最も活性が強い2,3,7,8-TCDDを1とし，2,3,7,8-TCDFは0.1，1,2,3,7,8-pentachlorodibenzofuranは0.05，などとして表す．なお，TEFの値は，ほぼ，AhR結合能に比例していることが経験的に知られている．

ダイオキシン毒性は，受容体原性毒性の典型であり，その説明には“AhRノックアウトマウスがダイオキシン投与に対し事実上無反応”であることが用いられる．すなわち，このノックアウトマウスでは，体中に広がったTCDDはそこにある酵素や膜などの生体分子に対して何の影響も与えないということを示している．野生型のマウスがTCDDで死ぬのはAhRが存在するからであり，言い換えれば，AhRからの異常なシグナルによるという

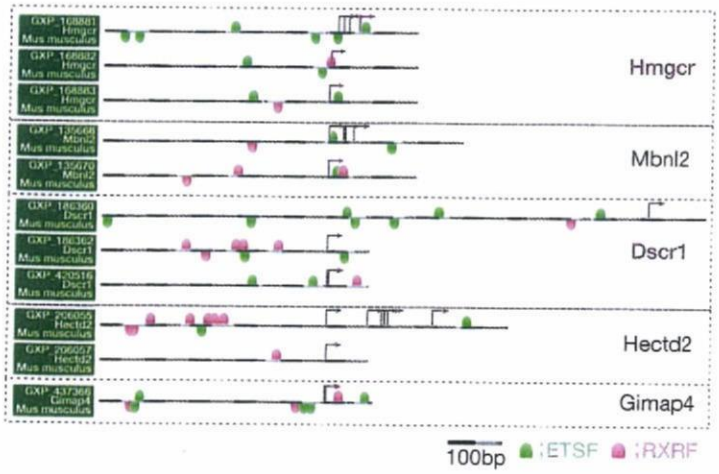

図6：TEF非依存遺伝子の *in silico* プロモーター解析

TEF非依存遺伝子約20のうち，TCDF優位の5遺伝子を絞り込み，Genomatix Software GmbHの提供する *in silico* プロモーター解析の結果を示す．5つの遺伝子に共通して，ETSファミリーとRXRファミリーの転写因子の結合配列を認めた．Hmgcr；3-hydroxy-3-methylglutaryl-coenzyme A reductase，Mbnl2；muscleblind-like 2，Dscr1；Down syndrome critical region homolog 1 (human)，Hectd2；HECT domain containing 2，Gimap4；GTPase，IMAP family member 4.

ことになる．この際の毒性も概してTEFに従うことが知られており，TCDDの1に対してTCDFの10が同等の影響を及ぼす．しかし，リガンド分子個有の作用には受容体毒性学上，興味があるところであり，培養細胞に対する影響を検討した際にこの値が逆転する場合があることを見いだしたことから，TCDF特有の作用がある可能性をマウス肝において遺伝子発現レベルで検討することとした．

TCDDとTCDFについて，以下のような同一プロトコールを用いての実験を行った．12週齢雄C57BL/6マウスの1群3匹，20群を用意し，0，1，3，10，および30 μg/kgの用量で単回強制経口投与後，2，4，8，および24時間後に肝を採取し，Affymetrix GeneChip，MOE430 2.0によりPercellome遺伝子発現データを得た．2時間目の反応を見やすくするために，仮想0時間に2時間溶媒対照群の値を流用し，用量軸5点，時間軸5点から成る5×5の三次元Surfaceを作製した．さらに，TCDD＝1，TCDF＝0.1というTEF値に従った反応を示す遺伝子を抽出するために，TCDDの0，1，3 μg/kg群から成る3×5のSurfaceとTCDFの0，10，30 μg/kg群から成る3×5のSurfaceを用意した．そして，この3×5のSurface同士について，上述のtmfアルゴリズムにより類似度を計算し，類似性の十分に高い遺伝子のリストを得た．次にコピー数が同等であるか，反応が投与依存的変動として生物学的蓋然性があるかを3×5および5×5のSurfaceにより確認し，TEFに忠実に従うTEF依存遺伝子（図1，2）を約140，従わないTEF非依存遺伝子を約20得た．TEFに従うと判定された遺伝子群を，Ingenuity Pathway Analysis (Ingenuity Systems, Inc.) により既知情報と照合するとAhRの下流の第1相代謝酵素やNrf2下流の第2相代謝酵素を中心に，AhRを中心としたPathwayの構成要員であることが示され（図3），上述したTEFについて現在想定されている分子背景に合致するものであった．従わない遺伝子についても，5×5のSurface同士を比較し，TCDDとTCDFで反応のパターンが異なるもの，および類似していてもTEF値の10倍差を説明できないもの，すなわち，TCDFが同等あるいはより強い反応を示すものを抽出した（図4）．TEF非依存遺伝子群は既知情報との照合で予想どおりAhRを含まないPathwayを描き出した（図5）．*In silico* プロモーター解析ソフトウェア (Genomatix Software GmbH) に甘い条件で遺伝子リストを投入した結果，すべてに共通するものとして多数のエレメント，例えば，E2F，EKL，ETS，HES，NR2，RXR，SP1，TBPなどのファミリーが見いだされたが，AhR結合配列は抽出されなかった．さらに，非依存遺伝子のうちTCDF優位の5遺伝子を絞り込みパスウェイ解析を行った結果，TNFを中心とし，ESR1やABCA1を含むネットワークが描かれ，*in silico* プロモーター解析では5遺伝子に共通するものとしてETSファミリーとRXRファミリーの結合部位が選択された（図6）．ETSはERK/MAPKシグナル系の下流に位置し，その1つであるETS2の強制発現系の実験などからp53系を介する胸腺系のアポトーシス，あるいはダウン症候群との関連性などが指摘される．これらの既知情報ベースの解析結果は限られた共通の公開情報源を基にしているため，概して同じリストに収束する．しかし，得られたリストのうち，この検索に投入しなかった遺伝子（図5中の灰色）について，再度Surfaceを吟味すると選定基準ぎりぎりで排除されていた遺伝子が見つかる．ここでは，図5中のTgfb1 (transforming growth factor beta 1)，Hspa1a (heat shock protein 1A)，およびFdft1 (farnesyl diphosphate farnesyl transferase 1) が該当する．このような既知情報と実際のデータとの往復が，データ解釈の向上と今後の検証実験の計画立案に役立つものと見込まれる．

おわりに

このTCDDとTCDFの実験結果の比較によるダイオキシン類化合物の生体影響に関わる分子メカニズム解析はいまだ途上にあり，追加としてAhRノックアウトマウスを用いた投与実験やChIP（クロマチン免疫沈降）解析などによる確認作業が考えられる．ここでは，Percellome Projectの投与実験の組み合わせと，それらに対するPercellome法の利点を生かした網羅的な解析が，環境化学物質をはじめとする外来性化学

物質（Xenobiotics）の生体影響に関する分子生物学的メカニズム解明研究のユニークな糸口を提供する手段としても利用可能であることを示すことができたと考える．誌面の都合上，他に譲るが，ヒトに対する催奇形性があり使用禁止となっていたが，癌や難治性炎症性疾患の治療薬として再登場したサリドマイドについて，成獣雄マウスの肺に及ぼす影響と経胎盤的にマウス胎仔に及ぼす影響とをPercellome解析により対比すると，間葉系成分に対する共通の抑制シグナルの存在が示唆される事例を見いだした．異なったプロトコールで異なった組織に対して行われた実験の間でも，このように共通のメカニズムを抽出しうる可能性を見ており，今後の複合的展開に大きな期待を抱いているところである．今後，本法の利点を生かした解析をさらに進めるとともに，データ・解析ツールの公開Webサイトの充実，および，実験のみならずデータ解析・データマイニングについての共同研究を含めた展開を加速させていきたい．

謝辞 本Percellome Projectの遂行にあたっては，当毒性部の全メンバー，特に松田菜恵，辻 昌貴，森田紘一，今井あや子，安東朋子，安部麻紀，森山紀子，近藤優子，青柳千百合，相原妃佐子，渡辺 忍の各氏の卓越した働きに深謝する．三次元Surface可視化およびそれを用いた解析ツール群のアルゴリズム開発は共筆者の相崎健一主任研究官による．データベース関連，MADIC実装などのIT開発はNTTコムウェア，日本NCR（日本テラデータ）との共同研究に負うところ大である．本研究は厚生労働科学研究費補助金H13-生活-012，H13-生活-013，H14-トキシコ-001，H15-化学-002，H18-化学-一般-001などによる．

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Special Review

Percellome Projectによる毒性トランスクリプトミクスの新しい試み

Percellome Project as a New Approach to Toxicology Transcriptomics

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身の回りの物質の毒性（有害性）を予測し，その被害を未然に防ぐのが毒性学の役割である．この精度向上を目指したトキシコゲノミクス研究を実施する際に，マイクロアレイなどから細胞１個当たりのmRNAコピー数を得るPercellome法を開発した．90化合物のマウス肝初期応答データを採取し終え，新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectを展開している．

key words
トキシコゲノミクス，分子毒性学，遺伝子発現カスケード，標準化，Percellome法，3次元多層(Millefeuille) データ

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1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌かく乱関連などの分子毒性学研究，トキシコゲノミクスプロジェクトなどを厚生労働所掌業務との有機的連携のもとに推進．

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はじめに

医薬品，食品，化粧品，生活関連用品など，身の回りの物質が我々の身体に取り込まれた際に生じる可能性のある毒性（有害性）を予測し，それらの使用に際しての被害を未然に防ぐのが毒性学の役割である[注1]（図1）．具体的には，人々の安全を確保するために使用法（用途）や使用量（残留量）を制限したり，場合によっては禁止したりするための科学的根拠を提供するが，その際，人の身代わりとして実験動物を用いる場合が多い．このような毒性学の精度向上の一環として，従来からの毒性研究（毒性症候学，毒性病理学，など）に加えてのトキシコゲノミクス（Toxicogenomics）研究が進められている．

トキシコゲノミクスでは，物質が生体に及ぼす影響をトランスクリプトームとして観測・解析する．その際，①分子毒性学を構築し種差や個体差の問題，複合暴露の問題などを解決するためには，遺伝子発現カスケードの全容解明を目指す必要がある，②形態学的に変化が現れた段階のトランスクリプトームは，遺伝子発現カスケードの最終段階に過ぎない，③形態変化の現れないごく初期段階を含む遺伝子発現カスケードを描出するためにはまとまった量のデータの蓄積が必須である，との観点から，筆者らは，マイクロアレイや定量PCRから細胞１個当たりのmRNAコピー数を得るPercellome手法と，そのデータ解析のための３次元多層（Millefeuille）システムを開発・実用化した．遺伝子発現量が共通の尺度，すなわち“コピー数／細胞”で表現されることから，検体間，実験間，マイクロアレイのバージョン間，異なったプラットホーム間，などのデータ比較が直接的に行えるようになり，数年かけて蓄積したデータの有機的活用が可能となった．現在，90種類の化学物質によるマウス肝の初期応答データを採取し終えたところである．新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectの概要を紹介する．

注1　環境への配慮も含まれる．

Ⅰ．Percellome法：細胞１個当たりのmRNA絶対量を得る方法

原理は単純である．サンプルの細胞数を計測し，外部標準mRNA（スパイクRNA）を細胞１個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出，測定に移る．サンプルのRNAの測定値を，スパイクRNAの値を基準に，細胞1個当たりのコピー数に換算する．実際には細胞数を直接計測するのが困難なことが多いため，その代替指標として細胞核内のゲノムDNA量を用いる[1), 2)]．定量性・直線性の検証にはLBM標準サンプル（肝[L]と脳[B]を100：0，75：25，50：50，25：75および0：100に混合した5サンプルから成るセット）を用いる．なお，スパイクRNAは，5種類の枯草菌遺伝子のmRNAを濃度公比3で混合したカクテル（dose-graded spike cocktail；GSC）として用意した．高精度を要求されるDNA定量法は手作業プロトコールおよび自動ロボット（PerkinElmer JANUS）のプロトコールを準備

図1. 毒性学の対象

毒性学は，身の回りの物質が引き起こす障害を予測し，その発生を未然に防ぐことを目的としている．トキシコゲノミクス（毒性ゲノミクス）は，最先端の網羅的遺伝子発現解析技術を用いて，従来の毒性学の予測の精度を著しく向上，迅速化させることで，国民の健康安全の確保にさらに貢献することを目指している．

中である．カクテルとも共同研究ベースで供給可能である（連絡先：kanno@nihs.go.jp）．また，ERCC（The External RNA Control Consortium）と連絡をとるとともに，国際的標準化への関与を深めるため平成18年度厚労科研費「医薬品などの有効性・安全性評価に資する遺伝子発現解析の国際的標準化に関わる研究（H18-特別-指定-023）」を立ち上げた．現在，この他にシックハウス症候群を考慮した低用量域での吸入毒性トキシコゲノミクス，1匹のマウスから多臓器を採取しそれらの連携状況をトランスクリプトームから解析する多臓器トキシコゲノミクスを開始し，特徴的な遺伝子について組織内の発現分布を*in situ*ハイブリダイゼーションで確認する作業を並行している．また，下記の3次元データをweb公開するサーバを整備し，一部の化合物から3次元多層（Millefeuille）データを順次閲覧可能とした（http://toxicomics. nihs.go.jp/db/）．

Ⅱ．3次元多層（Millefeuille）データシステム：生物系研究者に優しいデータ可視化と解析

医薬品を含む毒性既知の90化合物について単回経口投与後のトランスクリプトームデータを取得して，初期応答遺伝子カスケードを解析するための基盤データベースを構築した．現在，第二段階として反復暴露データ集積を開始した．データは，用量軸，時間軸，および遺伝子発現軸から成る3次元表示により，遺伝子発現の用量および時間に依存した変化を1枚の曲面として表すことで可視的に変化を判別しやすいように配慮した（図2）．これにより，コンピュータが選び出した遺伝子クラスターの中身を確認する際，特に，mRNAの合成分解のスピードなどの知見から生物学的にありえないパターン（用量軸の方向にも時間軸の方向にもジグザグな変化など）を排除する際に威力を発揮している．

1つの実験から排出されるGeneChip約50枚のデータを一括処理する能力を持ったPercellome自動換算・データ品質管理（QC）に関わるソフトウエアに加えて，3次元多層（Millefeuille）データに最適化した，発現パターン類似性による候補遺伝子検索，およびそれを発展させた教師無しクラスタリング[3]を中心とした解析システム（MF System, MFシリーズ，開発：相﨑健一）を独自に実用化し，開発継続中である（図3）．これらにより，データQCはその日のうちに，基本的な発現情報検索から全遺伝子の教師無しクラスタリングまでを3日間で完遂できるものとなっている．

この基本解析を用いて，発現パターンによって分類された候補遺伝子リストが多数生成される．一部の幸運な例ではただちに新規と思われる毒性関連反応を見いだすことができた．またそうでない場合のための1つの補強手段とし