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REVIEW

DNA microarray-based gene expression profiling of estrogenic chemicals

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Abstract We summarize updated information about DNA microarray-based gene expression profiling by focusing on its application to estrogenic chemicals. First, estrogenic chemicals, including natural/industrial estrogens and phytoestrogens, and the methods for detection and evaluation of estrogenic chemicals were overviewed along with a comprehensive list of estrogenic chemicals of natural or industrial origin. Second, gene expression profiling of chemicals using a focused microarray containing estrogen-responsive genes is summarized. Third, silent estrogens, a new type of estrogenic chemicals characterized by their estrogenic gene expression profiles without growth stimulative or inhibitory effects, have been identified so far exclusively by DNA microarray assay. Lastly, the prospect of a microarray assay is discussed, including issues such as commercialization, future directions of applications and quality control methods.

Keywords DNA microarray · Gene expression profiling · Estrogen · Phytoestrogen · Focused microarray · Signal transduction

Introduction

Expression profiling of estrogen-responsive genes

Almost 2 decades have passed since the first report of a miniaturized, high-throughput DNA microarray for gene expression profiling [1], and a number of DNA microarray-based applications, including for diagnostic use, have been developed [2–4]. A DNA microarray is a collection of DNA spots containing picomoles of DNA with specific sequences attached to a solid surface to measure the expression levels of a number of genes simultaneously (gene expression profiling) or to genotype multiple regions of a genome (genotyping). There are two major classes of applications of DNA microarrays for gene expression analyses: screening of a gene or a set of genes for further use, and profiling of phenomes, such as genetic phenotypes, diseases, responses to chemicals and clinical annotations, by means of gene expressions [5]. For the latter use, a focused microarray, a DNA microarray containing a limited set of genes for a specific purpose, would be desirable because statistical significance established by a set of reliable genes but not by their functional importance needs only a limited number of genes, generally about a hundred. Such sets of genes, collectively called a signature or a module, have been identified for various applications including cancer diagnosis and risk assessment of chemicals as they are the fields having a profound impact on people's lives. For example, the US Food and Drug Administration (FDA) approved DNA microarrays as in vitro diagnostic devices for predicting breast cancer risks [6] and typing cancer of unknown origins [7]. Meanwhile, it has become an urgent issue to develop in vitro assay systems for the risk assessment of chemicals because of increased emphasis on the safety management of commercial chemicals and their products, such as manifested by REACH, a European Union Regulation, [8], and of intensive demands to replace animal tests for toxicity testing with pathway-based in vitro assays [9].

Xenoestrogens or exogenous estrogenic chemicals are considered as a major source of endocrine disruptors, a class of chemicals in the environment having the ability to disrupt human developmental and reproductive systems;

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therefore, they attracted extensive attention in the late 1990s. Since this timing coincided with the development of DNA microarrays, their application for screening and testing environmental chemicals became an immediate reality. Thus, while a high-throughput DNA microarray assay was not considered as a major method for detecting xenoestrogens in early 2004 [10], its promising future was discussed, especially as one of the key assays for the evaluation of xenoestrogens based on a mechanism and pathway-based system to connect transcriptomic information with genomic, proteomic and metabolomic information [11, 12].

Assays for detecting estrogenic activity

Several assays have been developed to detect estrogenic activity, and they are classified according to the molecular and cellular mechanisms of estrogen action: ligand-binding assay (ligand-receptor interaction), reporter-gene assay (receptor-DNA interaction), yeast two-hybrid assay (receptor-coactivator interaction), DNA microarray assay (comprehensive analysis of transcripts), enzyme-linked immunosorbent assay (ELISA; detection of gene products), proteomics analysis (comprehensive analysis of proteins), cell-growth assay and various animal tests including the life-cycle test and uterotrophic assay [11]. More recently, new techniques have been developed, such as those based on serial analysis of gene expression (SAGE) [13] and next-generation sequencing-based gene-expression profiling (RNA-Seq) [14], for comprehensive analysis of transcripts. These assays have advantages and disadvantages [10], and the most suitable assay should be selected by considering the time, cost, sensitivity, accuracy/reliability, safety/ethics and purpose. For example, while the ligand-binding assay is easy to perform, rapid and relatively cheap, it cannot give information about the accessibility of the ligand to its receptor within a cell; moreover, it is difficult to tell whether the ligand is an agonist or an antagonist. Importantly, although RNA-Seq gives mechanism-based information about transcripts within a cell, it is still too early to conclude that it can replace older technologies including DNA microarray-based gene expression profiling because, apart from its technical immaturity [15], no new pathways or markers applicable to diagnostics or toxicity testing have yet been found.

Estrogenic chemicals and phytoestrogens

A number of natural and synthetic chemicals are known to show weak to strong estrogenic activity [16]. Estrogenic activity includes anti-estrogenic activity or antagonistic activity against estrogen receptors (ERs) because of the presence of selective estrogen-receptor modulators (SERMs), a group of chemicals with agonistic or antagonistic activity depending on cell or tissue types [17], and because of the nature of the assay system; for example, the ligand binding assay cannot distinguish between agonists and antagonists but has been used to evaluate estrogenicity [11]. Phenolics and polyphenolics containing at least one phenol ring are abundantly present in the environment and food; thus, they are the sources of beneficial, toxicological and/or optionally endocrine-disrupting activities [18, 19]. Industrial phenolics, such as alkylphenols, bisphenols, chlorinated phenols, parabens, benzoylphenols and pharmaceutical phenolics (ethinylestradiol and diethylstilbestrol, for example), and natural phenolics, such as endogenous estrogens (estrone, estradiol and estriol), phytoestorgens and some mycotoxins (mycoestrogens), are known to show estrogenic activity [19–24] (summarized in Table 1). Phytoestrogens are plant-derived chemicals (phytochemicals) that can mimic estrogenic effects and thus are useful for hormone-replacement therapy [22], and they are characterized by their basic structure, such as a flavonoid, chalconoid, phenylpropanoid (stilbenes and lignans) or coumestan structure, which contains phenol rings. Mycoestrogens are a group of fungal metabolites with estrogenic activity and include zearalenone and α-zearalanol [25]. Hydroxycinnamic acid derivatives, such as caffeic acid and ferulic acid [26], and phenolic derivatives of terpenoids, such as glyceollin [27] and carnosol [28], also show estrogenic activity. On the other hand, some industrial chemicals having no phenolic/aromatic hydroxyl groups also show activity and include perfluorinated compounds [29]; pesticides and herbicides such as dichlorodiphenyltrichloroethane (DDT), atrazine and endosulfan [23]; environmental pollutants such as dioxin, polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) [23]; plasticizers such as phthalate esters [23]; pharmacological estrogens such as tamoxifen [17]; metalloestrogens, a class of inorganic xenoestrogens, such as metal/metalloid anions (arsenite, nitrite, selenite, and vanadate) and bivalent cationic metals (cadmium, calcium, cobalt, copper, nickel, chromium, lead, mercury and tin) [30]. Natural chemicals without a phenolic structure but with estrogenic activity include terpenoids, such as β-sitosterol [31], withaferin A [32] and ginsenoside Rg1 [33], and indoles, such as indole-3-carbinol [34] and melatonin [35]. Some of them may bind to ERs and/or other receptors, and others may show activity after modification or degradation within a cell, although most of the mechanisms have not been fully understood.

Gene expression profiling of estrogenic chemicals

Protocols for DNA microarray-based gene expression profiling

DNA microarray-based gene expression profiling for the evaluation of estrogenic chemicals has been reported by

Table 1 List of estrogenic chemicals

Chemical		Reference
Group	Example	
Phenolics with estrogenic activity		
Industrial phenolics		
Alkylphenols	Nonylphenol	Watanabe et al. [122]
Bisphenols	Bisphenol A	Koda et al. [123]
Chlorinated phenols	Pentachlorophenol	Terasaka et al. [53]
Parabens	Butylparaben	Routledge et al. [124]
Benzoylphenols	2,4-DHBP, 2,2′,4,4′-THBP	Koda et al. [123]
Pharmaceutical phenolics	Ethinylestradiol	Koda et al. [123]
	DES	Odum et al. [125]
Natural phenolics		
Endogenous estrogens	Estrone, estradiol, estriol	Hilf et al. [126]
Phytoestrogens and mycoestrogens		
Flavonoids (flavonols/flavanones/flavones/isoflavones)	Genistein, daizein, biochanin A	Jefferson et al. [25]
Chalconoids (chalcones/dihydrochalcones)	Phloretin	Ise et al. [52]
Phenylpropanoids (stilbenes and lignans)	Resveratrol (a stilbene)	Ashby et al. [127]
	Secoisolariciresinol (a lignan)	Saggar et al. [128]
Coumestans	Coumestrol	Jefferson et al. [25]
Mycotoxins	Zearalenone, α-zearalanol	Jefferson et al. [25]
Other phytochemicals		
Hydroxycinnamic acids (phenolic acids)	Caffeic acid, ferulic acid	Serafim et al. [26]
Phenolic terpenoids	Glyceollin	Ng et al. [27]
	Carnosol	Johnson et al. [28]
Estrogenic chemicals without a phenolic ring		
Industrial chemicals		
Perfluorinated compounds	PFOS, PFOA	Kjeldsen et al. [29]
Pesticides and herbicides	DDT	Bulger et al. [129]
	Methoxychlor	Laws et al. [130]
Environmental pollutants	TCDD	Hutz et al. [131]
	PCBs	Salama et al. [132]
Plasticizers (phthalate esters)	Dibutyl phthalate, butylbenzyl phthalate	Zacharewski et al. [133]
Pharmacological estrogens	Tamoxifen, toremifene	Carthew et al. [134]
Metalloestrogens (metal/metalloid anions and bivalent cationic metals)	Arsenite	Davey et al. [135]
	Cadmium	Garcia-Morales et al. [136]
Natural chemicals		
Terpenoids	β-sitosterol (a sterol)	Orrego et at. [31]
	Ginsenoside Rg1 (a saponin)	Shi et al. [33]
Indoles	Indole-3-carbinol	Chen et al. [34]
	Melatonin	Martínez-Campa et al. [35]

Only a few examples are shown for each chemical group. Note that not all chemicals in the groups listed have estrogenic activity. For more information including other examples of the above groups of chemicals, see [17, 19, 20, 22–24, 29, 30]

DDT dichlorodiphenyltrichloroethane, *DES* diethylstilbestrol; 2,4-*DHBP* 2,4-dihydroxybenzophenone, *PCBs* polychlorinated biphenyls, *PFOA* perfluorooctanoate, *PFOS* perfluorooctane sulfonate, *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,2′,4,4′-*THBP* 2,2′,4,4′-tetrahydroxybenzophenone

several groups [36–43]. However, only our group continued to develop a protocol for the profiling of estrogen-responsive gene expression by means of a focused microarray [44], and the protocol is run on two types of DNA microarray platforms: a cDNA microarray, named EstrArray, and an oligonucleotide-DNA (oligo-DNA) microarray, both containing a set of 172 estrogen-responsive genes and 31 markers [44, 45]. DNA microarray

Table 2 Summary of the correlation of expression profiles between estrogen and chemicals revealed by DNA microarray assay

Chemical	Cell line	*R* value	*p* value	Reference
High correlation ($R = 0.8$–0.9)				
Zearalanone	MCF-7	0.96	<0.01	Parveen et al. [56]
Zearalenone	MCF-7	0.95	<0.01	Parveen et al. [56]
α-Zearalanol	MCF-7	0.95	<0.01	Parveen et al. [56]
β-Zearalanol	MCF-7	0.94	<0.01	Parveen et al. [56]
α-zearalenol	MCF-7	0.93	<0.01	Parveen et al. [56]
Bisphenol B	MCF-7	0.93	<0.01	Terasaka et al. [53]
Estriol	MCF-7	0.93		Terasaka et al. [44]
Genistein	MCF-7	0.93		Ise et al. [52]
p-Hydroxybenzophenone	MCF-7	0.92	<0.01	Terasaka et al. [53]
Genistein	MCF-7	0.91		Terasaka et al. [44]
Nonylphenol	MCF-7	0.90	<0.01	Terasaka et al. [53]
Bisphenol A	MCF-7	0.89	<0.01	Terasaka et al. [53]
C-heavy oil (TK09 W, 73d)	MCF-7	0.88		Zhu et al. [55]
Estrone	T-47D	0.87	<0.01	Inoue et al. [57]
Diethylstilbestrol	T-47D	0.87	<0.01	Inoue et al. [57]
Nonylphenol	MCF-7	0.86		Terasaka et al. [44]
Butylbenzyl phthalate	MCF-7	0.85	<0.01	Parveen et al. [58]
Estrone	MCF-7	0.85		Terasaka et al. [44]
Agaricus blazei extract	MCF-7	0.84	<0.01	Dong et al. [45]
Estriol	T-47D	0.83	<0.01	Inoue et al. [57]
4*n*-Heptylphenol	MCF-7	0.82	<0.01	Terasaka et al. [53]
β-Zearalenol	MCF-7	0.82	<0.01	Parveen et al. [56]
C-heavy oil (TK09 W, 101d)	MCF-7	0.82		Zhu et al. [55]
Medium correlation ($R = 0.4$–0.8)				
Biochanin A	MCF-7	0.79		Ise et al. [52]
Phloretin	MCF-7	0.78		Ise et al. [52]
4-*t*-octylphenol	MCF-7	0.75	<0.01	Terasaka et al. [53]
Daidzein	MCF-7	0.74		Ise et al. [52]
Coumestrol	MCF-7	0.74		Ise et al. [52]
Propylparaben	MCF-7	0.74	<0.01	Terasaka et al. [53]
Bisphenol A	T-47D	0.72	<0.01	Inoue et al. [57]
Naringenin	MCF-7	0.70		Ise et al., 2005 [52]
C-heavy oil (HP-7, 73d)	MCF-7	0.70		Zhu et al. [55]
Diethylstilbestrol	MCF-7	0.69		Terasaka et al. [44]
Kaempferol	MCF-7	0.68		Ise et al. [52]
Apigenin	MCF-7	0.67		Ise et al. [52]
Chrysin	MCF-7	0.66		Ise et al. [52]
Ginsenoside F1	MCF-7	0.66	<0.01	Dong and Kiyama, 2009 [60]
Bisphenol A	MCF-7	0.65		Terasaka et al. [44]
Luteolin	MCF-7	0.64		Ise et al. [52]
Brefeldin A	MCF-7	0.61		Dong et al. [54]
Butylparaben	MCF-7	0.60	<0.01	Terasaka et al. [53]
Glycitein	MCF-7	0.59		Ise et al. [52]
C-heavy oil (TK09 W, 28d)	MCF-7	0.57		Zhu et al. [55]
Methoxychlor	MCF-7	0.56		Terasaka et al. [44]
Quercetin	MCF-7	0.53		Ise et al. [52]
Diethyl phthalate	MCF-7	0.52	<0.01	Parveen et al. [58]
Genistein	T-47D	0.52	<0.01	Inoue et al. [57]

Table 2 continued

Chemical	Cell line	*R* value	*p* value	Reference
Diisopropyl phthalate	MCF-7	0.49	<0.01	Parveen et al. [58]
Nonylphenol	T-47D	0.49	<0.01	Inoue et al. [57]
G. glabra root extract	MCF-7	0.47	<0.01	Dong et al. [59]
Low or no correlation ($R < 0.4$)				
4-*n*-ethylphenol	MCF-7	0.39	<0.01	Terasaka et al. [53]
Ginsenoside Rb1	MCF-7	0.38	<0.01	Dong and Kiyama [60]
Dibutyl phthalate	MCF-7	0.36	<0.01	Parveen et al. [58]
Pentachlorophenol	MCF-7	0.32	<0.01	Terasaka et al. [53]
4-chloro-3,5-dimetylphenol	MCF-7	0.26	<0.01	Terasaka et al. [53]
Ginsenoside Rh1	MCF-7	0.22	0.01–0.05	Dong and Kiyama [60]
Ginsenoside Rg1	MCF-7	0.21	0.01–0.05	Dong and Kiyama [60]
Dioxin (TCDD)	MCF-7	0.21		Terasaka et al. [44]
C-heavy oil (control, 73d)	MCF-7	0.21		Zhu et al. [55]
Ipriflavone	MCF-7	0.19		Ise et al. [52]
Ethylparaben	MCF-7	0.19	0.01–0.05	Terasaka et al. [53]
4-chlorophenol	MCF-7	0.15	>0.05	Terasaka et al. [53]
p-cresol	MCF-7	0.12	>0.05	Terasaka et al. [53]
2,4-dichlorophenol	MCF-7	0.03	>0.05	Terasaka et al. [53]
Methylparaben	MCF-7	−0.21	0.01–0.05	Terasaka et al. [53]
Glycyrrhizin	MCF-7	No correlation		Dong et al. [59]

Data were reproduced from the references shown on the right. The gene expression profile of each chemical was obtained after either MCF-7 or T-47D cells had been treated with the chemical and compared with that of 17β-estradiol (E_2) obtained from the same cell line. Coefficients of correlation (*R* values) between each chemical and E_2 on the basis of linear regression and *p* values for the statistical significance of the correlation were calculated

analysis was performed using a two-dye (for cDNA) or a single-dye (for oligo-DNA) approach. In the cDNA microarray assay, target cDNA was synthesized using mRNA extracted from the control or the test sample, labeled with different fluorescent dyes, and then two samples were mixed and hybridized with cDNA probes on a single microarray. In the oligo-DNA microarray assay, cDNA from the control and test samples was respectively labeled with the same fluorescent dye, such as Cy and Fluolid dyes [46], and used for hybridization with oligo-DNA probes on different DNA microarrays (summarized in Fig. 1).

The signal intensity was averaged between duplicated spots and the ratio of the mean signal intensity for the test sample to that for the control sample was calculated for each gene. The ratios of the signal intensity for all genes were normalized against the mean ratio of the 28 calibration markers [44]. To analyze the relationship of gene expression between different chemicals, average-linkage hierarchical clustering was performed (cluster analysis) and/or correlation coefficients based on linear regression between the profiles was calculated (correlation analysis).

Genes used for monitoring estrogenic gene expression

It is crucial to use a set of appropriate genes to reliably monitor estrogenic gene expression attributable to a chemical. We screened a set of estrogen-responsive genes by profiling the gene expression in estrogen receptor-positive human MCF-7 breast cancer cells using two large-scale cDNA microarrays, a Human UniGEM v 2.0 microarray (IncyteGenomics) containing 9,128 genes and a GeneChip U95A (Affymetrix) containing 12,625 genes [42, 44]. After the reproducibility of the expression was confirmed by repeated cDNA microarray and/or RT-PCR assays, we constructed a customized DNA microarray, EstrArray, which contained 203 genes, including 108 upregulated and 64 downregulated genes, along with 28 calibration markers, which did not show any response to estrogen, and three functional markers [44]. Some have been reported to be estrogen inducible, such as *TFF1* (or *pS2*; [47]), *PDZK1* [48] and *IGFBP4* [49]. However, many (119/172, 69 %) are not reported to have any relationship with estrogen, and, after our report about gene expression profiling [42], we and others further characterized estrogen-related functions for 65 genes (38 %), including *EGR3* [50]; *BSN* and *AREG* [51]; and *ATF3*, *TP53I11*, *SH3BP5* and *TRIB3* [52].

Because genes with poor reliability may result in inaccurate profiling of the chemicals tested, it is important to select a set of highly reliable genes. Reducing the number of genes would result in less statistical stability to differentiate a variety of chemicals, especially when focusing on specific cellular functions. So, to select a set of genes with more statistical stability, ten sets of cDNA microarray data were statistically examined, and the correlation coefficients (*R* values) were compared between one assay and each of the other assays using sets of genes ranked by

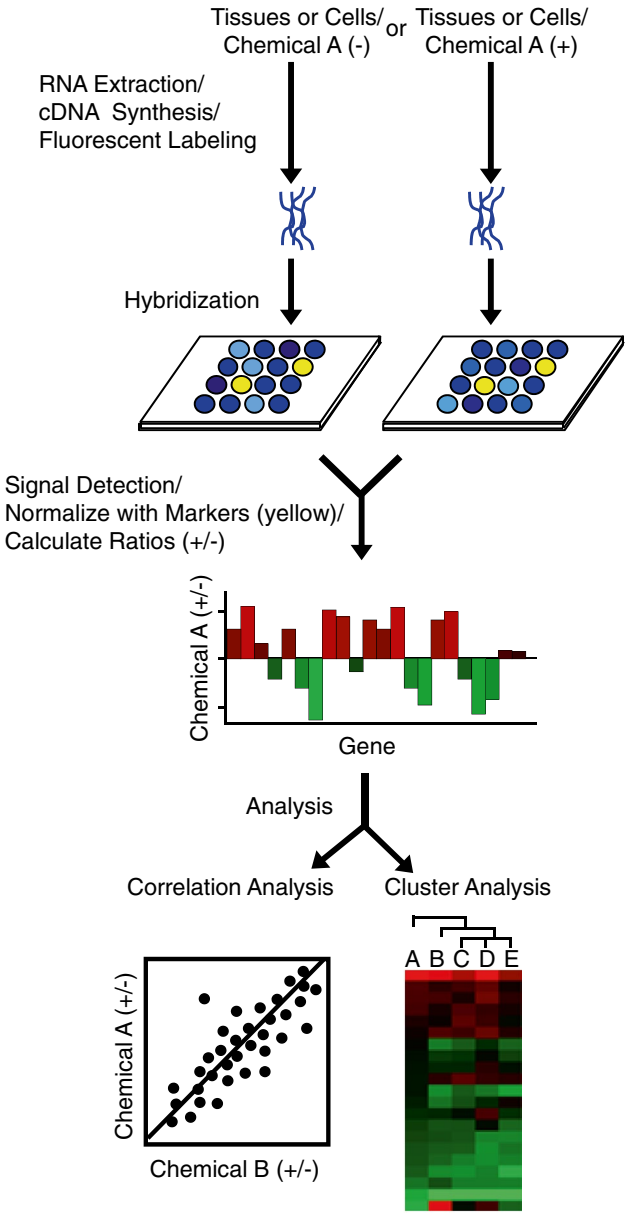

Fig. 1 Protocol of a single-dye DNA-microarray assay. For microarray assay, tissues or cells are treated with a chemical A or the vehicle (DMSO, control). Total RNA is isolated and used for cDNA synthesis. Target cDNA (control and test samples) is labeled with the same fluorescent dye and used for hybridization with the probes on the microarray. The signal intensity of each spot is detected and quantified by a fluorescent scanner. For data processing, the signal intensity is averaged among duplicated spots, and the ratio of the mean signal intensity for the test sample to that for the control is then calculated for each gene. The ratios of signal intensity for all genes are normalized against the mean ratio for the marker genes (*yellow spots*). Normalized values are used for correlation and/or cluster analyses

reproducibility or R values [53]. Among the sets of 203, 172, 150, 120, 90, 60 or 30 genes selected from the top of the rank, a set of 120 genes was adopted because it significantly improved the R value from the 150-gene set without losing much variability. The 120 genes selected were further classified into six tentative functional groups (enzyme, signaling, proliferation, transcription, transport and others), based on their annotations in the Entrez database [53]. The number in each functional group was 12–30, which was enough to maintain statistical stability. Likewise, a set of 150 genes was selected for the oligo-DNA microarray and was used to evaluate estrogen-like gene expression of the extract of *Agaricus blazei* (*A. blazei*), an edible mushroom originating from Brazil [45, 54], and C-heavy oil [55].

Gene expression profiling of estrogenic chemicals using estrogen-responsive genes

Using the focused microarray mentioned above, estrogenic gene-expression profiles of various chemicals, including natural/synthetic estrogens, industrial/environmental chemicals, phytochemicals and plant/fungus extracts, were examined (summarized in Table 2). The profiles obtained were compared with that of a natural estrogen, 17β-estradiol (E_2), by examining the correlation coefficient (R value) between the profiles for each chemical and E_2. Natural/synthetic estrogens, such as estrone, estriol and diethylstilbestrol, showed moderate to high levels of correlation. Phytoestrogens are a diverse group of plant-derived chemicals (phytochemicals) and, due to their structural similarities with E_2, they have the ability to cause estrogenic and/or antiestrogenic effects. Among the examined phytoestrogens, such as isoflavones, flavonols and flavones, and mycotoxins, such as zearalenone, most showed moderate to high levels of correlation, except ipriflavone ($R = 0.19$) [52, 56]. In particular, genistein and five of a group of zearalenone and its analogs showed very high R values ($R = 0.93$–0.96), which were close to those of natural estrogens, such as estriol. Some were analyzed with a different type of breast cancer cell line, T-47D, and showed quite different gene expression profiles from those in MCF-7 [57], suggesting differences in signaling within cells.

A number of chemicals and their degradation products have structural similarities to natural estrogens, and thus, they could act as xenoestrogens such as endocrine disruptors. Some chemicals showed profiles similar to that of E_2, such as nonylphenol ($R = 0.86$), bisphenol B ($R = 0.93$), *p*-hydroxybenzophenone ($R = 0.92$) and butylbenzyl phthalate ($R = 0.85$). On the other hand, the majority of the industrial chemicals examined showed only moderate ($R = 0.4$–0.8) or low ($R < 0.4$) correlations, such as 4-*t*-octylphenol ($R = 0.75$), methoxychlor ($R = 0.56$), diethyl phthalate ($R = 0.52$), dibutyl phthalate ($R = 0.36$), dioxin ($R = 0.21$) and *p*-cresol ($R = 0.12$) [44, 53, 58]. Furthermore, estrogenic gene-expression profiles were observed for bacterial-degradation products of C-heavy oil [55]. Oil samples, after they were cultured with complex

microbes (TK07 W) or a single bacterial strain (HP-7) for a longer period of time (73 days), showed gene-expression profiles similar to that of E_2 ($R = 0.88$ or 0.70, respectively), suggesting that components such as polycyclic aromatic hydrocarbons (PAHs) could gain estrogenic activity during their degradation process.

We also examined estrogenic gene-expression profiles of several extracts/ingredients including two traditional Chinese medicinal herbs, licorice (*Glycyrrhiza glabra* or *G. glabra*) [59] and ginseng [60]. While the profiles between E_2 and the extract of *G. glabra* root showed moderate correlation ($R = 0.47$), one of the major components in the extract, glycyrrhizin, did not show any correlation with E_2, suggesting the presence of other estrogenic components. Meanwhile, four components of ginseng had various degrees of estrogenic activity; ginsenosides F1 ($R = 0.66$), Rb1 ($R = 0.38$), Rg1 ($R = 0.21$) and Rh1 ($R = 0.22$) [60]. The extract of *A. blazei* was also examined, which showed a high estrogenic gene-expression profile ($R = 0.84$) [45]. A component of the activity was identified as brefeldin A (BFA) [54]. Since the correlation coefficient between the profiles of BFA and E_2 ($R = 0.61$) was significant but apparently less than that for the extract of *A. blazei*, BFA would need additional components to stabilize and/or enhance the activity (see "Silent estrogens for medical use"). Many estrogenic phytochemicals show poor water solubility and thus need modifications, such as glycosidation, and/or interaction with appropriate carriers.

Global and focused microarrays

Microarray-based gene expression profiling can be categorized into two types of microarray systems, global (or genome wide) and focused microarrays. We previously discussed the characteristics of the analysis using focused microarrays [5]. Focused microarrays typically contain a set of tens to hundreds of genes as a molecular signature for characterizing a specific type of phenomes, and some have been developed for clinical use (see "Prospect of DNA microarray-based gene expression profiling"). The reliability or reproducibility of microarray data, especially those obtained with global microarrays, has been questioned by several groups including microarray developers (see Inoue et al. [5]) and statisticians/clinicians [61–63]. Ioannidis noted that microarray technology performs no better than flipping a coin or horoscopes [63]. Focused microarrays use limited numbers of genes selected and validated in advance for specific purposes, and the genes selected are mostly uncharacterized but are expected to be signal target genes and/or genes associated with signal cascades (see "Silent estrogens"). Focused microarrays may efficiently eliminate noise and identify the relationship between gene expression and the signaling status with less time and labor.

As described above ("Gene expression profiling of estrogenic chemicals using estrogen-responsive genes"), a focused microarray was applied for evaluation of estrogenic activity based on the expression profile of the genes responding to estrogen or estrogen-like chemicals. Among the chemicals tested, zearalenone and its analogs showed expression profiles identical to that of E_2, while their structure was not particularly similar to that of E_2 [56], suggesting that the microarray assay can be used for analyzing the structural basis of estrogenic activity. Even though some chemicals belong to a group based on their structure, they may show quite different expression profiles, such as phthalate esters and ginsenosides [58, 60], suggesting that structural characteristics other than a phenolic basis, such as the mass and hydrophobicity of side chains, are essential for estrogenic effects. Mixtures of compounds, such as those extracted from plants, the extract of *G. glabra* root for example, which showed moderate correlation with E_2, can be analyzed, and the unique profile shown by a major component, glycyrrhizin, suggests that estrogen-responsive genes may respond differently from those of most estrogenic chemicals [59]. An important finding was that some chemicals showed differences between estrogenic cell proliferative effects and gene expression profiles: some chemicals have no positive effects on cell proliferation, but show estrogenic gene expression profiles (see "Silent estrogens"). Accordingly, focused microarrays could be an efficient method for evaluating various cellular activities and thus would be advantageous for analyzing natural or industrial chemicals and purifying chemicals from various extracts.

Silent estrogens

Signals for estrogen-induced cell proliferation

Cell proliferation is a state in which the number of cells is increased as a result of cell growth and cell division. Cell proliferation is stimulated by various chemicals such as mitogens, growth factors and survival factors [64], and specific receptors mediate the intracellular signals originating from these chemicals to regulate the timing and extent of protein synthesis, cell growth, cell division, cell movement and/or cell survival processes. Estrogen signals within a cell are initiated by specific receptors, estrogen receptors α or β (ERα or ERβ), or membrane receptors including a G-protein-coupled receptor (GPCR), GPR30, and membrane-bound ERα/β, upon their binding to estrogen [65, 66]. ERα and ERβ show significant overall sequence homology, and both are composed of five domains, A/B, C, D, E and F [67]. Among them, the A/B and E domains are responsible for the activation functions, AF1 and AF2, respectively,

to transactivate gene transcription in the absence (AF1) or presence (AF2) of bound ligands [68]. These domains regulate ER-mediated transactivation of target genes through the interaction with coactivators, and this process is collectively referred to as "the genomic pathway" (Fig. 2). A recent study suggested that AF1 of ERα is necessary and sufficient for estrogen-induced cell proliferation [69], although some beneficial estrogenic effects, such as atheroprotective effects, are attributable to AF2 but not AF1 [70].

On the other hand, a class of specific signaling pathways, the so-called "nongenomic pathway" (Fig. 2), has been found in association with estrogen-induced cell proliferation, in which signals are mediated through protein modifications and protein–protein interactions, such as Src/JNK/MAPK [71], PI3 K/Akt [72, 73], MEK/ERK/RSK [74], Rac [75] and Wnt signaling [76]. As a result of the activation of these pathways, downstream targets, such as c-Myc, β-catenin, cyclins and Rho-family small GTPases, involved in cell proliferation, cell survival, cell movement and transcription, are activated [77, 78]. Note that such a non-genomic pathway can directly lead to gene expression without the interaction of ERs with gene promoters [79].

Activation of GPR30 and membrane-bound ERα by bound estrogen mediates rapid cell signaling to activate a variety of second messengers and signal mediators [65, 66]. Furthermore, crosstalk has been implicated in the ER pathway with the membrane receptor pathways for epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and transforming growth factor α (TGFα) [77, 80], or in the GRP30 pathway with those for EGF and TGFα, both of which bind to the EGF receptor (EGFR), by direct interaction between GPR30 and EGFR [81], eventually leading to biological responses such as gene expression and cell proliferation. While ERs and GPR30 are involved in the same estrogen actions on the cardiovascular system, including reduced vascular smooth muscle cell proliferation, GPR30 agonist G-1 showed different responses in endothelial cell proliferation from estrogen [82, 83], suggesting the ligand-dependent response of cell proliferation.

IGF-I and BRCA1 play key roles in estrogen-induced cell signaling. BRCA1 negatively regulates the transcription of IGF-I as part of the transcription machinery, and loss of function of *BRCA1* results in constitutive activation of the IGF-I receptor (IGF1R) pathway, possibly causing breast cancer phenotypes [84]. Estrogen transactivates *BRCA1* and *IGF1R* promoters, and BRCA1, but not mutant BRCA1, inhibits the estrogen-induced activation of ERα transcription.

Silent estrogens: a new category of estrogenic chemicals

A series of extensive analyses of chemicals by means of DNA microarray assay of the estrogenic transcriptional response revealed that some chemicals show estrogenic gene expression profiles without showing positive effects on cell proliferation, and we termed them "silent estrogens" [54] (Fig. 3). The first of such chemicals was BFA, a lactone antibiotic originally found in a fungus, *Eupenicillium brefeldianum*, but currently well known for its inhibitory activity of intracellular protein transport from the endoplasmic reticulum to the Golgi apparatus. BFA showed a significant level of correlation (correlation coefficient $R = 0.61$) of the expression profile of estrogen-responsive genes in breast cancer MCF-7 cells with that of E_2, while there was no positive effect on cell proliferation at any concentration [54]. Apart from the difference in the expression of some estrogen-responsive genes, *TTF1*, for example, there are significant differences between BFA and E_2, in the effect of estrogen antagonist ICI 182,780, which suggests the involvement of discrete signaling pathways for BFA and may explain its absence of growth-stimulating

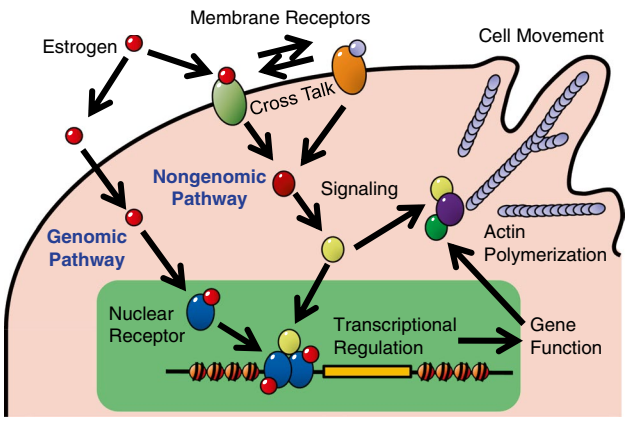

Fig. 2 Summary of the scheme of estrogenic response and cell signaling directing to cell functions. Actin polymerization and cell movement are representative

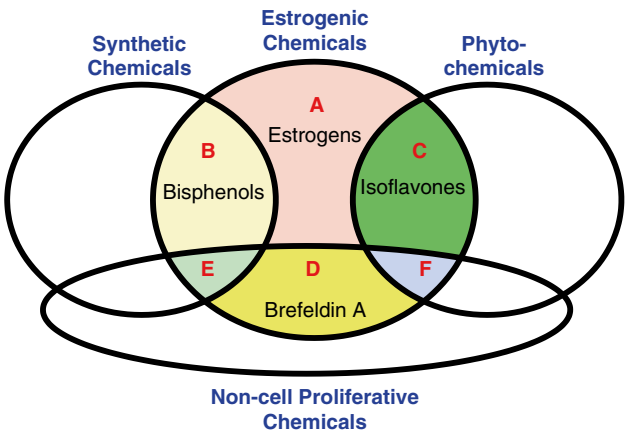

Fig. 3 Groups of chemicals classified by estrogenicity and cell proliferation activity

activity. We found that BFA is not the only chemical that can be categorized as a silent estrogen (data not shown). Since the extract of *A. blazei*, from which estrogenic activity of BFA was found, showed an estrogenic gene expression profile together with growth-stimulating activity [45], similar approaches could be explored to separate these two activities and find silent estrogens from natural and artificial sources. Such activity was found in licorice extracts [59] and oil-degradation products [55], where cell proliferation-independent gene expression profiles were observed.

The mechanism of silent estrogens has not been explored much. As mentioned above, there might be several ways of modulating estrogenic activity such as through (1) the alteration in activation functions of ERs, (2) the modulation of membrane receptor-mediated signaling pathways, (3) separate and independent pathways responsible for estrogenic expression profiles and the lack of cell proliferation or (4) as yet unidentified pathways, or (5) any combination of (1) to (4). Since BFA has quite a different structure from that of E_2, although its molecular weight is similar, activation functions of ERs are modulated by altered interactions of BFA and the domains affecting AF2, such as that observed for SERMs [16]. Meanwhile, there is a possibility of alterations at the receptors. We observed a clear difference in the effect of ICI 182,780 between E_2 and BFA. While BFA showed rapid activation of Erk1/2, Akt and P70S6 K, which was seen within 15–60 min after stimulation with E_2 or BFA, the inhibitory effect of ICI 182,780 was observed only for E_2 [54]. A similar effect was observed for another estrogenic chemical, bisphenol A, which mediates the rapid estrogenic signal through GPR30 [85], suggesting the involvement of membrane pathways. Note that bisphenol A has cell-proliferation activity, and therefore, it is not a silent estrogen.

Cell proliferation induced by phytoestrogens

Phytoestrogens consist of four major groups of chemicals, isoflavones, stilbenes, coumestans and lignans [86], and attention has been increasing because of their benefits such as reducing cancer risk, preventing cancer growth, improving atherosclerotic symptoms and supplementing endogenous hormones [87–89]. However, their characteristics are diverse because there are so many types of chemicals with various degrees of estrogenic activity [90], along with various additional activities [87], and, more importantly, estrogenic activity itself is diverse. It is known that tamoxifen acts as an antagonist against breast cancer but an agonist to uterine cancer, and chemicals such as tamoxifen or SERMs are characterized by their selective inhibition or stimulation dependent upon tissues [17]. Phytoestrogens are considered as SERMs and their beneficial as well as harmful effects have been discussed [88, 89]. Furthermore, a number of

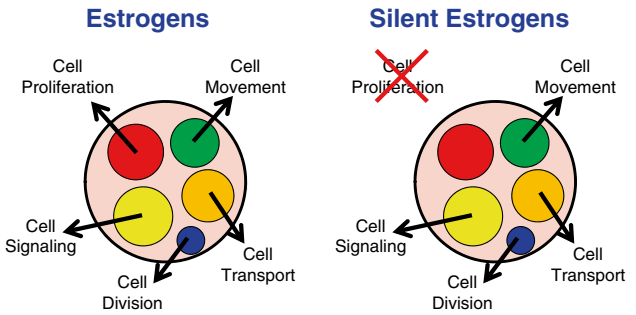

Fig. 4 Estrogenic responses of estrogens and silent estrogens. Estrogenic responses at the cellular level (*outside the boundary*) or the gene-expression level (*inside the boundary*) are illustrated

phytoestrogens are characterized by their suppressive but not stimulative effects on cell proliferation, especially at higher concentrations, because they bind more to growth-suppressive ERβ than growth-stimulative ERα [86]. A class of chemicals specifically lacking some physiological effects can therefore be anticipated, such as cell proliferation discussed here. However, as cell proliferation is one of the major roles of estrogen, assays for phytoestrogens often include analysis of whether they have positive or negative effects on cell proliferation [91]. Expression profiles of estrogen-responsive genes have been used as a set of standardized markers representing overall estrogenic actions such as cell proliferation, cell movement, cell signaling, cell division and cell transport (Fig. 4), and cell proliferation alone was examined at the cellular level, but is not necessarily related with gene transcription. Therefore, it is not difficult to imagine that estrogenic gene expression profiles are maintained while signals specifically directing cell proliferation are abrogated.

Silent estrogens for medical use

Information regarding medical potentiality is so far limited. While we observed an improvement of artificial atherosclerotic legions in rabbits fed a high-cholesterol diet after uptake of the extract of *A. blazei* containing BFA [45], such a clear improvement was not observed for BFA alone, probably because of its poor water solubility [54]. Such a problem occurs in many hydrophobic chemicals including estrogen, which is present as a bound form with carriers such as sex-hormone binding globulin (SHBP) due to its poor water solubility and its effective bioavailability [92]. Furthermore, an improvement of the level of oxidized low-density lipoprotein (LDL) in hyperlipidemic patients, the effect of reducing reactive oxygen species (ROS) and a positive effect on nitrogen-oxide (NO) production within the cell were observed for estrogen and the extract of *A. blazei* [45], while they have not been examined for BFA.

However, activation of Erk1/2, Akt and P70R6 K was observed for both materials [54], suggesting that some cell functions could be attributable to BFA.

Silent estrogens are mostly what we expect from phytoestrogens. However, it is important to note that BFA has never been classified as an estrogenic chemical. Indeed, all the phytoestrogens that we examined for estrogenic gene-expression profiles, including isoflavones, flavones, flavanols, phloretin, coumestrol, naringenin [52], ginsenosides [60], and zearalenone and their derivatives [56], were reported to have growth-stimulating activity. Therefore, silent estrogens could be a new category of chemicals that have not been recognized for their estrogenic effects, and lacking growth-stimulating activity would be advantageous as controlling cell proliferation is critical for the prevention of cancer growth. In addition, BFA is known to have various effects, which are not necessarily estrogenic, such as its anti-viral and anti-tumor effects, and more importantly, binding to Sec7, a guanine-nucleotide exchange factor (GEF) of small GTPase Arf1, and inhibiting the Arf1-mediated formation of vesicles for protein transport by stabilizing an abortive complex between Arf1-GDP and the catalytic domain in Sec7 [93]. Since the concentrations exhibiting the inhibition of cell transport are at least 100 times higher than those used for gene-expression profiling and intracellular signaling, estrogenic effects, if any, would not be damaged.

Prospect of DNA microarray-based gene expression profiling

Industrial applications

Applications of DNA microarray-based gene expression profiling have been explored in several industrial fields, including pharmaceuticals and foods, diagnostic and prognostic tests, and environmental technology. First, gene expression profiling by means of microarrays was used to develop drugs for the treatment of inflammation [94], heart failure [95] and cancer [96]. Microarray technology would be beneficial in this field to reduce sample size and trial time, and to increase the number of marker genes used to predict their efficacy as well as toxicity. For example, expression profiling has been used to distinguish genotoxic from non-genotoxic effects and to provide information on the mechanism of action [97]. Second, the technology was used in the development of functional foods [98] and food safety assessment [99, 100]. Microarrays have been listed as one of the key strategies to detect alterations in the gene expression of genetically modified organisms (GMOs) for safety evaluation [101]. Third, an important field in its application is the technology of medical devices, such as diagnostic and prognostic tests. However, quite a number of researchers noted that many published reports need further lines of extensive statistical evaluation based on appropriate sets of parameters and algorithms [62, 102]. Fourth, another important field is toxicological studies (toxicogenomics) [103, 104] and environmental risk assessment [105, 106]. From a survey of toxicogenomic studies, Fent and Sumpter [107] summarized that microarray data can give a set of unbiased, comprehensive and sometimes novel information about the gene expression of toxicants or their mode of action, although there are some technical difficulties and problems of cost performance, quality of data, standardization of protocols and consistency with physiological data.

Microarray technology needs significant care of the samples taken during surgery (treatment of tissues and short-term storage), handling (transport and long-term storage), preparation (extraction, purification and storage) and quality evaluation step (pre-analysis step) even before the microarray assay or the analytical step. Since sample preparation affects the quality of gene expression data, significant attention has been paid to the usage of formalin-fixed paraffin-embedded (FFPE) samples [108]. However, although collection and storage of FFPE samples are routinely practiced in pathology laboratories, the quality of RNA is often questionable, and it may not be suitable for microarray assay without appropriate quality control protocols with standard values. As was often argued about the reliability of microarray data, variations derived from biological, experimental and technical differences should be examined [109]. Performance such as false positive rates and predictability, and the strength for commercialization such as cost and automation, were examined for immunohistochemistry, fluorescence in situ hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR) and microarray technology, and two commercial products, RT-PCR-based Onco*type* DX and microarray-based MammaPrint, were cross-examined [110]. While there are various advantages as well as disadvantages of microarray technology, an important point distinguishing these technologies is the number of markers used; 21 genes in Onco*type* DX and 70 genes in MammaPrint, for example. Generally, the number of markers is one to a few in immunohistochemistry and FISH, a few to tens in RT-PCR, and tens to hundreds or more in microarray technology [4, 110, 111]. Thus, none of these technologies are superior, but they may co-exist depending on the necessity and the situation.

Pathway-based technological applications

DNA microarray-based gene expression profiling has been used for pathway analysis because the recent progress in

the study of specific signaling pathways needs comprehensive analysis of complex higher orders of networks consisting of various major or minor pathways, which were revealed by the analysis of ligand/receptor interaction, receptor crosstalk, bypassing cascades, inhibitor/stimulator activity and agonistic/antagonistic effects, and thus, we can see quite a number of higher orders of networks identified by microarray assays. For example, microarrays have been used for various signaling pathways/cascades, such as MAPK, angiogenesis, nuclear receptor, ErbB/HER, ubiquitin/proteasome signalings, various cellular functions, such as chromatin/epigenetic regulation, apoptosis, autophagy, cellular metabolism, translational control, cell cycle/DNA damage, and cytoskeletal regulation and adhesion, and various higher levels of functions, such as immunology and inflammation, neuroscience, and development and differentiation (detailed in Table 3).

On the other hand, specific signatures consisting of the genes representing pathways of signal mediators, hormones/growth factors and oncogenes/tumor suppressors are useful for some applications, such as breast cancer diagnosis [112]. However, this approach needs more pathway-based analysis of gene expression profiles to find the best signatures for the purpose and only a few have been commercialized so far. It should be noted that transcriptomics is not the sole technology used to understand the pathways related to applications, and other 'omics' technologies, such as proteomics and metabolomics, will be used in the future separately or in combination with the transcriptomic data. Likewise, utilization of epigenetic changes such as those observed by DNA methylation, histone modifications and non-coding RNAs has also become a reality [113].

Innovation, specification, generalization and standardization

Innovation is not always followed by immediate commercialization or success in the market. The difficulty between the technological development and successful commercialization is called the valley of death [114]. Such difficulty was successfully overcome by microarray technology in the late 1990s partly but significantly because of increased demands from various genome projects for high-throughput technologies for screening genes with specific functions and for analyzing newly identified expressed sequence tags (ESTs), or pieces of cDNA of unknown origin, to find their potential functions [115]. For such purposes, global microarrays were useful, and several companies, Affymetrix, Agilent Technologies and Illumina, for example, manufactured this type of microarray. However, the efforts of a number of researchers have been focused on finding specific sets of genes, or signatures, which can be used in respective applications. Specific signatures will gain more importance because of the trend toward personalized medicine, and information at the molecular level, such as that provided by various 'omics' technologies, will form a new healthcare paradigm. Such specification is an important step for technologies to cross the valley of death and to enter the next stage, generalization, which can be achieved by governmental approval of diagnostic devices or risk-assessing tools, for example, and public familiarity as a useful tool as well as stability and competitiveness in the market. Immunoassays such as ELISA and its variations have gone through this process [116].

An important step for a technology to gain stability in public or industrial acknowledgement is its standardization. Standardization is the process of developing and implementing technical standards to achieve commoditization (independence of single suppliers), compatibility, interoperability, safety, repeatability and/or quality. Standardization of DNA microarrays was started by the MicroArray Quality Control (MAQC) Consortium, a consortium of 51 academic, government and commercial institutions organized by the US Food and Drug Administration in 2006 [117] after criticism of the reliability of microarray data [62] and, through two periods of activity (MAQC-I and II), MAQC provided the international microarray community with quality control tools, guidelines for protocols, thresholds/limitations in assessing the performance and models for best practices in pharmacogenomics and toxicogenomics. The MAQC Consortium reported a framework for assessing microarray technologies as a reliable tool for clinical and regulatory purposes, such as a median coefficient of variation (CV) of 5–15 % and a concordance rate of 80–95 % among the platforms tested (MAQC-I [118]), and examples of genomic classifiers, gene signatures representing specific pathological conditions, by examining data preprocessing and normalization methods, and algorithms and protocols for discerning patterns and identifying signatures using clinical and toxicological data (MAQC-II [119]).

MAQC activity was immediately followed by other countries, including European countries and Japan, to further standardize the analytical and pre-analytical steps for the quality control of clinical samples in gene-based diagnostic tests. SPIDIA (Standardisation and improvement of generic pre-analytical tools and procedures for in vitro diagnostics) is a 4.5-year project, funded by the European Union, to standardize and improve pre-analytical procedures for in vitro diagnostics. SPIDIA's activities are intended to create evidence-based guidelines and tools, and protocols for pre-analytical tests, and to develop biomarkers for quality control [120]. In a recent report, the pre-analytical phase of blood samples from 102 European laboratories was assessed by the stability and quality of RNA after extraction with different tubes under different

Table 3 Microarrays and signaling pathways

Pathway/cascade/function	Key molecule(s) identified[a]	Reference
MAPK signaling		
GPCR/MAPK	Small GTPase/apoptosis signaling genes	MAPK Chang et al. [137]
NFκB/MAPK/ERK	PI3 K/Akt/mTOR	Mendes et al. [138]
MAPK/JNK	Mitochondrial function related genes	Cízková et al. [139]
Other signaling		
Angiogenesis signaling	HIF-1α-dependent genes	Copple et al. [140]
Nuclear receptor signaling	PPARγ-associated genes	Lee et al. [141]
ErbB/HER signaling	Scaffolding adaptors	Nakaoka et al. 2007 [142]
Ubiquitin/proteasome signaling	NF-κB/IκB signaling genes	Granese et al. [143]
Chromatin/epigenetic regulation		
Histone modification	Hdac1 (histone deacetylase)	Reichmann et al. [144]
Heterochromatin	HP1-regulated genes	Lee et al. [145]
DNA methylation	SCARA5 (scavenger receptor)	Khamas et al. [146]
Apoptosis		
Infectious response	NF-κB/p53/RB/JUN/apoptosis genes	Faherty et al. [147]
Virus induced apoptosis	p53/caspase-1	Nasirudeen and Liu [148]
Death receptor signaling	FAS-mediated apoptosis genes	Wang et al. [149]
Autophagy		
Starvation stress	Autophagy-related genes	Burgess et al. [150]
PI3 K/Akt/FOXO/mTOR	Glutamine synthetase	van der Vos et al. [151]
Cellular metabolism		
Insulin receptor signaling	Metabolic process-related genes	Bolukbasi et al. [152]
AMPK signaling	AMPK activators	Solskov et al. [153]
Translational control		
eIF2 signaling	tRNA processing genes	Saikia et al. [154]
eIF4/p70S6 K signaling	eIF4 factors	Villas-Bôas et al. [155]
mTOR signaling	TGF-β/MNK1/SMAD2	Grzmil et al. [156]
Cell cycle/DNA damage		
G1/S checkpoint	EIF2 activator	Stockwell et al. [157]
G2/M DNA damage checkpoint	RB/E2F/ECT2	Eguchi et al. [158]
Cytoskeletal regulation and adhesion		
Actin dynamics	PKCδ/cofilin	Wada-Kiyama et al. [159]
Microtubule dynamics	Formin1	Simon-Areces et al. [160]
Adherens junction dynamics	Diabetes-related genes	Caramori et al. [161]
Immunology and inflammation		
Inflammatory response	miR-155/Jak/STAT	Kutty et al. [162]
Cytokine receptor signaling	TLR/IL-1R	Abend et al. [163]
T-cell activation	NF-κB/Rel	Chang et al. [164]
TLR-sinduced inflammation	TLR4-responsive genes	Yang et al. [165]
B-cell receptor signaling	Cytokines	Franke et al. [166]
Rheumatoid arthritis	T-cell receptors	Kim et al. [167]
Neuroscience		
Alzheimer's disease	Neurological disease-related genes	Walker et al. [168]
Parkinson's disease	FOXO1	Dumitriu et al. [169]
Development and differentiation		
Wnt/β-catenin signaling	Wnt-associated genes	Nguyen et al. [170]
Notch signaling	RB family genes	Viatour et al. [171]
Hedgehog signaling	Eyeless/hedgehog-regulated genes	Nfonsam et al. [172]
TGF-β signaling	Corneal dystrophy-associated genes	Choi et al. [173]

Classification of pathways/cascades/functions was based on that by Cell Signaling Technology (http://www.cellsignal.com/). A representative case is listed for each category

AMPK AMP-activated protein kinase, *eIF* eukaryotic initiation factor, *FOXO* forkhead box O, *GPCR* G-protein-coupled receptor, *IGF-1* insulin-like growth factor-1, *IL-1R* interleukin-1 receptor, *JNK* c-Jun N-terminal kinase, *MAPK* mitogen-activated protein kinase, *mTOR* mammalian target of rapamycin, *PI3 K* phosphatidylinositol 3-kinase, *PPARγ* peroxisome proliferator-activated receptor γ, *RB* retinoblastoma, *TLR* Toll-like receptor

[a] Proteins and/or cellular factors identified by microarray assay and subsequent characterization, such as RT-PCR and Western blotting

conditions [121]. On the other hand, the Japanese government also provided companies, academic institutions and public agencies with guidelines and guidance for industrial development (http://www.meti.go.jp/policy/mono_info_service/service/iryou_fukushi/) and governmental drug-approval (http://www.pmda.go.jp/operations/shonin/info/iryokiki/iryokiki-list.html) purposes. Meanwhile, global standardization of DNA microarrays and other gene expression-based in vitro diagnostic devices has been pursued by Technical Committee 212 of the International Organization for Standardization (ISO/TC 212), which focuses on clinical laboratory testing and in vitro diagnostic test systems, and includes quality management, pre- and post-analytical procedures, analytical performance, laboratory safety, reference systems and quality assurance, or by the newly organized ISO/TC 276, which focuses on analytical methods based on 'omics' technologies.

During the standardization process, however, severe competition among industries occurs, and commercialization could stop because their products are not included in the tools or protocols subjected to standardization, not because their quality, performance, cost or even patent strength are inferior to others. In this sense, the roles of governments and regional consortia, such as those of MAQC and SPIDIA, will become greater than ever.

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