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Construction and Validation of Human cDNA Microarray for Estimation of Endocrine Disrupting Chemicals (KISTCHIP-400 ver. 1.0)

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Abstract

Transcript profiling is a particularly valuable tool in the field of steroid receptor biology, as these receptors are ligand-activated transcription factors and therefore exert their initial effects through altering gene expression in responsive cells. Also, an awareness of endocrine disrupting chemicals (EDCs) and their potential screening methods to identify endocrine activity have been increased. Here we developed an in-house cDNA microarray, named KISTCHIP-400 ver. 1.0, with 416 clones, based on public database and research papers. These clones contained estrogen, androgen, thyroid hormone & receptors, sex hormone signal transduction & regulation, c-fos, c-myc, ps2 gene, metabolism related genes etc. Also, to validate the KISTCHIP-400 ver. 1.0, we investigated gene expression profiles with reference hormones, 10^{-8} M 17β-estradiol, 10^{-7} M testosterone and 10^{-7} M progesterone in MCF-7 cell line. As the results, gene expression profiles of three reference hormones were distinguished from each other with significant and identified 33 17β-estradiol responsive genes. This study is in first step of validation for KISTCHIP-400 ver. 1.0, as following step transcriptional profile analysis on not only low concentrations of EDCs but suspected EDCs using KISTCHIP-400 ver. 1.0 is processing. Our results indicate that the developed microarray may be a useful laboratory tool for screening EDCs and elucidating endocrine disrupting mechanism.

Keywords : endocrine disrupting chemicals, transcriptional profile, hormones, mechanism

Many chemicals that mimic naturally occurring hormones or are antagonistic to their modes can be found in the environment and have been shown to potentially disrupt the endocrine system in wildlife and human[1,2]. Some effects attributed to these contaminants include reduced fertility, hatchability and viability of offspring, as well as impaired hormone activity and altered sexual behavior. The endocrine system can be influenced by a wide range of chemical compounds[3]. Bioactive compounds are found in most major classes of pollutants, including dioxins and furans, halogenated organic compounds, polychlorinated biphenyls, phthalate esters, pesticides (both banned substances, such as DDT, and others currently in use, such as atrazine), and a number of other pollutants, such as polyaromatic hydrocarbons, tributyl tin, and heavy metals. Many of these compounds are highly persistent in the environment and are capable of bioaccumulation and biomagnification in living organisms. Human activities also introduce a number of natural endocrine signalers into the environment at abnormally high concentrations. For instance, municipal sewage and agricultural runoff following field application of manure can contain elevated levels of animal hormones[4]. Many pharmaceutical products and medical wastes likewise influence the endocrine system, and disposal of these substances can introduce into the environment. As mentioned above, the compounds that bind steroid hormone receptors including estrogen receptors (ERs), progesterone receptor (PR) or androgen receptor (AR), and induce or modulate a steroid hormone receptor-mediated response could be defined as endocrine disrupting chemicals (EDCs)[5,6].

Currently, there are no standard methods to determine whether a chemical is an endocrine disruptor or not although a wide variety of testing methods have been developed to investigate EDCs[7]. These include physical and chemical fractionation methods[8-10], the study of biomarkers in sentinel species, and single mode of action (MOA) oriented in vivo and in vitro assays, and life cycle or multigenerational in vivo tests.

Development of novel approaches for high-throughput screening for potential EDCs a major goal in the environmental health research. The need for advancing prediction the adverse biological effects of EDCs

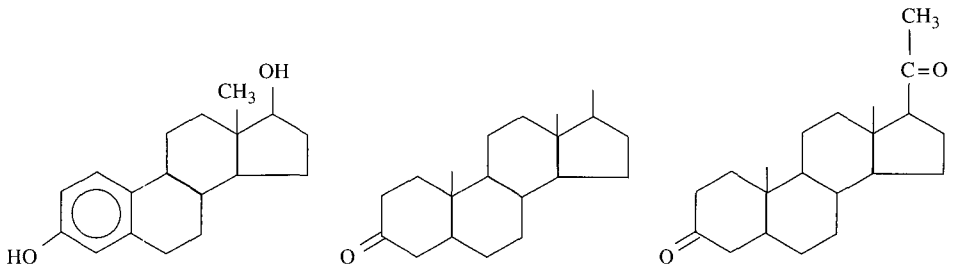

Fig. 1. Structure of Three Reference Hormones: (A) 17β-estradiol, (B) Testosterone, (C) Progesterone

in mammalian has made technologies exploiting advances in molecular techniques. Current molecular-level techniques rely on ligand-binding assays[11], enzyme-linked immunosorbent assay (ELISA)[12], and more recently, gene expression profiling[13,14]. In the future, more reliance will be placed on the development of gene expression assays to determine the intricate interactions between genes that are affected by the exposures. A new screening assay has several advantages over other in vitro techniques: 1) a high sample throughput, 2) a compact size allowing small sample volume, and 3) a sensitive determination based on the expression profile of hormone responsive genes. These new experiments that rely primarily on cDNA microarray technology have the potential of defining changes in the entire transcriptome. Initial experiments with this technology suggest that specific contaminants may have their own specific profiles of gene expression, perhaps making it possible to define in molecular terms the effects of exposure. It has the potential to implicate previously unsuspected estrogen, androgen and thyroid hormone-sensitive genes that may later become molecular markers of endocrine disruption[15,16]. Once gene expression patterns for multiple hormones are established, a custom microarray comprised of identified hormone-responsive genes will be constructed to make data analysis and handling more manageable.

With this concept, we designed a high throughput bioassay system, which responses to EDCs may be detected by gene expression using DNA microarray analysis, to assist in the identification of potential EDCs and to understand molecular mechanisms of EDCs. And then to validate whether genes spotted upon constructed cDNA microarray responds to reference hormones as like estrogen and androgen, we observed genes and its expression profiles affected by treatment with 17β-estradiol, testosterone and progesterone.

As the first step toward validation of "KISTCHIP-400 ver. 1.0", we identified gene expression profiles in MCF-7 human breast cancer cells after treatment with 17β-estradiol, testosterone and progesterone.

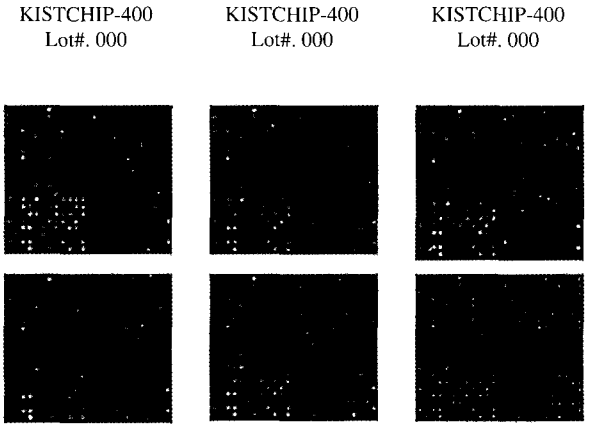

Fig. 2. Image displaying the human cDNA microarray related to endocrine system (KISTCHIP-400 version 1.0) comprised of 416 genes and cDNA array of MCF-7 cell samples treated three reference hormones: (A) 10^{-8} M 17β-estradiol, (B) 10^{-7} M testosterone, (C) 10^{-7} M progesterone. Colors represent hybridization to probes labeled with Cy3 (green) or Cy5 (red). Yellow spots indicate common hybridization between both probes.

Three sets of data were generated for this cell line after 24 h of treatment: gene expression profile for cells treated with vehicle ethanol (control); 10^{-8} M 17β-estradiol; 10^{-7} M testosterone; 10^{-7} M progesterone. In each case, concentrations of compounds used were based on studies published previously.

The total RNA obtained from MCF-7 cells with each reference hormone and solvent (0.1% ethanol) was used to prepare cDNA probes labeled with Cy3 and Cy5, and hybridized to the KISTCHIP-400 ver. 1.0. Each slide of KISTCHIP-400 ver. 1.0 was spotted with duplicated sets of 416 unique genes. As shown in a color-image of the slide in Fig. 2, the signal profiling of two slides exhibited the same patterns, and the fluorescent intensities derived from duplicated spots were also almost the same. In pre-

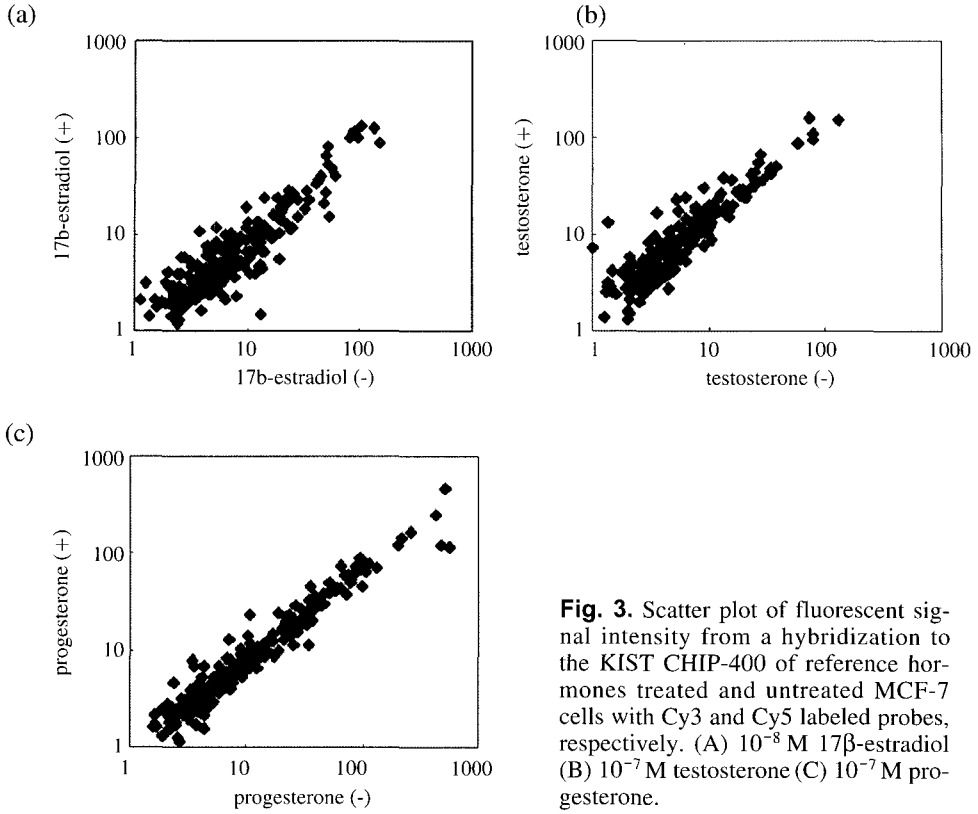

Fig. 3. Scatter plot of fluorescent signal intensity from a hybridization to the KIST CHIP-400 of reference hormones treated and untreated MCF-7 cells with Cy3 and Cy5 labeled probes, respectively. (A) 10^{-8} M 17β-estradiol (B) 10^{-7} M testosterone (C) 10^{-7} M progesterone.

sent study, the median values of the intensities of each spot in the four experiments were calculated, and plotted in Fig. 3 in this experiment. The results of hybridization with chemical plus sample and chemical minus sample labeled with Cy3 or Cy5 yielded a linear scatter plots that indicated excellent reproducible results.

To determine the significantly differential genes from MCF-7 cells with each three reference hormones, cutoff value (fold change in expression level) was chosen > 1.5 for up-regulated genes and < -1.5 for down-regulated genes in each three reference hormones (Table 2). In the case of 17β-estradiol, 33 genes exhibited up-regulated genes, and 23 genes exhibited down-regulated genes. From this data, we identified 33 responsive-genes for 17β-estradiol (Table 3). These genes were classified into eight functional categories based on biological functions: apoptosis, cell adhesion, metabolism, regulation of cell cycle, regulation of transcription, regulation of translation, signal transduction and others (including EST, tight junction, protein folding, kinesin complex). Also, identified 20 genes were up-regulated and 15 genes were down-regulated by testosterone. But, above 50% genes appeared different gene expression pattern compared testosterone to 17β-estradiol. In progesterone, only 13 of 50 genes were similar to 17β-estradiol and the others showed the gene expression which is unique. Table 2 shows gene exp-

Table 1. A Category of 416 Unique Element Function on the cDNA Microarray (KISTCHIP-400 ver. 1.0)

Description of 416 gene functions for probe DNA	Gene category	Number of genes
House keeping genes	M	22
Drug metabolism related genes	N	6
Estrogen related genes		
estrogen associated genes	A	26
up-regulated genes by estrogen	P	19
Androgen related genes		
androgen associated genes	B	21
regulation of androgen genes	O	35
Thyroid hormone related genes	C	76
Sex hormone signal transduction genes	G	3
Sex hormone regulation genes	F	3
Endocrine signal transduction genes	E	3
Endocrine regulation genes	D	13
c-fos related genes (Tumor associator)	H	7
c-myc related genes (Tumor associator)	I	16
PS2 related genes	K	2
Overlapping genes	L	36
Other genes	Q	128
Total		416

Table 2. List of Down (A), Reciprocal (B) and up (C) Regulated Genes by 10^{-8} M 17β-estradiol, 10^{-7} M Testosterone and 10^{-7} M Progesterone in MCF-7 Cell Lines

Fold change vs. Control			Gene name	Gene symbol	Accession No.
17β-estradiol	Testosterone	Progesterone			
(A)					
−3.4326	−1.7765		G protein-coupled receptor 30	GPR30	AA810225
−3.0602	−1.6690		BCL2-like 1	BCL2L1	Z23115
−2.4250	−1.6964		retinoid X receptor, alpha	RXRA	AA464615
−2.1160	−1.6016		BCL2-antagonist/killer 1	BAK1	U16811
−1.9597	−1.6415		integrin-linked kinase	ILK	U40282
−1.6833	−1.6696		nuclear receptor subfamily 1, group H, member 2	NR1H2	AA629265
−1.5746	−1.7106		hypothetical gene supported by AK093437	na	AA485677
	−1.6512	−1.6104	hypothetical protein LOC283126	LOC283126	AA846573
−2.5402			claudin 3	CLDN3	AA434144
−2.4857			integrin, alpha 3	ITGA3	M59911
−2.1218			clusterin	CLU	NM_001831
−2.1179			phosphorylase kinase, alpha 2 (liver)	PHKA2	AA677340
−2.1000			nuclear receptor subfamily 3, group C, member 1	NR3C1	AA664219
−2.0950			cyclin-dependent kinase (CDC2-like) 10	CDK10	X78342
−2.0364			ninjurin 1	NINJ1	U72661
−1.7491			GATA binding protein 3	GATA3	X58072
−1.6901			cytochrome P450, family 2, subfamily J, polypeptide 2	CYP2J2	U37143
−1.6651			G protein-coupled receptor 56	GPR56	AA775249
−1.6190			early growth response 1	EGR1	AA486628
−1.6118			ribosomal protein S3	RPS3	S42658
−1.5990			cofactor required for Sp1 transcriptional activation	CRSP2	R40567
−1.5988			faciogenital dysplasia (Aarskog-Scott syndrome)	FGD1	AA902269
	−2.3255		Sapiens cDNA FLJ36787 fis, clone ADRGL2003585,		R16838
	−2.1533		potassium voltage-gated channel, shaker-related subfamily,	KCNA1	AA018214
	−1.9869		lipase, hormone-sensitive	LIPE	NM_005357
	−1.8283		von Willebrand factor	VWF	AA487787
	−1.7833		retinoblastoma binding protein 6	RBBP6	X85133
	−1.7142		trefoil factor 3 (intestinal)	TFF3	NM_003226
	−1.6061		amyloid beta (A4) precursor protein	APP	AA128553
		−3.5323	tuberous sclerosis 1	TSC1	AA455968
		−2.9600	zinc finger protein 276	ZFP276	AA644129
		−1.6484	calcyphosine	CAPS	AA858390
(B)					
−1.7431		1.9148	nuclear receptor subfamily 4, group A, member 1	NR4A1	N94487
−1.7175	1.7183	1.7069	similar to restin	na	AA070226
(C)					
1.6865	2.3628	1.8386	PDGFA associated protein 1	PDAP1	U41745
1.8117	1.9298	1.9803	caveolin 2	CAV2	AF035752
1.9306	2.0048	1.6009	small nuclear ribonucleoprotein polypeptide G	SNRPG	X85373
2.2161	1.7907	2.1710	chaperonin containing TCP1, subunit 2 (beta)	CCT2	NM_006431
2.3198	3.3776	5.1814	caspase 9, apoptosis-related cysteine protease	CASP9	U60521
2.3778	1.9145	1.7592	fibroblast growth factor receptor 1	FGFR1	X66945
2.5042	2.6972	2.4710	anterior gradient 2 homolog (Xenepus laevis)	AGR2	AF038451
2.6057	2.9363	3.8719	luteinizing hormone beta polypeptide	LHB	AI051683
1.7589	6.5499		prostaglandin E synthase	PTGES	AF010316
2.9540	1.7237		H2A histone family, member X	H2AFX	X14850
1.8815		2.2756	bromodomain containing 8	BRD8	AA481621

Table 2. Continued

Fold change vs. Control			Gene name	Gene symbol	Accession No.
17β-estradiol	Testosterone	Progesterone			
(C)					
1.9467		2.1217	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	U03688
2.1155		2.9492	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	AA464600
2.1165		1.9829	replication factor C (activator 1) 4, 37kDa	RFC4	M87339
2.9766		2.0157	androgen receptor	AR	NM_000044
1.5907			FK506 binding protein 4, 59kDa	FKBP4	NM_002014
1.5956			tripartite motif-containing 28	TRIM28	H41030
1.6126			transcription factor A, mitochondrial	TFAM	NM_003201
1.6197			Homo sapiens transcribed sequence		R33030
1.6411			phosphodiesterase 8B	PDE8B	AA455365
1.6427			dyskeratosis congenita 1, dyskerin	DKC1	AA052960
1.6671			splicing factor, arginine/serine-rich 7, 35kDa	SFRS7	L22253
1.7156			lactate dehydrogenase A	LDHA	NM_005566
1.7310			mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	MLH1	U07418
1.7886			integrin beta 3 binding protein (beta3-endonexin)	ITGB3BP	U37139
1.7917			exportin 1 (CRM1 homolog, yeast)	XPO1	D89729
1.9059			X-ray repair complementing defective repair in CH cells 5	XRCC5	M30938
2.0289			calmodulin 2 (phosphorylase kinase, delta)	CALM2	NM_001743
2.0639			tumor protein D52-like 1	TPD52L1	NM_003287
2.4863			solute carrier family 7, member 5	SLC7A5	NM_003486
2.6858			matrix Gla protein	MGP	AA155913
3.1933			H2A histone family, member Z	H2AFZ	NM_002106
8.2858			polo-like kinase (Drosophila)	PLK	U01038
	1.6398	2.1164	trophoblast-derived noncoding RNA	TncRNA	AA400234
	1.6415	1.6588	myotubular myopathy 1	MTM1	AA491225
	2.1916	2.1515	guanine nucleotide binding protein (G protein)	GNAI2	AA071330
	7.4719	4.0526	membrane cofactor protein	MCP	X59408
	1.6027		ribosomal protein L10a	RPL10A	R01139
	1.6313		chaperonin containing TCP1, subunit 4 (delta)	CCT4	AF026291
	1.7709		tyrosine 3-/tryptophan 5-monooxygenase activation protein, zeta	YWHAZ	M86400
	1.8987		protein kinase, DNA-activated, catalytic polypeptide	PRKDC	U47077
	2.3432		discs, large (Drosophila) homolog 5	DLG5	AA478949
		1.5799	bone morphogenetic protein 6	BMP6	AA424833
		1.6148	B lymphoma Mo-MLV insertion region (mouse)	BMI1	AA478036
		1.6554	nuclear receptor interacting protein 1	NRIP1	X84373
		1.6739	cytochrome P450, family 2, subfamily B, CYP2B6 polypeptide 6	M29874	
		1.6745	FK506 binding protein 5	FKBP5	AA653318
		1.6797	nuclear receptor coactivator 1	NCOA1	AA489785
		1.7621	procollagen-proline, 2-oxoglutarate 4-dioxygenase	P4HA1	AA457671
		1.7704	tumor protein p53 binding protein, 2	TP53BP2	U58334
		1.7792	CBF1 interacting corepressor	CIR	U03644
		1.8064	CASP2 and RIPK1 domain containing adaptor with death domain	CRADD	U84388
		1.8548	insulin-like growth factor 1 (somatomedin C)	IGF1	X57025
		1.8702	X-ray repair complementing defective repair in CH cells 2	XRCC2	AF035587
		1.8854	TRAF family member-associated NFKB activator	TANK	U59863
		1.8911	aminomethyltransferase (glycine cleavage system protein T)	AMT	N59532

Table 2. Continued

Fold change vs. Control			Gene name	Gene symbol	Accession No.
17β-estradiol	Testosterone	Progesterone			
(C)					
		1.9429	gap junction protein, beta 1, 32kDa	GJB1	N62394
		1.9525	pescadillo homolog 1, containing BRCT domain (zebrafish)	PES1	U78310
		2.0073	nuclear receptor subfamily 2, group C, member 1	NR2C1	H68838
		2.0268	caspase 10, apoptosis-related cysteine protease	CASP10	U60519
		2.0539	homeo box B13	HOXB13	U81599
		2.0735	adenomatosis polyposis coli	APC	AA455997
		2.1283	zinc finger protein 161 homolog (mouse)	ZFP161	AA459007
		2.2103	transcription elongation factor A (SII)-like 1	TCEAL1	AA451969
		2.2380	potassium large conductance calcium-activated channel, sub M	KCNMA1	AA904544
		2.2962	Rap1 guanine-nucleotide-exchange factor directly activated by cAMP	EPAC	U78168
		2.4729	hydroxysteroid (17-beta) dehydrogenase 3	HSD17B3	AA437291
		3.0362	BCL2-like 2	BCL2L2	D87461
		3.1258	nuclear receptor co-repressor 1	NCOR1	AA926832

The results of KISTCHIP-400 analysis are shown as values of fold change (fluorescent intensity for chemical plus/fluorescent intensity for chemical minus). Genes presented in shadow was selected on 17β-estradiol responsive genes.

ression was consistently altered by 17β-estradiol, testosterone and progesterone. Genes presented in shadow was selected on 17β-estradiol specific genes. All of the data described above clearly demonstrate that the quantitative expression profiling using cDNA microarray (KISTCHIP-400 ver. 1.0) could be applied to the indicator of endocrine relating and disrupting phenomenon.

DNA microarray analysis is a powerful technology for global gene expression profiling[17,18]. Using traditional methods in molecular biology, researchers were able to survey a relatively small number of genes at a time. However, microarrays allow scientists to analyze expression of many genes in a single experiment quickly and efficiently. This technology is still considered to be in its infancy. Therefore, many initial studies using microarrays have represented simple surveys of gene expression profiles in a variety of cell types. Nevertheless, these studies represent an important and necessary first step in our understanding and cataloging of the human genome. Microarray technology can, in principle, be used to study the toxicology of anticancer drugs and of environmental and dietary agents potentially involved in cancer development[19-21].

Also, an increased awareness of endocrine disrupting chemicals (EDCs) and their potential to affect wildlife and humans has produced a demand for practical screening methods to identify endocrine activity[22-24]. Despite the exciting prospects of this methodology, a scan of the literature reveals very few toxicogenomic studies. Here, we have provide data from a human endocrine related cDNA microarray constructed from clones generated in our laboratory which we believe will serve as a template for future studies in toxicogenomics for performing EDCs monitoring. To facilitate these analyses, a so-called "KISTCHIP-400 ver. 1.0" has been developed that contains many of the human genes known or proposed to be involved in endocrine system (Table 1). The goal of these studies is to identify the patterns of alteration of gene expression characteristic of exposure to each class of EDCs in cultured human cells. Once signatures are identified, the patterns of altered gene expression induced by unknown agents might identify their mechanism of action. This approach could also be applied to identify signatures for various types of tissue-specific EDCs, providing a more rapid test for the possible toxicological effect of drugs or unknown agents and less need to use animals.

To assess the qualities of the KISTCHIP-400 ver. 1.0 microarray, we investigated transcriptional profiles of three reference hormones: 17β-estradiol, testosterone, and progesterone. 17β-Estradiol is a steroid hormone produced mainly in the ovaries, by the placenta during pregnancy, and to a lesser extent in the adrenal cortices, testes and peripheral tissues. The hormone is synthesized enzymatically from acetate, cholesterol, progesterone and testosterone. In addition to the well-described anatomic and physio-

Table 3. Functional Classification of 17β-estradiol Responsive Genes

Gene function	Gene category[a]	Accession No.	Fold changes vs. control
Apoptosis			
clusterin	O	NM_001831	−2.1218
lactate dehydrogenase A	P	NM_005566	1.7156
exportin 1	O	D89729	1.7917
Cell adhesion			
integrin, alpha 3	Q	M59911	−2.4857
ninjurin 1	Q	U72661	−2.0364
integrin beta 3 binding protein	Q	U37139	1.7886
Metabolism			
phosphorylase kinase, alpha 2	C	AA677340	−2.1179
cyclin-dependent kinase 10	Q	X78342	−2.0950
similar to restin	C	AA070226	−1.7175
cytochrome P450, family 2, subfamily J, 2	Q	U37143	−1.6901
G protein-coupled receptor 56	C	AA775249	−1.6651
matrix Gla protein	C	AA155913	2.6858
Regulation of cell cycle			
transcription factor A, mitochondrial	O	NM_003201	1.6126
dyskeratosis congenita 1, dyskerin	B	AA052960	1.6427
mutL homolog 1	Q	U07418	1.7310
solute carrier family 7, member 5	P	NM_003486	2.4863
polo-like kinase (Drosophila)	Q	U01038	8.2858
Regulation of transcription			
nuclear receptor subfamily 3, group C, 1	L	AA664219	−2.1000
GATA binding protein 3	Q	X58072	−1.7491
early growth response 1	H	AA486628	−1.6190
cofactor required for Sp1 transcriptional activation	C	R40567	−1.5990
tripartite motif-containing 28	O	H41030	1.5956
X-ray repair complementing defective repair in CH cells	Q	M30938	1.9059
Signal transduction			
faciogenital dysplasia (Aarskog-Scott syndrome)	G	AA902269	−1.5988
phosphodiesterase 8B	C	AA455365	1.6411
calmodulin 2 (phosphorylase kinase, delta)	P	NM_001743	2.0289
Tight junction			
claudin 3	B	AA434144	−2.5402
Protein folding			
FK506 binding protein 4, 59 kDa	P	NM_002014	1.5907
EST			
Homo sapiens transcribed sequence	C	R33030	1.6197
Kinesin complex			
tumor protein D52-like 1	A	NM_003287	2.0639

[a] Genes spotted on KISTCHIP-400 categorized through the papers & OMIM database. Description of each category was presented in Table 1.

logical regulation of reproduction and secondary sex characteristics, it also influences diverse activities such as bone growth, brain development and maturation and the intracellular concentrations of calcium and certain second messenger molecules[25]. The principal androgen is testosterone. This steroid is manufactured by the interstitial (Leydig) cells of the testes. Secretion of testosterone increases sharply at puberty and is responsible for the development of secondary sexual characteristics of men. It is also essential for the production of sperm. Finally, progesterone is a major steroid hormone secreted in large amounts by the corpus luteum and results from the extracellular conversion of cholesterol, cholestryl esters, adrenal steroids, prenenolone and pregnenolone sulfate. It has many effects in the body, some having nothing to do with sex and reproduction.

In the case of 17β-estradiol, 33 genes exhibited up-regulated genes, and 23 genes exhibited down-regulated genes. From this data, we have identified 33 responsive-genes for 17β-estradiol (Table 3). These genes were classified into eight functional categories based on biological functions. Some of the gene expression changes induced by 17β-estradiol identified in these studies are consistent with reported observations described in the literature[26,27]. These included

the induction of FK506-binding protein 4 known as chaperones group[28]; lactate dehydrogenase A of apoptosis associated gene[29]; solute carrier family 7 of cell cycle related gene[30]; calmodulin 2 of signal transduction[31,32]; tumor protein D52-like 1 of well-known tumor associated protein[33]; and H2A histone family of chromatin associated gene[34]. Although Leung *et al.*, reported that BCL2 related genes were down-regulated by estradiol in MCF-7 cells[35], our results presented these genes were down-regulated by estradiol but also testosterone. Therefore BCL2 related genes may be not estradiol responsive genes but genes related to cell death by sex hormone. We also identified 20 genes were up-regulated and 15 genes were down-regulated by testosterone. But, above 50% genes appeared different gene expression pattern compared testosterone to 17β-estradiol. In progesterone, only 13 of 50 genes were similar to 17β-estradiol and the others show the gene expression which is unique. Table 2 shows gene expression was consistently altered by 17β-estradiol, testosterone and progesterone. Genes presented in shadow was selected on 17β-estradiol specific genes. All of the data described above clearly demonstrate that the quantitative expression profiling using cDNA microarray (KISTCHIP -400 ver. 1.0) could be applied to the indicator of endocrine relating & disrupting phenomenona. Therefore, it can be concluded that the KISTCHIP-400 ver. 1.0 prepared in the present study is of a high enough quality so that it can be used for further analysis of the gene expression profiles. We have been proposed that microarray profiling could be used to monitor environmental exposure to EDCs in humans. The rationale is that, as gene expression is a sensitive endpoint, alterations in microarray expression profiles may act as useful biomarkers to define more precisely the nature and level of exposure to hazardous chemicals.

Methods

Construction of Human cDNA Microarray Related to Endocrine System (KISTCHIP-400 ver. 1.0)

A human cDNA microarray with 416 unique elements of genes was constructed. The array consisted of clones generated from hormone related genes, factors, and expressed sequence tags (ESTs), based on public database and research papers. Theses clones contained estrogen, androgen, thyroid hormone & receptors, sex hormone signal transduction & regulation, c-fos, c-myc, ps2 gene, metabolism related genes etc. A functional category of 416 unique elements on the cDNA microarray (KISTCHIP-400 ver. 1.0) can be viewed in Table 1. Among these clones, approximately 200 unigenes were purchased from Invitrogen Life Technologies as I.M.A.G.E. clones. The others were purchased from Korean Collection for Type Cultures (KCTC) and Digital genomics Inc. as plasmid. These clones transformed to DH-5α strain, followed by isolation of plasmid DNA of these clones using the Nucleogen plasmid prep kit. The quality and quantity of plasmid DNA were confirmed using agarose gel electrophoresis and spectrophotometer. Individual DNA fragments were amplified by polymerase chain reaction (PCR) using 50 to 100 ng plasmid DNA as template, GF200 primer set from Invitrogen Life Technologies and Taq DNA polymerase (TaKaRa Ex Taq^{TM}) in 100 μl reaction mixture. PCR products were purified using Nucleogen PCR purification kit and quantified with spectrophotometer. More than 99% of the clones gave a single band. Purified PCR products were dried and resuspended in distilled water, and then mixed with 3 × SSC. cDNA solutions were spotted onto superamine-coated CMT-GAPS2 slide glass (Corning) by Digital genomics Inc. using a pin type arrayer. Each slide was spotted with duplicate sets of about 416 unique cDNAs on the upper and lower sides of the array (twin chip). For the validation of chip, we checked the DNA spot quality using Syto61 dye (Sigma). This cDNA microarray was termed as KISTCHIP-400 version 1.

Cell Culture and Chemical Treatment

MCF-7 cell line originated from human breast cancer cell was a gift from the Prof. Soto (Tufts University School of Medicine). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (Invitrogen Life Technologies), penicillin 100 IU/ml and streptomycin 100 μg/ml. Subculturing was conducted every 3 day so as not to exceed 1×10^6 cells/ml. For validation of constructed microarray, 17β-estradiol, testosterone, and progesterone were used as reference hormones. These hormones were dissolved in absolute ethanol. Vehicle concentrations were less than 0.1% in all experiments. At 80% confluence, cells were treated with 10^{-8} M 17β-estradiol, 10^{-7} M testosterone and 10^{-7} M progesterone in phenol-red-free DMEM/F12 supplemented with 5% charchol/dextran fetal bovine serum culture media for 24 h and total RNA was extracted for analysis. Chemical structures are shown in Fig. 1.

RNA Extraction

Total RNA was isolated from MCF-7 cells with

three reference hormones using Trizol reagent (Invitrogen Life Technologies) and purified using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen) during RNA purification. The amount of each total RNA sample was measured by a spectrophotometer, and its quality was checked by agarose-gel electrophoresis.

cDNA Microarray Experiments

We examined transcriptional profiling of three reference hormones: 17β-estradiol, testosterone, and progesterone. For cDNA microarray analysis, total RNA was isolated from MCF-7 cells with each three reference hormones treatment. Labeling and hybridization were performed by instruction of MICROMAX direct cDNA microarray system (PerkinElmer) with minor modification. Briefly, the RNA samples from MCF-7 cells of treated reference hormone were labeled with Cy3-dUTP (NEN), and those of non-treated reference hormone were labeled with Cy5-dUTP (NEN). The two color probes were then mixed, purified using Microcon YM-100 column (Millipore). Hybridization and washes were performed according to the Digital Genomics Inc.'s instruction. Hybridization (hybridization buffer; 25% formamide, 5 × SSC, 0.1% SDS, 0.5 mg/ml polyA, 0.5 mg/ml Cot-1 DNA) was performed in a hybridization oven at 58°C for 16 h. After washing (2 × SSC/0.1% SDS for 5 min at 58°C, 0.1 × SSC/0.1% SDS for 10 min at RT, 0.1 × SSC for 1 min at RT), the slide was dried by centrifugation at 650 rpm for 5 min. Hybridization images on the slides were scanned by ScanArray Lite (PerkinElmer). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments) to obtain gene expression ratios. The overall intensities were normalized using a correction coefficient obtained from the ratios of housekeeping genes.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[36]. The statistical significance of the differential expression of genes was assessed by computing a q-value for each gene. To determine the q-value we used a permutation procedure, and for each permutation, two-sample t statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in four independent hybridizations were more than 0.65 or less than -0.65, i.e., 1.5-fold difference in expression level, and when the q-values were < 5.

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