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Toxico-Genomiics

Functional Genomics Towards Understanding of the Mechanisms of Life

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The impact of the recent output of genome analysis is enormous. The genome sequencing of dozens of microorganisms showed that the genome structure has changed dynamically, leading to rethinking of the definition of species in microorganisms. The genome sequencing of model organisms such as nematode, fruit fly and Arabidopsis and human is providing us clues to understanding of the 'strategy of life' in the evolutionary history. I believe that the consideration of evolutionary aspect of genome is essential for understanding of the mechanisms of life, which provides a basis for both basic and applied sciences including toxicogenomics.

In the long history of evolution, it is thought that multicellular organisms were produced through gene duplication and that vertebrates were generated through genome duplication. The gene numbers of prokaryote and lower eukaryote genomes are about 1,000 to 4,000 and about 7,000, respectively, while those of invertebrates such as nematode and fly increases to 10,000-20,000. It was surprising that the gene number of fly genome were less than that of nematode, although fly is obviously more complex than nematode. Although still debated, the gene number of the human genome is estimated about 40,000, which is only 2-3 times of that of invertebrates. It is also suggested that the gene number of vertebrates is essentially the same from fish to human. This is much more surprising. How 'life' manages to generate different species from similar sets of genes? Various mechanisms, including alternative splicing, difference in expression pattern (spatial and temporal), imprinting and genome rearrangements in somatic cells, are expected to work for generating the diversity in gene expression. Invertebrate genome is the prototype of the human genome. The genomes of fish are in the same family with the human genome. Thus, comparison among invertebrate, fish and human genomes with respect to their expression and function should provide great clue to understand the mechanisms of life.

Here I will present the current status of functional genomics of the nematode C.elegans. This tiny creature has the basic body plan of animal but consists of only about 1,000 somatic cells, whose entire cell lineage from fertilized egg to adult has been described. Thus, it is a good model system for functional genomics at the level of single cell. C.elegans belongs to lower organisms, but its many genes are similar to human genes and some genes are interchangeable with the human counterparts. Based on the systematic cDNA analysis, we have performed the integrated analysis of the structure, expression, function and evolution of the genome, aiming at the understanding of the gene network system that governs its development. We are also trying to construct a computer modeling of the early embryogeneis. All the data are stored in NEXTDB (Nematode Expression Pattern DB), and many of the data are available at http://helix.genes.nig.ac.jp/db/.

Toxicogenomics and Its Potential for Assessment of Pollutant Impact in Fish

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Toxicogenomics combines information and material from genomics and bioinformatics in order to identify and characterise mechanisms of toxicity from known or suspected toxicants. The main emphasis of this area of research has concerned the screening of novel compounds, such as drug candidates, for potential toxicity and initial studies on the mechanisms by which toxicants exert their effects. These techniques are now beginning to be applied to environmental monitoring using sentinel species, in order to assess the impact of anthropogenic pollution.

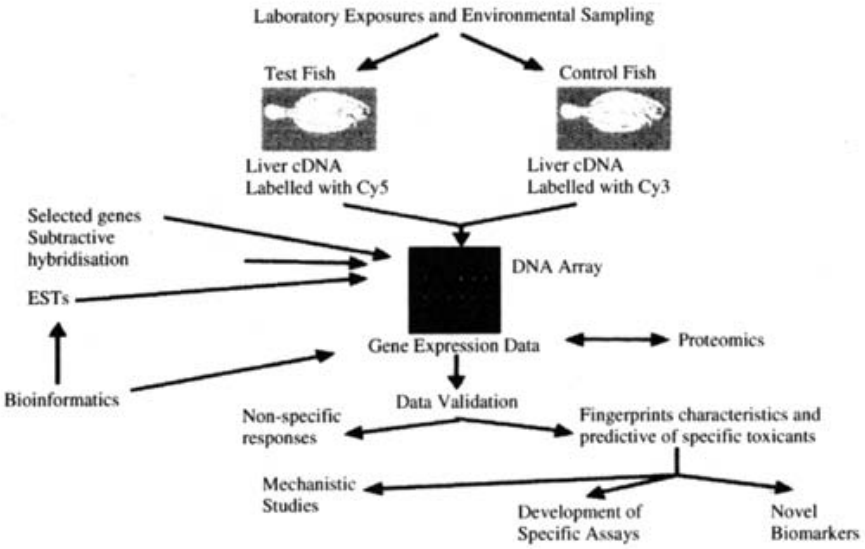
The marine environment may be contaminated with a wide range of toxicants, these include xenoestrogens, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and dioxins, usually in a complex mixture. Fish species have often been used as sentinels to detect the impact of such pollutants. We study European flounder *(Platichthys flesus),* a flatfish species which inhabits estuaries around Western Europe, feeds on invertebrates located in the sediment and is thus exposed to pollutants. It is closely related to Japanese flounder *(Paralichthys olivaceus).* Using this environmentally relevant species presents difficulties as its genome is not currently being sequenced. The two fish species, Fugu *(Takifugu rubipres)* and Zebrafish *(Brachydanio rerio),* currently being sequenced are inappropriate for monitoring in this environment, but provide valuable data on fish genes which aid our research. Techniques are, however, available to apply toxicogenomics to non-model species.

Gene expression changes can serve as "early warning markers" of pollution impact. We are studying gene expression using DNA microarrays, these allow the monitoring of the expression of thousands of genes simultaneously and thus constitute an extremely powerful technique for the identification of differentially regulated genes. This technique has not yet matured sufficiently for routine use in environmental monitoring. We aim to characterise responses to individual pollutants and mixtures and to distinguish between responses indicative of harm to the organism and those due to other environmental influences. We anticipate that this approach will lead to the identification of novel biomarkers of response, which could be incorporated into reporter gene assays or ELISAs and to focussed arrays, utilising the genes with most reproducible responses to pollutants. (see Figure)

The first step towards microarraying is to isolate gene fragments. Gene targets for arraying may be randomly picked from a cDNA library, preferably made from stress-exposed fish, characterised by sequencing to produce ESTs (Expressed Sequence Tags) and used directly for arraying. Alternatively, differentially regulated genes (both up- and down- regulated during stress) may be isolated using techniques such as SAGE, differential display or subtractive hybridization. Another option is to clone fragments of specific genes of toxicological interest using primers designed to homologous regions of orthologous genes from different species.

Our initial experiments followed this third approach. We isolated 120 fragments of toxicologically interesting genes and others, to create a mini-array. Our next input of gene targets has been prepared by subtractive hybridisation (PCR-Select, Clontech) using fish from the polluted Tyne estuary (UK) and the reference estuary, the Aide (UK). We are initiating a European flounder EST sequencing project which will provide up to 10,000 targets for further expression studies.

We used cDNA targets arrayed onto glass slides by a high precision robot at the University of Birmingham Genomics Laboratory, an in-house facility (BBSRC grant 6/JIF 13209). We labelled cDNA from flounder livers with fluorophores Cy3 and Cy5, in dual labelling experiments (eg. Polluted=Cy5, Reference=Cy3). After hybridization to the arrays, slides were scanned and the relative amounts of each fluorophore indicated the relative gene expression in each pair of samples. Initially livers of feral fish from clean and polluted locations were compared. Subsequently we are extending our studies to include fish exposed to specific compounds or mixtures in laboratory based exposures.



Our data from the environmental samples showed differential regulation of a number of genes. Some of the genes up-regulated in polluted fish were metallothionein, indicating heavy metal contamination, NADP-menadione oxidoreductase (NMO), which responds to a wide range of organic toxicants, and a transcript similar to Zona Pellucida protein C (ZPC), an egg membrane protein (often called ZRP in fish). ZPC has been used as a biomarker of xenoestrogen exposure in fish, as it can be regulated by the estrogen receptor. In collaboration with Dr. D. Hughes (University of Birmingham Women's Hospital) we intend to elucidate how many genes of this family are expressed in flounder and their evolutionary relationships to mammalian ZP genes. We are also interested in the expression of these genes in liver and possible transport of the proteins to the ovary, the site of the bulk of ZPC expression. This illustrates how microarray data can be used to focus attention on specific systems involved in the response to specific classes of toxicants such as xenoestrogens. This work has been funded by the NERC and the European Union.

Progress in Medaka Genomics

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Medaka is a small freshwater fish which is native in Japan, Korea and China. This fish is widely used as a material for various biological fields such as physiology, genetics and developmental biology. In the toxicology field, this fish is one of the standard materials for the environmental risk assessment.

After the publication of the human genome draft sequence, the genome sequencing analysis of the small fishes are also accelerated. In October 2001, the draft sequence of Japanese puffer fish, Takifugu rubripus are determined by JGI (USA) and collaboration with IMCB(Singapore) and HGMP-RC(UK). The large scale EST analyses of medaka were done in French, Germany and Japan. Over 13000 medaka sequences are already deposited in the DDBJ/Genbank/EMBL DNA bank. The EST analyses are still continued in several laboratories. After several months, over 50000 sequences will be open in the public DNA bank. We sequenced over 8000 cDNAs and performed the bioinformatic analysis such as the clustering of each sequence and searching homology with blast program. We identified that about 2000 clusters have the significant homology with cDNA of other species. We successfully mapped over 400 EST markers showing the homology with cDNA sequences of other species and compared the linkage relationship between human and medaka. Based on our criteria about the conserved synteny (at least 3 orthologous genes are located on the same linkage group), about 40 synteny conserved regions are detected. This result suggests that the medaka and human have the similarity of the genome structures in terms of the synteny.

In 1953, late Prof. Toki-o Yamamoto succeeded the artificial induction of sex-reversal by the sex-steroid treatment. This is the first successful case of the artificial induction of sex-reversal in vertebrate. This is also the first study of endocrine disruption in fish. He used the orange-red and white varieties of medaka. These strains are belonging to the Southern population. There is another medaka population in Japan. This is called the northern population. The fish in the northern population is less sensitive to estrogen. If he used the medaka in the northern population, we never succeeded the artificial induction sex reversal. The fishes in the southern and northern population have no sexual segregation. This indicates that these fishes are the same species. These results clearly showed that the genetic background influences the response to the chemicals.

Although the structural similarity of genome between medaka and human support the usefulness of medaka as an experimental model, the fishes, those have almost the same genomic structures, have the different response to the environmental chemicals such as endocrine disrupter. These results suggested that the understandings about the “genetic” variations of population are important for the true assessment of risk of environmental chemicals.

Status of Xenopus laevis and Xenopus tropicalis microarrays

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Xenopus laevis is a unique resource for several important areas in vertebrate biology including early embryonic development, cell biology and toxicology. Xenopus laevis has led the way in identifying the mechanisms of early fate decisions, patterning of the basic vertebrate body plan, and early organogenesis. Contributions in cell biology and biochemistry include seminal work on chromosome replication, chromatin and nuclear assembly, control of the cell cycle, *in vitro* reconstruction of cytoskeletal dynamics, and signaling pathways. This wealth of knowledge will facilitate toxicogenomic studies designed to understand the mechanisms through which toxicants act.

Xenopus laevis and tropicalis EST projects are underway. Nearly 120,000 ESTs are now available from Xenopus laevis (12,000 unique genes) together with an additional 7000 from Xenopus tropicalis. A number of laboratories worldwide are engaged in developing microarrays based on the cDNA clones identified. The status and availability of these microarrays will be reported together with perspectives on the expected future progress in this area.

Toxicogenomic Assessment of Endocrine Disruptors

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In order to fully assess the effects of chronic and subchronic exposure to synthetic and natural endocrine disruptors, a more comprehensive understanding of the molecular, cellular and tissue level effects is required within the context of the whole organism, its genome and its proteome. The rapid advancement of various genome projects has generated full or partial sequence data for thousands of genes in a number of different organisms. It is now possible to analyze the response of these genes following exposure to exogenous substances and infer potential consequences. Toxicogenomics, an emerging field that integrates the awesome potential of bioinformatics and genomics into toxicology, has been proposed to screen synthetic and natural endocrine disruptors for potential toxicity and to provide mechanistic information about these substances. This presentation will provide an overview of the toxicogenomic strategies used to assess endocrine disruptors and the infrastructure required to fully employ these approaches including, the development of a relational database (dbZACH) to support toxicogenomic studies, the construction of model specific arrays, study design considerations and data analysis strategies will be discussed in relation to the following three studies, 1) the effect of gestational and lactational exposure to diesthylstilbestrol (DES) on sperm quality, *in vitro* fertilizing ability and testicular gene expression in the mouse using cDNA arrays enriched for testicular genes, 2) the effect of synthetic and natural endocrine disruptors on global gene expression in cAMP-induced differentiating human SVG astroglial cells using a custom SVG cDNA array , and 3) the temporal effects of ethynyl estradiol on global gene expression in the mouse uterus using the Affymetrix MullKsubA GeneChip. Details of each study are provided below.

Study 1: Effects of Gestational and Lactational Exposure to Diethylstilbestrol on Testicular Gene Expression Using cDNA Microarrays and Real-Time PCR.

To explore the molecular events underlying the adverse effects on sperm quality following developmental exposure to estrogenic chemicals, we have constructed cDNA microarrays to examine testicular gene expression in B6D2F1 offspring of mice exposed by gavage to 10 μg/kg diethylstilbestrol (DES) from gestational day 12 to postnatal day 21. Replicate gene expression profiles were examined in male offspring at 3, 15 and 45 weeks of age to determine if the effects on gene expression paralleled the long term decreases in testis weight, sperm count, and sperm fertilizing ability, as previously described. A mouse cDNA microarray containing approximately 1948 genes was constructed and used to compare testicular gene expression in the offspring of DES-exposed mice to that of vehicle-exposed mice. An independent reference design coupled with paired t-tests was used to identify genes significantly ($p<0.05$) altered in expression by DES. Based on adjusted p values, there was only one gene significantly altered at 3 weeks of age (a component of the 20S proteasome). At 15 weeks of age, there were 46 genes significantly altered in expression. There were no significant changes at 45 weeks of age. Raw p values and functional annotation were used to prioritize the selection of genes for verification. Using real-time PCR, both age and dose-dependent changes in gene expression were examined. There was a significant decrease in the expression ofER alpha mRNA at 3 weeks of age ($p<0.01$), while mRNA expression was below the limits of detection in 15 and 45 week old mice. Other genes selected for verification include inhibin, the orphan receptor TR2, and Xmr, a component of the synaptonemal complex. These results suggest that the adverse effects on sperm fertilizing ability may be due to

altered expression of ER alpha, and possibly other genes, in the testis.

Study 2: Retinoic Acid Disruption of cAMP Induced Human SVG Astroglial Cell Differentiation: Morphology and Global Gene Expression Effects

Previous work has shown that treatment of human SVG cells with 5 μ M forskolin (F) and 200 μ M 3-isobutyl-1-methylxanthine (iBMX) increases cAMP levels resulting in differentiation and dramatic morphologic changes. We show that co-treatment with 0.5 μ M retinoic acid (RA) enhances these morphologic changes as measured by increases in the total number of processes ($p = 0.03$) and the length of processes contacting an adjacent cell (LPT; $p<0.01$). At 36 hrs, RA inhibited regression of LPT ($p = 0.02$) with increases in mean process length ($p = 0.04$) and the longest primary process ($p<0.01$). A custom, SVG-specific cDNA microarray (2,990 genes) was used to investigate the temporal changes in gene expression during cAMP induced differentiation. Two different exploratory filtering approaches were used to identify changes in gene expression. First, an incomplete block design and individual gene mixed model ANOVA was used for analysis, followed by t-tests using a step-down Bonferroni adjustment for multiple comparisons. The mixed model included treatment, time, dye and treatment x time as fixed effects, and spots as random effects. To identify genes with significant expression changes during differentiation, least squares means of F/iBMX treated cells were compared to time-matched vehicle control samples utilizing t-tests. For comparison, a second technique utilizing Shannon Entropy filtering of normalized microarray data from treated, vehicle control, and untreated/time 0 h control cells. Filtered data was then analyzed using Principal Components Analysis (PCA) to identify time-effect relationships amongst genes. Results from the two divergent approaches were comparable and identified time and treatment effects on gene expression, although some differences were observed.

Study 3: Temporal Effects of Ethynyl Estradiol on Global Gene Expression in the Mouse Uterus

Estrogens are classically defined as compounds that are capable of inducing a trophic effect on the uterus. This physiological change has been linked to alterations in the expression of estrogen-responsive genes, but the cascade of molecular events remains unknown. In the present study, the effects of ethynyl estradiol (EE), a synthetic estrogen, on global gene expression in the uterus were examined in immature, ovariectomized C57BL/6 mice. Mice were gavaged with 0.lmg/kg EE or vehicle, with uteri harvested at 0, 2, 8, 12, or 24 hrs. Overall, no differences in uterine weight were found between treatment groups ($p=0.296$) from 0 to 24 hrs. However, significant changes in global gene expression levels were observed during this period using MullKsubA Affymetrix GeneChips. The relative expression levels of over 6000 genes in uterine tissue of two animals were assessed from each treatment and time-matched vehicle treated (control) group. Data were then screened using t statistics and a Kruskal-Wallis test to identify gene expression changes outside the normal range of variation for the control group. In both methods, treated values were compared to i) a global control assuming control observations did not significantly change during the course of the experiment, or ii) only the average for that specific time matched control. These methods were found to give different results, with comparisons to the time-matched control consistently resulting in the identification of fewer active genes. The greatest number of significant changes in gene expression was found with the 24 hr treated uteri. The nature of these changes over time was characterized using a parametric model based on the general shape of the time-relationship effect. Importantly, many of the genes in the vehicle-treated groups also showed significant temporal fluctuations in expression, underscoring the importance of incorporating time-matched controls into the study design.

Molecular Profiling of Genes Showing Altered Expression in the Livers of Rats Treated with Non-Genotoxic Carcinogens for 28 Days

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Short-term *in vitro/in vivo* genotoxicity assays are usually employed for the prediction of carcinogenicity and have proved useful in the initial identification of potential genotoxic agents. However, their value is limited by the observation that approximately 60% of chemicals identified as carcinogens by *in vivo* long-term carcinogenicity studies produce mainly negative findings in genotoxicity tests. There is currently no reliable rapid means of evaluating the carcinogenic potential of new chemicals that fall into this latter group of compounds, termed non-genotoxic carcinogens.

Although the molecular events in initial stage of chemical action in a target organ may vary with the carcinogen, there may be some common mechanism by which carcinogenic response is triggered. One approach to addressing this issue is molecular profiling to identify common factors/mechanisms that can serve as early biomarkers of carcinogenic potential for new chemicals. In the present investigation, we performed global gene expression analysis using microarray technique in the rat livers to clarify common molecular events that appeared in response to repeated oral administration of representative non-genotoxic liver carcinogens for 28 days.

Six-week-old male SD:IGS/DuCrj rats were fed diet containing 600 ppm-phenobarbital (PB), 600 ppm-thioacetamide (TAA), or 20,000 ppm-diethylhexyl phthalate (DEHP). As a negative control for carcinogenicity, a group of animals were fed hepatotoxic dose (10,000 ppm) of non-carcinogenic acetaminophen (APAP). After 28 days of experiment, mRNA expression analysis was performed in the livers using GeneChip® Rat Genome U34A Array (Affymetrix Inc.) with three animals in duplicate in each group.

In the livers of untreated rats that fed basal diet alone, approximately 3,000 genes were shown to be expressed among the 8,000 genes included in the chip. Numbers of genes up-regulated twice or more by treatment with PB, TAA, DEHP, or APAP as compared to the levels in untreated controls were 58, 566, 230, and 50, respectively. Genes down-regulated twice or less were 90, 368, 290, and 79, respectively. In every chemical examined in the present study, majority of genes showing up-regulation were classified into the category of Metabolism, and majority of down-regulated genes were classified as those for Cell signaling/Communication, Metabolism, and Cell/Organism defense. However, majority of genes identified to be down-regulated were different between chemicals, and therefore this difference may determine the phenotype of biological responses induced by each chemical. On the other hand, among the genes those expression levels were unchanged by APAP-treatment, a group of genes showed similar expression pattern between PB, DEHP, and TAA. Since these genes could be candidate biomarkers for detecting non-genotoxic carcinogens, expression analysis is now in progress in the livers of rats treated with these chemicals for 1 and 2 years.

Fetal Transcript Profiles for Endocrine Disrupters

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The Procter & Gamble Company

Developmental exposure to endocrine disrupters may result in persistent effects on function; however, these effects may be undetectable until maturation. The long latency period for the manifestation of effects is a hindrance to screening environmental agents for endocrine disrupting activity. Therefore, proposed screening methods have not been developed in embryos and fetuses, even though it is generally acknowledged that developing life stages have the potential to be the most sensitive to endocrine disruption.

We postulate that prenatal exposures to estrogens, anti-androgens or thyrotoxicants produce stereotypical patterns of gene expression (transcript profiles) that presage alterations in functional development. If true, the profiles could be used as a molecular fingerprint of hormonal action. We are testing this hypothesis by exposing pregnant rats to known estrogens, anti-androgens or thyrotoxicants of varying potencies, and evaluating transcript profiles in responsive tissues of term fetuses. For estrogens, those tissues are uterus, ovary and testis; for anti-androgens, testis and epididymides; for thyrotoxicants, lung and brain. The transcript profile for estrogens has been completed; an overview of the experimental procedure and results is presented below.

Pregnant Sprague Dawley rats were given sc injections of 17-alpha-ethinyl estradiol (EE), a potent estrogen, genistein, a moderately potent estrogen, or bisphenol A (BPA), a weak estrogen, daily on gestation days 11-20. Dosages were 0.5, 1, or 10 ug/kg/day EE; 0.1, 10 or 100 mg/kg/day genistein; or 5, 50 or 400 mg/kg/day BPA. Concurrent controls for each study were treated with vehicle. Fetal uteriand ovaries together, or testicles with excurrent ducts, were collected two hours after the last dose and mRNA extracted (samples pooled within litters). Gene expression profiles were determined using the Affymetrix GeneChip system, which assesses approximately 8000 rat genes or ESTs. The expression of approximately 1% of the probed genes is changed in the estrogen-treated samples, with results being comparable in the high dose EE and BPA groups. A clear dose-response pattern was detected for most of the genes. Genes that were upregulated include progesterone receptor, steroid metabolizing enzymes, growth factors and various kinases and phosphatases. Many of the genes in the profile were already known to be regulated by estrogens in adult tissues, but the profile also contains characterized genes and ESTs not previously associated with estrogens.

The results support the notion that prenatal exposure to estrogens produces a replicable fingerprint of gene expression. This transcript profile could serve as the basis for estrogen screening. Results with anti-androgens and thyrotoxicants, not presented here, are also consistent with the premise that each produces characteristic transcript profiles that are diagnostic of these modes of action. Duration of dosing and sacrifice time are consistent with Segment II protocols and endocrine disrupter screening could be feasibly added to Segment II studies or be conducted as stand-alone assessments.