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Workshop 2.4

Transcript profiles elicited by developmental exposure to endocrine-mediated toxicants*

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Abstract: Genomics can be applied in toxicology as a means of identifying modes of action, for generating hypotheses on the relationships of gene activity and toxicity, and for better characterizing the nature of dose–response relationships at low doses. This paper illustrates each of these applications with examples from our research on endocrine active compounds. We have determined that agents that bind estrogen receptors produce a characteristic transcript profile in estrogen-responsive tissues of the fetal and juvenile rat. The transcript profile is diagnostic of the mechanism of action. The transcripts that are up- or down-regulated by estrogens belong to a number of functional groups, such as growth factors, pro-apoptotic factors, transcription factors, and steroid metabolizing enzymes. There are a number of testable hypotheses that can be generated from these data regarding the relationships between changes in these genes and developmental or physiological responses to estrogens. We have determined that the sensitivity of gene expression changes is high, making it possible to define the shape of the dose–response curve at dose levels of estrogen several orders of magnitude below those that cause a physiological response (in this case, a uterotrophic response). The shape of the dose response is monotonic: both the number of genes changed and the intensity of the up- or down-regulation decreases with decreasing dose.

Our lab is using toxicogenomics to identify molecular fingerprints, which we call transcript profiles, that are specific for steroid hormone receptor-mediated mechanisms of action. The main reason for conducting this research is to develop screening methods based on these profiles. However, the work also has general utility in understanding the life-stage specificity of gene expression; characterizing dose–response relationships, particularly at the low end of the dose–response curve; and in providing the basis for testing hypotheses on the role of individual genes and combinations of genes in development and physiological processes.

Regulatory agencies around the globe are engaged in validating screening systems for endocrine-mediated toxicants. The screening batteries are extensive, but few of the screens use developing organisms and none use developing mammal embryos. This is despite the general belief that early development is the most sensitive to endocrine-mediated toxicity, and the fact that manifestations after developmental exposure tend to be permanent. One reason why screening assays have not used mammalian embryos is that the adverse effects produced by endocrine mechanisms are often latent and cannot be detected using traditional toxicology methods until well after exposure occurs, often not until the animal becomes sexually mature. The long lag time between exposure and outcome has made development-based screening infeasible.

Although the ultimate manifestations of toxicity, which occur at the tissue and organ level, take a long time to be manifested, it is likely that there are much more rapid, and perhaps persistent, changes

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in gene expression that precede and are responsible for the biological response. In fact, gene expression is an integral part of the signal transduction pathway for steroid hormones. Therefore, we have stated the hypothesis that toxicants that act via interference with normal steroid hormone receptor function will produce a characteristic pattern of gene expression in sensitive tissues. If true, then it should be possible to use these profiles as a basis for screening for endocrine active agents in developing tissues, because the gene expression response will be rapid and specific to a particular mechanism of action. The general support for this hypothesis is the knowledge that gene expression is an integral step of the steroid hormone signal transduction process. Empirically, it has been reported that prenatal exposure to potent estrogens [e.g., diethylstilbestrol (DES)] elicits expression of a few genes in the mouse reproductive system that are known to be estrogen-responsive.

We tested the hypothesis by examining the gene expression changes induced by transplacental exposure of rat fetuses to agents with estrogenic activity. (Similar work is underway for antiandrogens and thyroid toxicants, but these will not be discussed here.) The agents we used were ethinyl estradiol, a potent estrogen; bisphenol A, a chemical with weak estrogenic activity; and genistein, a phytoestrogen with potency between that of ethinyl estradiol and bisphenol A (dosages are listed in Table 1). For each compound, the highest dosage was selected because it has been reported to have estrogenic activity in a uterotrophic assay, and the dosages were adjusted for potency such that the pharmacological activity of the compounds was roughly comparable across compounds, even though on a mg/kg basis the dosages were different.

Table 1 Dosages used to elicit estrogen-specific transcript profiles in fetal rats.

17α–EE	0.5	1	10	(µg/kg/day)
Bisphenol A	5	50	400	(mg/kg/day)
Genistein	1	10	100	(mg/kg/day)

Pregnant Sprague–Dawley rats were assigned to treatment groups randomly and administered the test compound daily from gestation day 11 to gestation day 20. The rationale for this treatment interval is that the reproductive system primordia arise on gestation day 12 and continue to develop and mature during this period. Gestation day 20 is the day of gestation when most segment II developmental toxicity studies are ended. In selecting this treatment regimen, it was our thought that, if successful, transcript profiling could be added to regular developmental toxicity studies to enhance their ability to detect endocrine-mediated toxicity. The rats were sacrificed 2 h after the final treatment on gestation day 20. Fetuses were evaluated for external abnormalities, sexed, and the uterus and ovaries of females, and testes and epididymides of males, were removed and immediately placed in RNALater. Subsequently, mRNA was isolated from the tissues and gene expression evaluated using Affymetrix U34A gene chips. These microarrays contain sequences for about 8000 annotated genes and about 1000 expressed sequence tags (ESTs), which represent mRNA sequences that have been isolated from a rat library, but for which the sequence (and function) of the gene have not been determined. Tissues from five fetuses within a litter were pooled to provide sufficient RNA. RNA from at least five litters per treatment group was hybridized to the microarrays, which provided us with sufficient statistical power to support our conclusions about which genes were induced or repressed by treatment.

We observed some apparent toxicity in the high-dose ethinyl estradiol group, in the form of vaginal bleeding in a number of the dams, and early parturition in one. Prominent nipples and areolae were observed in male and female fetuses exposed to the highest dosages of bisphenol A or ethinyl estradiol, and in female fetuses at lower dosages of these compounds. There were no effects on uterine or ovarian morphology or histology of the fetuses.

Each estrogenic compound significantly altered the pattern of gene expression in uterus and ovaries. The expression level of around 5 % of the genes was changed according to at least one statis-

tical criterion that we used. Applying extremely rigorous statistical criteria, we identified a subset of 66 genes that were changed by all three estrogenic compounds, in a dose-related manner. We consider this panel of 66 genes to be the transcript profile for estrogens in the fetal rat uterus and ovaries.

A partial list of genes that are upregulated (Table 2) include many that have already been reported in the literature to be estrogen-responsive at some life-stage. This includes the progesterone receptor and intestinal calcium-binding protein. There were also a number of genes that are involved in steroid metabolism, as well as growth factors and other regulators of cell differentiation and function. In addition to the genes previously reported to be estrogen-responsive, we have discovered a number of other

Table 2 Partial list of genes regulated by estrogens in fetal rat uterus and ovaries.

Upregulated:
- Intestinal Ca-binding protein
- 11-β-OH steroid dehydrogenase
- Progesterone receptor
- Vascular a-actin
- Uterus-ovary-specific transmembrane protein
- FSH-regulated protein
- Asp aminotransferase
- Low-density lipoprotein R/LDLR
- Dermo-1 protein
- *trk* B (receptor Tyr-kinase)
- Growth potentiating factor
- Growth hormone receptor
- Interleukin 4 receptor/IL-4R
- Insulin-like growth factor 1
- Non-neuronal enolase
- C-CAM4
- Glucocorticoid-regulated kinase
- NGF-induced factor A
- Glutathione-*S*-transferase
- Na-K-ATPase-β-3 subunit
- NTAK α-2-1p (activator of Erb-kinases)
- Protein Tyr-phosphatase

Downregulated:
- Retinol-binding protein
- Glutathione-*S*-transferase M5
- Phosphodiesterase I
- Apolipoprotein CI
- Calmodulin-binding protein
- K-channel regulatory protein
- Distal-less 3 homeobox protein
- VLDL receptor
- Phosphofructokinase-C
- *Ssecks* 322*,* mitogenic regulatory gene
- *c-ret*, Tyr-kinase receptor
- Polymerase alpha
- Precursor interleukin 18
- Carboxypeptidase E

Data from J. M. Naciff, M. L. Jump, S. M. Torontali, G. J. Carr, J. P. Tiesman, G. J. Overmann, G. P. Daston. *Toxicol. Sci*. **68**, 184–199 (2002).

genes, including ESTs, that have not previously been reported to be estrogen-responsive. An analysis of the genomic sequence of the annotated genes indicates that not all of these contain consensus estrogen response element sequences, suggesting that their expression is secondary to changes in the expression of genes that are immediately activated by the receptor complex, or that they contain other response elements that bind the receptor.

There are also a number of genes that are downregulated by treatment (Table 2). Again, the genes identified represent a mixture of functions.

We have verified the results of the microarrays using an independent technique, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), for a subset of the genes in the transcript profile. The microarray results are reproducible, and the fold-changes observed by either technique are comparable.

We have compared the fetal transcript profile to the profile that is induced in the uterus and ovaries or rats that are mature enough to produce a uterotrophic response to estrogen exposure. For this experiment, we used a typical uterotrophic assay treatment regimen in which rats were treated daily on postnatal days 21–24, then sacrificed. We used ethinyl estradiol (0.1, 1, or 10 µg/kg/day) as the estrogen for this set of experiments. The dosages of ethinyl estradiol that we used produced a dose-related increase in uterine weight and uterine epithelial cell height, as well as accumulation of fluid in the uterine lumen at the highest dose level. The expression level of over 600 genes was changed according to some statistical criterion, and the transcript profile, composed of those genes that gave a consistent, dose-related response, consisted of 227 genes, much larger than the fetal profile for estrogens.

As with the fetal profiles, there were a number of genes identified in the juvenile profiles that have been previously reported to be estrogen-responsive, such as complement component 3. One impressive difference between the fetal and juvenile profiles was the extent to which gene expression could be changed by treatment. In the juvenile rat, the most robust increases in gene expression were on the order of 300–400-fold, whereas the most marked increases in the fetus were less than 20-fold. It is not clear at this point whether this represents some form of generalized repression of estrogen responsiveness in the fetus, or that the difference is attributable to a few genes in the juvenile profile that have particularly robust responses.

There is considerable, but not complete overlap between the fetal and juvenile uterine transcript profiles. Of the 80 most highly expressed genes in the juvenile profile, 87 % were also expressed in the fetus. On the other hand, only 45 % of the genes present in the fetal profile were also present in the juvenile profile. It is possible that the genes that are unique to the fetus are developmentally important, or that the genes unique to the juvenile are critical for the uterotrophic response (which is not mounted in the fetus), or both. The differences in the profile will serve as the basis for generating and testing hypotheses about the role of specific genes in physiological or developmental events. An example of one such hypothesis will be given later.

We have begun to use toxicogenomics to better characterize the nature of the dose–response curve, particularly at the low end of the curve. This is particularly important in that some labs have reported that estrogens at very low dosages produce qualitatively different changes in responsive tissues than are observed after exposure to exaggerated dose levels. Other laboratories have been unable to repeat these studies, and the weight of evidence is strongly on their side. However, a panel of scientists convened by the U.S. National Institute of Environmental Health Sciences (NIEHS) was unable to conclude, for a few examples of this low-dose phenomenon, which results were correct. They cited the many sources of experimental variability that could be confounding the results, thereby making it impossible at this point to come to a definitive conclusion.

One possible way forward is to evaluate responses at a more fundamental level of biological organization (i.e., gene expression), the thought being that changes in gene expression, being a more immediate response to the toxicant–receptor interaction, may be more sensitive than more traditional endpoints of organ weight and histology. If low-dose phenomena are real, then one would expect it to be reflected in the dose–response behavior of gene expression.

To test this hypothesis, we again used the uterotrophic assay protocol and ethinyl estradiol. In this instance, we used two experimental blocks, the first consisting of the relatively high dosages of ethinyl estradiol used in the previous experiment, and the second consisting of a block in which the high dose (0.1 µg/kg/day) was the same as the low dose in the first block, and the lowest dose being two orders of magnitude lower. We used different diets for the two blocks, standard alfalfa- and soy-based lab chow in the first instance, and a casein-based, phytoestrogen-free diet in the second. Comparisons of the two control groups, and the 0.1 µg/kg/day ethinyl estradiol groups that were in common between the two blocks were made to determine whether there was any effect of diet. We also reviewed data from the controls in our fetal transcript profile experiments, in which the phytoestrogen-free diet was used for the genistein experiment and standard lab chow for the other two compounds.

There were some differences in the profiles that were attributable to diet; however, they were subtle and the majority of the changes appear to be attributable to some aspect of the diet unrelated to estrogen content.

As for the dose–response curve for gene expression in the uterotrophic protocol, change in gene expression was a more sensitive endpoint than uterine weight or morphometry, in that a statistically significant number of genes was changed at a lower dose level than that for which a uterotrophic or histological change could be detected. Furthermore, a few elements of the transcript profile were detectable even at the lowest dosage of ethinyl estradiol used. However, both the number of genes and their level of expression decreased in a monotonic manner with decreasing dose, supporting the conclusion that, at least for this tissue and treatment paradigm, there is not a low-dose phenomenon.

One of the strong potential uses of toxicogenomic analysis is its use in formulating and testing hypotheses about the role of genes in particular physiological processes. One such example, for the uterotrophic response, is given here.

One of the genes that is strongly upregulated by estrogen in the juvenile uterus is matrilysin, a matrix metalloproteinase. One of the substrates for matrilysin is osteopontin, a secreted protein that has been reported to induce apoptosis. Not only is osteopontin degraded by matrilysin, but its expression is downregulated by estrogen; therefore, this signal that would promote an increase in apoptosis, manifested at the tissue level as a decrease in uterine weight, is doubly inhibited. Another gene that is upregulated by estrogen is CD44, a membrane protein that functions to bring matrilysin and the precursor of an epidermal growth factor, HB–EGF, into proximity. Matrilysin activated the growth factor, enabling it to interact with the receptor tyrosine kinase Erb B4, which functions to promote cellular proliferation and tissue changes such as angiogenesis. These changes promote tissue growth, and in this case may be partially responsible for the uterotrophic response. This hypothetical pathway is testable through the use of knock-out animals or other techniques that allow the temporary manipulation of mRNA levels. It is only one of the many hypotheses that can be formulated from the data we have generated thus far, and only serves to illustrate the power of toxicogenomic technology.

In conclusion, we have provided evidence that transcript profiles can be used to identify a mechanism of action, in this case, estrogenicity. We have shown that the response can be detected in the fetus, and that transcript profiles could serve as the basis for the development of improved screens for endocrine active compounds. One particular advantage is that transcript profiling provides a practical way to incorporate developing mammalian systems into a screening approach. We have also shown that transcript profiles can be generated in more mature life-stages. The profiles from fetus and juvenile rat uterus are both recognizable as being estrogen-induced, but their differences suggest different roles for gene expression in the fetus and adult (or near-adult, in the case of the juvenile model). We have used toxicogenomics to evaluate dose response and to formulate hypotheses as to how changes in gene expression may control tissue responses. These hypotheses are testable, and, together with dose–response information, may provide the basis for a quantitative, biologically based approach for understanding the relationship of changes in gene expression to toxicity, and for using this information to improve our predictions about human risk from endocrine-mediated toxicants.