Toxicogenomic Assessment of Endocrine Disruptors

Timothy Richard Zacharewski

Michigan State University

Thank you. Can I have the first slide please? Thank you. I will start with the second slide as well. Next slide.

With the development and the availability of global assessment technologies, there is really a new paradigm that is evolving within the toxicology community, and essentially that paradigm states that in order to fully assess the risk of chronic and subchronic exposure to synthetic as well as natural substances and their mixtures, a more comprehensive understanding of the physiological, cellular and molecular effects is required within the context of the whole organism, its genome, transcriptome, proteome, and metabolome.

Today, what I am going to talk about is our efforts in this area related to the transcriptome. Next please.

Currently, ongoing at our lab there are three projects that are involved in toxicogenomics or looking at the transcriptome. The first one is looking at the gestational and lactational exposure effects to endocrine disrupters on reproductive development and sperm quality. This is in collaboration with Karen Chou and Peter Saama.

The second one is the effect of synthetic and natural endocrine disrupters on *in vitro* human neural cell differentiation.

The last one is to establish gene expression profiles for endocrine disrupters and their mixtures using *in vitro* and *in vivo* models.

For the sake of time, what I am going to talk about today is more of what we are doing in the area of toxicogenomics using this first study as an example of the kinds of things that are ongoing and the infrastructure that is being developed in the lab in order to carry out this project.

The idea here really is two things. One is to determine whether endocrine disrupters — specific model compounds have an effect on reproductive development and sperm quality.

Two is to identify biomarkers that can be subsequently used to identify other compounds that may have effects on spermatogenesis, and then take those biomarkers and extrapolate those to other species, whether they are wildlife species: amphibians, reptiles, avian or aquatic species, and see whether similar responses are observed there. Next slide, please.

In terms of male endocrine disruption there are really three different issues that have brought this to public attention. The first one and probably the one that is getting the most attention is the decreasing sperm counts and semen volume. The second one is reproductive tract abnormalities, and that is also getting considerable attention and more attention as of late. And the third is the increased incidence of testicular cancer.

In each one of these cases, it is believed that exposure to endocrine disrupters during critical periods of development predisposes individuals to adverse health effects at later stages of life, primarily when they reach sexual maturity. Next slide, please.

The Sharpe-Skakkebaek hypothesis illustrates this, and there is actually a mechanistic basis for this support as well. It is illustrated in this slide, where we are looking at sperm and decreasing sperm counts and the reason for that.

Essentially, sperm and sperm counts are dictated by the number of Sertoli cells. The number of Sertoli cells is really regulated by FSH. FSH causes proliferation of those Sertoli cells. Sertoli cells act as

the nurse cells for sperm. So the number of Sertoli cells really dictates how much sperm is actually produced.

This FSH regulation is under negative feedback control from estrogens. So the hypothesis is that during developmental periods if you are exposed to estrogenic compounds or increased estrogenic burden as a result of exposure to endocrine disrupters, you are going to have an increased level of estrogens, you are going to have a higher level of negative feedback, less FSH secretion, fewer Sertoli cells, and as a result, as an adult male, you will have lower sperm counts.

With this project what we decided to do was to essentially try and test this hypothesis and identify potential markers of testicular gene expression that would be predictive of this kind of adverse effect. Next slide, please.

We looked at this using a comprehensive assessment strategy where we were examining these effects at four different levels of organism.

At the developmental level, we were looking at secondary sex characteristics: body weight, organ weight, and developmental landmarks like anogenital distance.

At the tissue level, we were examining the testis, and we were doing this at the histopathology level, and hopefully we will be able to return to this level by doing *in situ* hybridizations.

At the cellular level we were looking at spermatogenesis or sperm quality, and we were looking at sperm counts, motility, and a measure of quality through *in vitro* fertilization.

At the genome level we were examining the expression of genes in the mouse testis and we were doing this with microarray technology, a cDNA microarray that was developed in our lab, and then verifying those genes that look to be of interest using real time-PCR. Next slide, please.

The study design is outlined here. Three compounds were overproduced to be investigated: diethylstilbestrol, the compound that has been known to cause adverse effects in both male and female offspring to women that were prescribed that drug during pregnancy; genistein, the phytoestrogen found in soy; and ethynyl estradiol, the estrogenic component of the contraceptive pill.

For today's presentation, I am going to focus on diethylstilbestrol as the results that will be presented. The genistein results are just being completed now, and with the ethynyl estradiol we are still in the treatment phase.

The time period or the layouts for the experiments are illustrated in this timeline. Essentially we have C57BL6 and DBA/2 crosses. We needed to use that kind of a cross in order to ensure that well, it is the case that that model had been worked out the best for *in vitro* fertilization, which we consider to be our apical test in this study.

Treatment occurred through gestation and lactation, starting at gestational day 12 and stopping at postnatal day 21. At parturition we were measuring a variety of different parameters in the pups. At 21 we were looking at other ones, including anogenital distance, testis weight, histology, as well as gene expression. At 15 and 45 weeks we also included measures of sperm, which included sperm motility, sperm counts, and the *in vitro* fertilizing ability. Next slide, please.

This summarizes the results for the diethylstilbestrol. In terms of testis weight, you can see that there is a significant decrease at 3 weeks, 15 weeks and 45 weeks in those animals exposed to the high dose of diethylstilbestrol that was 10 μ g/kg. This is the dose that falls within the range that human exposures actually occurred.

We did not want to go to higher concentrations because at higher concentrations you start to get reproductive tract abnormalities which would confound the purpose of our study, which really was to look at sperm quality, not necessarily induce teratogenic events in those animals.

In addition, sperm counts again what you see is that there was a significant decrease at the 10 μ g/kg level and there does appear to be somewhat of a dose dependent decrease in sperm counts although it is only significant at the high dose. Next slide, please.

If we then examine sperm motility, there was no effect on sperm motility. There was a trend that was observed in the 15 week as well as the 45 week animals that was somewhat dose dependent, but again not statistically significant.

If you look at the apical test, this is the sperm fertilizing ability test, you see that there is a significant decrease at the high dose level. In this assay what was happening was that you would normalize sperm counts across all the animals and then incubate them with eggs from an individual female and then look for division.

What was interesting in this assay as well was that there was actually an increase in sperm quality at the lowest dose of diethylstilbestrol. What is interesting is that when we looked at the studies with genistein, which is a weak estrogen, at the high doses of genistein what we found was that there was also an increase in *in vitro* fertilizing ability from that sperm, suggesting that there might be an enhancement in activity as a result of low level exposures to estrogens. Next slide, please.

In order to take this into the toxicogenomics portion of the project, we started out using some commercial arrays. But we quickly found out that that was not going to be a viable mechanism in order to examine endpoints that we wanted to look out. The commercial arrays were not specific for the testis, and in general only contained about 5-10% of the genes expressed in the testis at any one time.

We then subsequently went on an ambitious plan to make our own cDNA microarray that was enriched for genes expressed in the testis. The array essentially involved genes from a variety of different areas and has been expanded now. It initially involved a 960 sequence of verified genes and most genes from the EPA Microarray Consortium through David Dicks. Approximately 300 of those are known to be expressed in the testis.

We then obtained approximately 1200 I.M.A.G.E. Consortium clones. From that we sequence verified those in-house and isolated specific plasmids from those, and we were able to obtain 1304 unique cDNAs and ESTs that were isolated and sequence verified. We also sort of what we call "cherry-picked" the kinds of things that we wanted to look at, and we were looking at selected genes. Here, we included 200 estrogen inducible genes, approximately 75 androgen inducible genes, and 400 genes containing dioxin responsive elements, and then a series of controls as well. Next slide, please.

This is Version 2 of our microarray. All spots represent a cDNA or EST. Each one is printed in duplicate on the array with labeling. Because we are working on the testis, we have sufficient RNA, so we are doing direct incorporation of both the Cy5 and Cy3 samples. The Cy5 was the treated animals and Cy3 was the control animals, and these are time-matched controls as well.

Image analysis actually occurred using the GenePix software, and then that software — the image that was obtained from that software — was essentially post-processed using what we call GP3, a script that was developed in our lab that does data correction and flagging, normalization and transformation as well as providing ratio and quality control reports.

Data analysis, or the results that come out of this GP3, then go into data analysis. There are two forms of the data analysis. The first form is the screening process: we are identifying those genes that are of interest that actually had some sort of affect as a result of treatment. Then we get into something more sophisticated to specifically examine trends or relationships among those genes with clustering.

Finally, all this information is going into dbZACH, which is a relational database for sample and gene tracking, data storage as well as retrieval. Next slide, please.

This slide illustrates more thoroughly the kind of effort we have been putting in, in terms of being able to track the vast amounts of data that this kind of project generates. Essentially, once you get your

image, the image as a TIFF is stored on file. This TIFF image is merged with another file that keeps track of all the clones, their location, and their identity.

This gets processed by GenePix, and the GenePix results into a GPR file. This goes back into the database, this goes back into the system in terms of analysis. The GenePix processing is then done in order to do the normalization and the standardization and the flagging.

Once we do that we go into the screening process. In the screening process that we are trying to do is, as I mentioned before, identify those genes that have undergone a significant change. After identifying those kinds of genes — and there are various ways of doing that — you can use cut-off values, you can use t-statistics, you can do Shannon Entropy, or ANOVAs.

For identifying relationships among those genes, it can be clustering, or principal component analysis, and some other measure that we are doing in collaboration with Chris Gennings, a statistician at the Virginia College of Medicine.

All of this information is being stored into dbZACH. dbZACH contains four subsystems: this represents one, the microarray subsystem. The other two are the gene subsystems, which contain all the information about the gene: its sequence, its function, and then the clone subsystem, which contains the sequence information and the unigene identification factors. Next slide, please.

These are some of the results we have obtained for the 15 week treatments. The three graphs represent controls, two controls and then a treatment effect. What you have is experimental variation, where you have control animal versus control animal. The spots that represent each one of the genes on the array fall pretty well along that line with a reasonable correlation of 0.97, indicating that there is good reproducibility in the assay that we are generating.

If we look at biological variation, we are looking at two different animals. Both of these animals were in the control group. Again, what you can see is that there is a little bit more scatter in this group, which would not be unexpected because of biological variation. Again, the correlation is still pretty good, so the assay and the variation between animals is not going to cause us too much grief.

If you look at a treatment-induced variation, where you are looking at Cy3 being the control and Cy5 being the treated animal, you can see that there is a significant — even visually — significantly greater amount of variation within the genes or their plot along that line, and that is reflected in that r value of 0.89.

This is really an exploratory approach to identify or determine whether the treatments that you performed actually had any kind of an effect on gene expression. Now what we want to do is we want to analyze those more thoroughly to identify those genes that actually did undergo a change as a result of gestational and lactational exposure to diethylstilbestrol. Next slide, please.

What we have done, as I mentioned before, we did paired t-test. I think this is 3 week, 15 week and 45 week animals. We used controls that were time matched in treatment animals as well. Solid lines here indicate changes in gene expression for animals that fall underneath the 0.05 p value, or at 95% confidence.

For 3 week animals that was approximately 641 genes. It split rather evenly in terms of the number of repressed as well as the number of induced genes.

For 15 week animals, the number of genes increased: it was about 1,200, again split rather equivalently, and then by 45 weeks — and remember that treatment actually ceased at 3 weeks of age — there still is a significant number of genes, 178 that were found to be changed. These are all raw values.

As a result of doing multiple comparisons from a single experiment, statistically you need to do some p value correction or adjustment. In this case, we use the Sidak step-down correction. What you find is that a lot of these genes drop out and are no longer significant in terms of being changed.

This is an extremely — I like to think of it as an extremely conservative — adjustment, but now you can see that 641 of those genes decreases to only one of the genes at 0.05 as being significant. There are 46 at 15 weeks, and actually at 45 weeks no genes were found to be statistically significant in terms of changes as a result of treatment. Next slide, please.

We used a combination of approaches in terms of trying to identify genes to determine whether they had any kind of predictive value or value as a biomarker. This involved using unadjusted values to derive this Venn diagram here, as well as the adjusted values to select a further subset from that, and then we even went one step further in terms of selecting genes for verification by determining if they had any known information regarding their role in spermatogenesis.

For the 3 weeks, 15 weeks, and 45 week data, there was an intersection of approximately 32 genes that exhibited a persistent change over that length of time. Several of those genes were down-regulated and/or up-regulated and had some role or some known response or function in terms of spermatogenesis. Next slide, please.

This is just one example of the RT-PCR verification; that has been ongoing. In this case we were looking at quantitation of estrogen receptor $-\alpha$ mRNA by RT-PCR in these groups. At the 3 week value there is a significant inhibition of estrogen receptor $-\alpha$ mRNA as a result of exposure to all doses of diethylstilbestrol. At 15 weeks and 45 weeks, there was no detectable measure of mRNA. Next slide, please.

In summary, what we have identified or been able to determine at this point in time is that developmental exposure to 10 μ g/kg of DES decreased testis weight, epididymal sperm counts, and *in vitro* sperm fertilizing ability in the absence of any histological lesions. I never talked about the histology there, but the testes looked normal. Second, DES compromised sperm fertilizing ability and it persisted from 15 to 45 weeks. So there seem to be a persistent effect, or grandfathered effect, in terms of on gene expression that resulted in a compromise in sperm fertilizing ability. Next slide, please. Correlation analysis confirmed that treatment increased the variability of gene expression above the level of biological variation seen within the control animals. We have been able to take that a step further, in that t-statistics and adjusted p values identified significant changes in gene expression.

I can actually summarize to some extent, because we have been able to further look at this data prior to presenting this material, that there seems to be that gestational and lactational exposure to DES causes the animals to be refractory to estrogen, which causes an inability to respond to endogenous estrogens within the system, which has compromised their sperm quality. Next slide, please.

Gogh Lab. Next slide, please.

My collaborators. I am happy to answer any questions. Thank you.

Q&A

Inoue: Thank you very much, Dr. Timothy Zacharewski. If you have any questions, we have time to accept questions and comments, please.

Daston: Tim, very interesting work. There are some advantages and disadvantages from following animals out so far from the actual insult. One of the questions that was whether the gene expression changes are through the primary effect of the DES treatment or some sort of long term response. Have you thought about? For example have you analyzed the gene sequences to see if whether there is estrogen response elements in the genes that are persistently changed?

Zacharewski: We have thought about that. It was never the intention trying to identify those genes that were a primary response to diethylstilbestrol or any kind of estrogenic response. It was more in terms of seeing what was going to be perturbed and whether that perturbation was actually going to be persistent that could subsequently be used as a biomarker in the adult animals.

So we were really looking at developmental exposures and trying to predict that exposure later on in life after exposure had ceased. To answer the second part of it, no we have not done any kind of promoter analysis to investigate whether there is estrogen response element in any of those promoters.

Inoue: Any other questions? We encourage you. No? OK, thank you very much, Dr. Zacharewski.