Response to NICEATM Request for Data and Information in Technologies Used for Identifying Potential Developmental Toxicants

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Toxicogenomics-Based Assay to Measure Chemical Effects on the Stem Cell Epigenome

Background:

Epigenetic abnormalities, potentially occurring during embryogenesis, puberty, or adulthood, have been implicated in embryo lethality, diverse developmental abnormalities such as Rubinstein-Taybi syndrome and Fetal Alcohol Spectrum Disorder, cancer, neurodegenerative diseases including Huntington's Disease, Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis, cardiac hypertrophy, asthma and inflammation, as well as memory impairment and cognitive dysfunction. Aberrant epigenetic function has also been linked to several psychiatric disorders, including schizophrenia, depression, and drug addiction. Epigenetic drugs exist at various stages of development for the treatment of cancer, psychiatric disorders, obesity, and other complex diseases. Many natural dietary polyphenols have been shown to modulate the epigenetic machinery; thus, synthetic modification of these polyphenols to increase potency and/or target selectivity as potential chemopreventive/chemotherapeutic agents is an active area of current research in the pharmaceutical arena.

The stem cell epigenome is responsible, at least in part, for the maintenance of pluripotency and differentiation processes. Disruption of this complex regulatory circuit by exposure to environmental agents and/or pharmaceuticals during embryogenesis can lead to spontaneous abortions and birth defects, as well as adverse health and cognitive effects later in life. Indeed, several known HDAC inhibitors induce embryo lethality and/or congenital malformations associated with hyperacetylation of histones in target organs. Gene deregulation, oxidative stress, DNA demethylation, and/or retinoic acid imbalance are some modes of action postulated for teratogenesis induced by HDAC inhibitors, some of which are in clinical use.

Chromatin in pluripotent stem cells is characterized by several unique properties. Compared to differentiated cells, embryonic stem (ES) cells have highly dispersed euchromatic nuclei, elevated and reduced amounts of histone modifications typically associated with open chromatin (e.g. acetylated histone H3 and H4) and heterochromatin (e.g., H3K9me3), respectively, and

loosely binding "hyperdynamic" architectural chromatin proteins. Notably, many silenced genes having roles in embryonic development and lineage specification carry acetylation and methylation marks normally associated with active transcription (i.e., H3K9ac, H3K4me3) juxtaposed with a repressive methylation mark (i.e., H3K27me3). During differentiation, these bivalent chromatin marks are typically resolved, correlating with transcriptional activation of tissue-specific genes and concomitant silencing of gene loci associated with alternative developmental pathways. The importance of the stem cell epigenome to maintaining pluripotency and regulating cell differentiation, and the demonstrated susceptibility to chemical modulation, makes human embryonic stem (hES) cells an ideal *in vitro* model system for testing chemicals for epigenotoxic potential, and are especially relevant as a model for developmental toxicity.

Overview of Work in Progress:

A biomarker panel capable of detecting chemicals with potential to alter the epigenome ("epigenotoxicants") at genes important for normal development will aid hazard identification. We are working to develop a medium throughput assay platform to detect chemicals that perturb histone marks at a panel of developmentally-relevant genes in hES cells. A comprehensive toxicogenomics evaluation of gene expression changes is being performed using a large (~60) set of chemicals representing various classes of chemicals known to affect enzymes that modulate histone acetylation and methylation, including some known teratogens. To derive signatures of gene expression changes reflecting exposure of hES cells to various classes of epigenotoxicants, RNA-Seq is being used to profile transcripts from WA09 (H9) hES cells exposed to ~$IC_{0\text{-}10}$ and ~$IC_{30\text{-}50}$ concentrations of chemical inhibitors/activators of histone methylation or acetylation for 24 hours.

Interestingly, using a published consensus set of genes in hES cells with bivalent methylation marks, we found that the most robust transcriptional changes (number of genes affected and magnitude of response) in bivalent genes were exhibited in response to histone deacetylase inhibitors (HDACi) rather than modulators of histone methylation (or histone acetyltransferase inhibitors); in some cases, >50% of bivalent genes were deregulated.

A variety of bioinformatic strategies are being used to derive signatures of epigenotoxicant exposure, including unsupervised and supervised learning bi-cluster, Nearest Shrunken Centroid, self-organizing map, and Principal Component Analysis approaches, as well as Weighted Correlation Network Analysis (WGCNA). There exist quite a few challenges for developing assays to test for epigenotoxicity. The major challenges we encountered for epigenotoxicant signature development were as follows:

1) making predictions for chemicals purported to affect multiple epigenetic targets/mechanisms to assess the accuracy of a gene expression signature for a specific epigenetic activity
2) cross-talk between epigenetic mechanisms
3) lack of bona fide negative control chemicals
4) discrepancies regarding chemical effects reported in the literature
5) apparent differences between chemical effects reported for immortalized cell lines versus our observations using normal hES cells

6) potential confounding effects of cytotoxicity

Despite these challenges, preliminary bioinformatic signatures generated for HDACi chemicals look particularly promising. To assist in interpretation of our HDACi signature development results, we are testing 5-6 concentrations of all known HDACi, as well as putative negative control (non-epigenetic) chemicals, in our training/validation set of chemicals for inhibitory activity in an HDAC assay performed directly in H9 cells following 4 hours of chemical exposure. Using HDAC assay results for 24 chemicals tested to date, our preliminary independently-derived HDACi signatures correctly distinguished 21-23 (88-96% accuracy) of those HDACi that exhibited inhibitory activity in H9 cells from those drugs and naturally-derived (e.g., botanical) HDACi and putative negative control chemicals that did not exhibit inhibitory activity in H9 cells. Notably, those HDACi active in the HDAC assay are largely known teratogens whereas the inactive HDACi are mostly non-teratogenic. The preliminary signatures also correctly identified 16/17 (94% accuracy) of the chemicals that target other epigenetic mechanisms as non-HDACi. Using pathway analyses and other bioinformatic approaches, the HDACi gene signature(s) will be refined to include genes that provide greatest contribution to predictivity and reflect pathways with greatest relevance to development. We are similarly working to derive a gene expression signature indicative of inhibitors of histone acetyltransferase (HAT), histone methyltransferase (HMT), and histone demethylase (HDMT).

Although our original goal was to develop a medium throughput (96-well) assay based on chromatin immunoprecipitation to monitor chemical-induced changes at gene promoters of developmentally relevant genes, the relatively large number of genes in our preliminary gene signatures to date suggest that an RNA-based approach will be more feasible. Our plan is to use the Temp-O-Seq (BioSpyder) platform to screen chemicals for epigenotoxicity using a panel of genes reflecting a consensus or combination of gene expression signatures. One major advantage of the Temp-O-Seq platform is the ability to go directly from cells to RNA sequencing without the need for RNA isolation. Temp-O-Seq flow cells can be customized to accommodate the gene panel used for chemical screening. The final assay platform is intended for rapid and efficient early screening of effects of environmental toxicants and drugs on the human epigenome that could lead to developmental defects and/or predispose an individual to disease or cognitive disorders.

[Note: This work has not yet been published in the peer-reviewed literature. Additional details are available upon request. RNA-Seq files will be made publicly available following the conclusion of this project. RNAs were prepared for multiple doses for most chemicals; after the completion of this project, these can be made available for dose response analysis, etc. for chemicals of interest to NTP/NICEATM.]