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Stage-specific signaling pathways during murine testis development and spermatogenesis: A pathway-based analysis to quantify developmental dynamics

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ABSTRACT

Shifting the field of developmental toxicology toward evaluation of pathway perturbation requires a quantitative definition of normal developmental dynamics. This project examined a publicly available dataset to quantify pathway dynamics during testicular development and spermatogenesis and anchor toxicant-perturbed pathways within the context of normal development. Genes significantly changed throughout testis development in mice were clustered by their direction of change using *K*-means clustering. Gene Ontology terms enriched among each cluster were identified using MAPPfinder. Temporal pathway dynamics of enriched terms were quantified based on average expression intensity for all genes associated with a given term. This analysis captured processes that drive development, including the peak in steroidogenesis known to occur around gestational day 16.5 and the increase in meiosis and spermatogenesis-related pathways during the first wave of spermatogenesis. Our analysis quantifies dynamics of pathways vulnerable to toxicants and provides a framework for quantifying perturbation of these pathways.

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1. Introduction

Increasing rates of reproductive disorders that have origins in early reproductive development demonstrate the need for methods to characterize and quantify perturbations of developmental processes in gonadal development and spermatogenesis. For example, there is mounting evidence for a consistent decline in semen quality in recent decades, accompanied by an increasing prevalence of male reproductive disorders, including hypospadias, undescended testes, and testicular cancer [1–6]. All of these adverse reproductive health outcomes are manifestations of testicular dysgenesis syndrome (TDS), a set of conditions believed to have common origins during early gonadal development [7]. Early testicular development and spermatogenesis are very sensitive processes that depend on a series of precisely timed steps regulated by hormonal cues and germ cell microenvironments [8,9]. These processes in male reproductive development are therefore particularly vulnerable to perturbation by genetic and environmental factors [10,11]. Indeed, the recent increase in TDS related conditions has been hypothesized to be a result of environmental factors that can influence early male reproductive development, such as exposure to endocrine disrupting chemicals [7].

Exploration of the complex interaction of environmental and genetic factors underlying reproductive disorders requires a systems-based framework for characterizing normal and perturbed pathway dynamics during critical windows of male reproductive development. The field of toxicology is increasingly shifting toward characterization of pathway perturbation as a sensitive indicator of toxicity [12]. A quantitative framework for measuring shifts from normal pathway dynamics would facilitate quantification of pathway perturbation by toxicants. Furthermore, incorporation of *in vitro* models into chemical screening, underscores the need to anchor pathway dynamics captured in these *in vitro* models to pathway dynamics driving *in vivo* development. The first step in being able place pathway perturbation measured *in vivo* and *in vitro* within the context of normal development is to define normal pathway dynamics *in vivo* in an easily translatable, quantitative framework.

Fortunately, much of the data needed to provide this baseline characterization of normal developmental dynamics is available in publicly available datasets. Microarray-based high-throughput

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gene expression analysis has proven to be an effective method for studying the changes in gene expression associated with the growth and development of mammalian tissues [13,14]. Many of the genetic drivers of male reproductive development have been characterized through mouse knockout models [15,16] and global gene expression analysis [17–21]. The Griswold lab at Washington State University has employed microarray-based gene expression analysis to characterize dynamic changes in global gene expression patterns over the course of several particularly sensitive processes of male reproductive development in mice [17,18]. The group identified genes with changing expression patterns throughout gonadal differentiation and development and the first wave of spermatogenesis [17,18]. The gene expression profiles observed by Small et al. reiterated the known functional activities of each cell type, and suggested the involvement of novel genes in the maturation of the testis and differentiation of germ cells. Their temporal microarray study provides a valuable resource for evaluating biological factors that influence testis maturation and spermatogenesis. However, as with all microarray data, the functional interpretation of such a vast set of genomic data presents a major challenge. In order to elucidate the biological consequences of these expression changes in single genes, gene expression data must be integrated with quantitative information on functional changes in whole gene networks and developmental signaling pathways over time.

Gene ontology (GO) analysis is a powerful tool for translating a vast amount of genomic data into a description of functional changes in gene networks and signaling pathways. The GO approach has been successfully combined with pathway analysis to generate an unbiased determination of the statistical significance of changes observed in pathways of interest [22–25]. For example, previous GO analysis of testicular gene expression has successfully identified pathways that are significantly changed throughout murine spermatogenesis [26]. However, standard GO analysis results in a list of enriched pathways with no quantitative description of how these pathways are changed. In addition these approaches did not retain quantitative information on the expression of individual genes and are limited to the evaluation of only two experimental dimensions.

In order to address the need to quantify changes in pathway dynamics through time or in response to an environmental exposure, our lab developed the GO-Quant approach [27]. GO-Quant incorporates gene expression data with Gene Ontology analysis in MAPPfinder [22] to calculate the average intensity of expression of all significantly altered genes associated with a given GO term. This allows the quantitative evaluation of the dynamics of entire gene pathways along a third dimension, such as developmental stage or toxicant dose. We first applied this quantitative pathway-based approach in a published dose- and time-dependent genomic dataset [28] and found that our systematic approach quantitatively described the degree to which functional gene systems changed across dose or time course [27,29]. We have subsequently used our quantitative pathway analysis for a genome-wide assessment of phthalate toxicity in an *in vitro* rat testis co-culture model [30] and for an assessment of time- and dose-dependent methylmercury toxicity in developing mouse embryos undergoing neurulation [31].

In the current study, we applied our established quantitative pathway-based approach to a publicly available dataset of murine male reproductive development [18] to quantify the dynamic functional changes in biological processes that characterize normal testicular development and the first wave of spermatogenesis *in vivo*. Through this analysis we demonstrate that our approach can quantitatively illustrate pathway dynamics throughout a complex developmental process *in vivo*, successfully capturing well characterized developmental milestones. The result provides a framework for quantifying perturbation of normal developmental pathways *in vivo* as well as anchoring emerging *in vitro* models of male reproductive development to *in vivo* pathway dynamics.

2. Materials and methods

2.1. Gene expression data set

For this analysis we obtained publically available temporal mouse genomic data during early testis development (gestational days (GD) 11.5, 12.5, 14.5, 16.5, and 18.5) and the first wave of spermatogenesis (postnatal days (PND) 0, 3, 6, 8, 10, 14, 18, 20, 30, 35, and 56). Gene expression intensity in testicular tissue at each timepoint was quantified using Affymetrix MGU74Av2, Bv2, and Cv2 arrays. Detailed methods of sample collection and microarray processing are available in the original papers [17,18]. NCBI's gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) was used to retrieve the raw dataset.

2.2. Identification and clustering of significantly changed genes

Microarray analysis was conducted based on our established quantitative pathway-based approach as shown in Fig. 1 [27]. Significantly changed probes were identified using BRB ArrayTools, developed by Dr. Richard Simon and the BRB ArrayTools Development Team. Data were normalized by gcRMA normalization and log2 transformed. In order to identify significantly changed probes, we conducted an ANOVA class comparison across timepoints. Genes that were significantly altered ($p \leq 0.001$) across time were selected and *K*-means cluster analysis was used to group genes based on the similarity of their patterns of mean expression though time. Since there are generally two directions of gene expression changes at a certain time (either up-regulation or down-regulation within a specific gene category), the average of these two different directions of gene expression alteration would mask the degree of absolute change in a pathway. For pathway analysis, we therefore separated significantly changed genes into two groups with patterns of expression tending toward consistent up- or down-regulation across time based on *K*-means cluster analysis [32].

2.3. Identification of enriched GO terms

We applied MAPPfinder to identify enriched Gene Ontology (GO) terms at $p \leq 0.001$ [33] in up- and down-regulated probes at each timepoint. Enriched GO terms were ranked by *Z*-score and permutation *p*-value [33]. As previously described, the *Z*-score, a statistical measure of significance for gene expression in a given group, was calculated by subtracting the number of genes expected to be randomly changed in a GO term from the observed number of changed genes in that GO term. This value was then divided by the standard deviation of the observed number of genes under a hypergeometric distribution. The equation is written out as

$$Z\,\text{score} = (r - n*(R/N))/((n*(R/N)) \times (1-(R/N))(1-((n-1)/(N-1)))^{1/2} \quad (1)$$

where N is the total number of genes measured, R is the total number of genes meeting the criterion that the gene be significantly changed based on an F test at significant $p \leq 0.001$ value, n is the total number of genes in each specific GO term, and r is the number of genes meeting the criterion in this specific GO term. Complete lists of GO terms enriched among significantly up- or down-regulated genes are available as supplemental data (Supplemental Tables 1 and 2).

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