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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.

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).

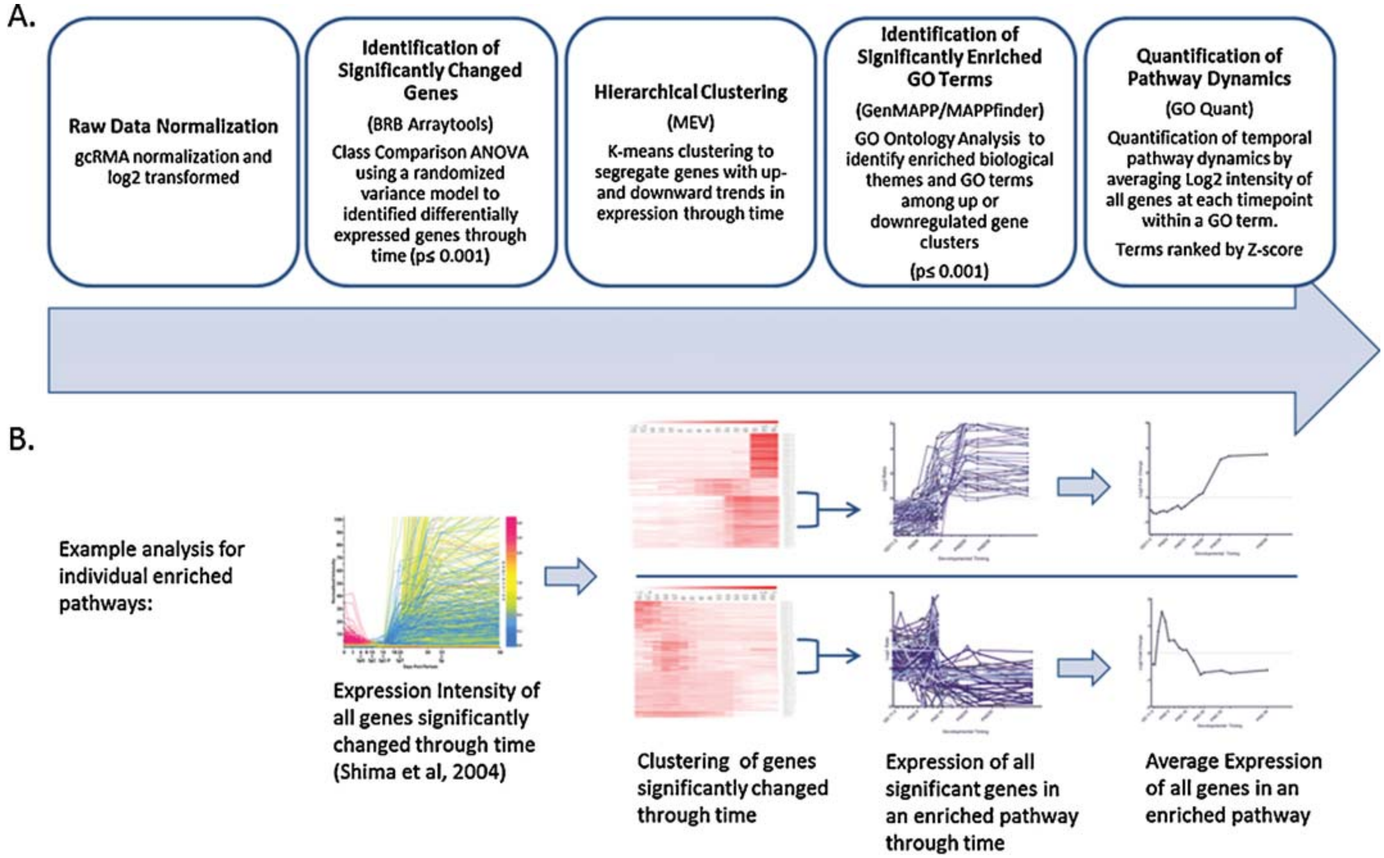

Fig. 1. Microarray analysis pipeline for quantification of pathway dynamics. (A) Summary of analysis pipeline. (B) Illustration of data analysis process. Starting with single gene expression in testis through time (Shima et al., 2004) we normalized data and identified significantly changed genes using BRB Arraytools software, clustered genes with significantly increasing or decreasing expression through time using Multiple Experiment Viewer software (MEV), then used GO Quant software to identify GO terms enriched among these clusters and average the expression of all significantly changed genes in that pathway to produce a quantitative summary of gene expression dynamics in each GO term through time.

2.4. Quantification of pathway dynamics throughout development

To quantify the dynamic changes in GO terms through time, we used GO-Quant to link enriched GO terms to the expression values of all the significantly up- or down-regulated genes contained within the pathway. By incorporating gene expression data into the pathway analysis, we could compute the average intensity of expression among genes in each enriched GO term at each timepoint. These values at individual timepoints were then normalized to 'average' expression across time by subtracting the average expression intensity of genes in the GO term across all the timepoints. This yielded a log2 ratio of mean expression at each timepoint relative to the average mean expression across all timepoints. Plotting the log2 ratio for each GO term through time illustrates temporal pathway dynamics throughout development. GO terms included in the figures and tables presented here are the ten biological processes with the highest *Z*-scores that are relevant to each of the dominant categories selected.

3. Results

Our quantitative pathway analysis (summarized in Fig. 1A) translates a vast amount of information on expression of single genes into a quantitative summary of temporal changes in activity across entire gene networks and biological processes described by GO terms (Fig. 1B). Of the approximately 36,000 probes on the array, 9599 probes were significantly changed through time. Significant genes were clustered into two groups based on their general expression trends using *K*-means clustering. This resulted in segregation of significantly changed genes into two sets: one set with overall upward trends in expression and another set with overall downward trends in expression. We then used MAPPFinder to identify GO terms describing biological processes that were enriched among each of these two gene clusters. We found 308 GO terms enriched among genes with downwards trends in expression and 112 GO terms enriched among genes with upward trends in expression. For each of these enriched GO terms, GO-Quant facilitated generation of a quantitative temporal summary of average gene expression dynamics at each timepoint among genes significantly changed in the pathway through time.

3.1. GO terms significantly enriched among genes with upward trends in expression

The GO terms most significantly over represented among genes with increased expression are dominated by terms associated with spermatogenesis and meiosis (Table 1). Indeed, 12 of the top 20 (60%) of significantly enriched GO terms ranked by *Z*-score are directly relevant to spermatogenesis or meiosis (Supplemental Table 1). Quantitative pathway analysis reveals that average gene expression in GO terms related to meiosis (Fig. 2B) begins increasing around PND 3, slightly preceding the spermatogenesis and spermatid maturation signal. We see a dramatic increase in the average expression of genes in GO terms relating to spermatogenesis and spermatid development throughout testis development, capturing gene expression dynamics that are closely aligned in

Table 1
GO terms enriched among genes with significantly increased expression through time.

GOID	GO name	# Genes in GO	% of present genes changed	% of genes present on array	*Z*-score	Parametric *p*-value
Spermatogenesis and sperm maturation						
48232	Male gamete generation	194	53.3	70.6	11.935	4.0E−05
7283	Spermatogenesis	194	53.3	70.6	11.935	4.0E−05
7276	Gamete generation	278	41.8	66.2	9.601	5.1E−05
30317	Sperm motility	15	75	106.7	6.427	3.7E−05
48515	Spermatid differentiation	36	56.2	88.9	6.202	7.7E−05
7286	Spermatid development	34	56.7	88.2	6.067	3.3E−05
1539	Ciliary or flagellar motility	6	100	66.7	4.574	1.7E−06
48240	Sperm capacitation	5	100	60	3.961	3.3E−05
Meiotic cell cycle						
7127	Meiosis I	32	63.2	59.4	5.597	1.9E−04
51327	M phase of meiotic cell cycle	76	37.7	69.7	4.309	1.5E−04
7126	Meiosis	76	37.7	69.7	4.309	1.5E−04
51321	Meiotic cell cycle	77	37	70.1	4.21	1.5E−04
7131	Meiotic recombination	17	75	23.5	3.212	6.4E−06
7128	Meiotic prophase I	4	60	125	2.677	1.0E−07
7130	Synaptonemal complex assembly	6	60	83.3	2.677	1.1E−04
Ubiquitin mediated processes						
6512	Ubiquitin cycle	433	25.3	83.1	4.837	7.7E−05
6511	Ubiquitin-dependent protein catabolic process	165	28.9	69.1	3.767	6.9E−05
19941	Modification-dependent protein catabolic process	167	28.4	69.5	3.654	6.9E−05
43632	Modification-dependent macromolecule catabolic process	167	28.4	69.5	3.654	6.9E−05
6464	Protein modification process	1609	19.5	78.3	3.535	8.7E−05
Epigenetic regulation						
43687	Post-translational protein modification	1375	19.1	79.1	2.895	8.5E−05
6338	Chromatin remodeling	52	28.9	73.1	2.169	8.6E−05
31497	Chromatin assembly	142	25.4	44.4	2.026	3.6E−05
6333	Chromatin assembly or disassembly	180	23.6	49.4	1.945	7.0E−05

time with known developmental outcomes (Fig. 2A). Average expression of genes involved in a host of GO terms relating to spermatid development and differentiation begin a gradual increase on PND 10 with the emergence of spermatocytes, increase around PND 20 when spermatids appear, and remain high through PND 56 as the first wave of spermatogenesis is completed and spermatozoa are produced. Simultaneously, average expression in GO terms associated with a panel of pathways related to ubiquitin-mediated processes (Fig. 2C) and epigenetic processes (Fig. 2D) increases in parallel, with over a quarter of the genes associated with chromatin remodeling and ubiquitin cycle changing significantly through spermatogenesis.

3.2. *GO terms significantly enriched among genes with downward trends in expression*

The dominant themes in GO terms enriched among genes with decreasing expression through time include developmental processes, metabolic processes, developmental signaling pathways, mitosis, and steroid regulation (Table 2). Quantitative pathway analysis illustrates dynamic changes in GO terms related to steroid regulation throughout testis development. For example, average gene expression in sterol metabolic and biosynthetic processes peak at GD 16.5, decrease before birth, and decrease further after spermatid development commences (Fig. 3A). Several other catabolic and metabolic pathways, including lipid metabolism, follow a similar pattern of expression (Fig. 3B).

Our analysis also reveals dynamic changes of signal transduction pathways throughout spermatogenesis (Fig. 3C). Average gene expression of many developmental signaling pathways, including BMP, Notch, and MAPK signaling, are elevated toward the end of gestation and during the earliest stages of spermatogenesis, peaking between GD 16.5 and PND 3. Average expression in these developmental signaling pathways decreases dramatically between PND 14 and PND 18, as testicular formation and development give way to functional adult tissue.

GO terms for developmental processes (Fig. 3D) and mitosis (Fig. 3E) display a similar pattern of dramatic upregulation during peak testicular development, dropping off as testes mature and spermatogenesis progresses. For example, pathways involved in cell growth and differentiation, mitosis, and cellular morphogenesis and development, are generally elevated during gestation and the first week of life. Then, around PND 8, expression of genes in these pathways begins a gradual decrease. This decrease corresponds to the end stages of testis development and maturation and the beginning of spermatogenesis.

4. Discussion

Our systematic pathway-based approach distils complex genomic data into a quantitative description of pathway dynamics over the course of gonadal development and spermatogenesis. This analysis successfully captures well characterized developmental processes, offering a quantitative framework for assessing normal temporal dynamics of reproductive development and measuring any perturbation of these dynamics that could lead to pathology. Finally, by defining *in vivo* pathway dynamics, this analysis facilitates evaluation of the ability of emerging *in vitro* systems to capture important developmental pathways.

4.1. *Pathway analysis captures dynamics of key events known to drive testicular development and spermatogenesis*

The trends highlighted by our analysis are consistent with well characterized developmental processes described in the literature. For example, the notable peak in expression of pathways related to steroidogenesis and hormonal regulation observed in our analysis at GD 16.5 corresponds to the well characterized peak in testosterone known to drive male reproductive development [16,34]. Furthermore, in this dataset, specific genes that are widely recognized in the literature for their roles in steroidogenesis, including, star, 3β-HSD, and C/EBPβ [35,36] follow temporal expression patterns that are consistent with the average expression patterns

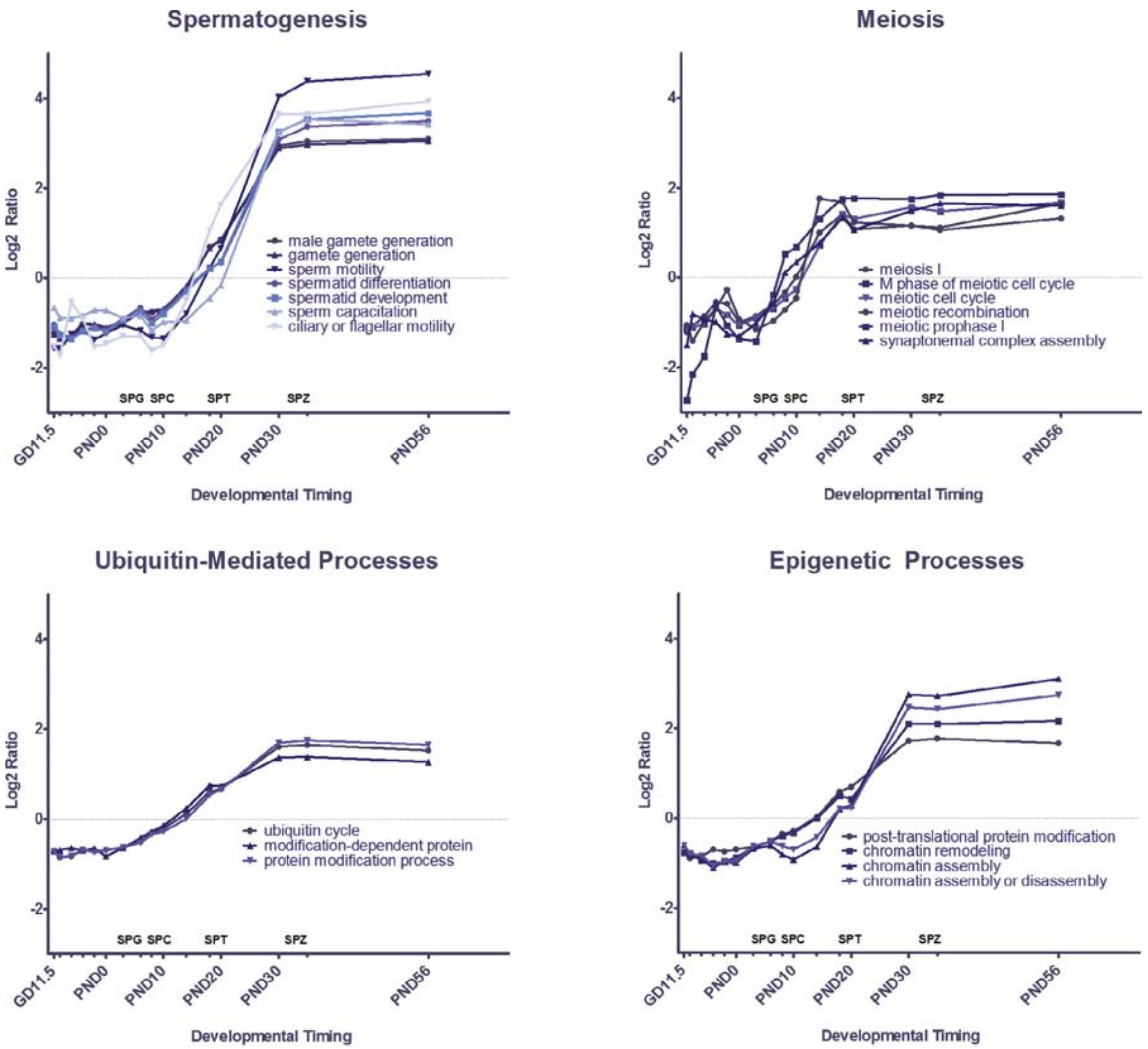

Fig. 2. Quantified pathway dynamics of GO terms that significantly increase through time. GO terms enriched among genes with significantly increasing expression ($p < 0.001$) through time were identified through MAPPfinder. Significantly enriched GO terms ($p < 0.001$) were ranked by Z-score and dominant themes among these terms were identified as (A) spermatogenesis, (B) meiosis, (C) ubiquitin mediated processes, and (D) epigenetic processes. Dynamic change in GO terms related to each dominant theme are plotted here as the ratio of average Log2 intensity of all significantly changed genes at each timepoint in a GO term over Average Log2 intensity across all timepoints for that GO term. Corresponding stages of spermatogenesis, including spermatogonia (SPG), spermatocyte (SPC), spermatid (SPT), and spermatozoa (SPZ) are indicated along the *X* axis.

reflected in pathway analysis. The significant increase in steroidogenesis during this phase has previously been identified as a critical initiator of testis development and masculinization of the fetus [16,34]. Expression of a host of genes in the mouse reproductive tract has been shown to be modulated by estrogen and testosterone [37].

The dramatic proliferation of somatic cells that occurs early in testis development plays a role in promoting the male fate by increasing the number of SRY-producing cells and marks a key difference between male and female development [38]. Accordingly, in this analysis, we see that pathways that promote proliferation and mitosis are expressed in developing testes and then downregulated as functionally mature testes begin spermatogenesis. Conversely, meiosis in the testis does not begin until around PND5, with the initiation of the first wave of spermatogenesis [39]. This is clearly illustrated at the pathway level in our analysis. In addition, several meiosis-specific genes described in the literature, such as the family of synaptonemal complex proteins [40] follow expression trends consistent with overall pathway trends.

The onset of meiosis in this analysis is followed by a dramatic increase in expression of a host of specific genes known to play an important role in spermatogenesis. For example, by PND 30 there is a sharp increase in gene expression of protamines 1 and 2 and the transition proteins that facilitate the switch between histones and sperm-specific protamines [40]. General expression dynamics of GO terms related to spermatogenesis are consistent with expression dynamics for these genes with well-characterized roles in spermatogenesis.

During spermatogenesis, chromatin restructuring facilitates sperm compaction and regulation of DNA methylation [41,42]. Precise regulation of these epigenetic processes is essential for sperm development as well as for appropriate expression of the paternal genome in the developing embryo [42]. The ubiquitin system plays an important role in performing the histone modifications that underlie this chromatin restructuring [43]. The prominent increase in expression of genes associated with ubiquitin mediated processes and epigenetic processes that is observed concurrently with spermatogenesis in our analysis illustrates the temporal dynamics of these well-described regulatory processes.

Our analysis also successfully captures the important role of signal transduction pathways in regulating development. At least 17 highly conserved signaling pathways are now recognized for their

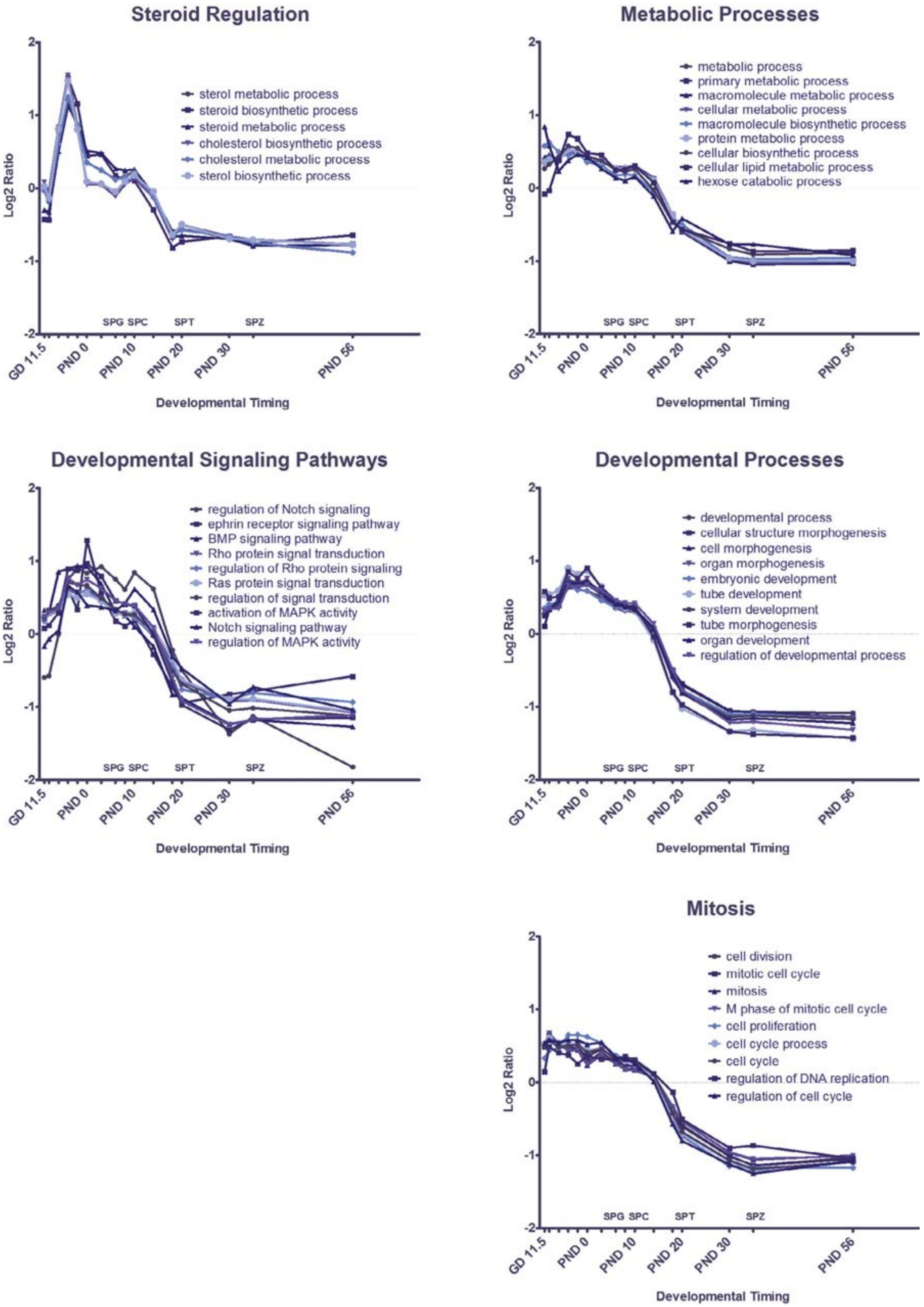

Fig. 3. Quantified pathway dynamics of GO terms that significantly decrease through time. GO terms enriched among genes with significantly decreasing expression ($p < 0.001$) through time were identified through MAPPfinder. Significantly enriched GO terms ($p < 0.001$) were ranked by Z-score and dominant themes among these terms were identified as (A) steroid regulation, (B) cellular metabolism, (C) developmental signaling pathways, (D) developmental processes, and (E) mitosis. Dynamic change in GO terms related to each dominant theme are plotted here as the ratio of average Log2 intensity of expression of all significantly changed genes at each timepoint in a GO term over Average Log2 intensity across all timepoints for that GO term. Corresponding stages of spermatogenesis, including spermatogonia (SPG), spermatocyte (SPC), spermatid (SPT), and spermatozoa (SPZ) are indicated along the *X* axis.

Table 2
GO terms enriched among genes with significantly decreased expression through time.

GOID	GO name	# Genes in GO	% of present genes changed	% of genes present on array	*Z*-score	Parametric *p*-value
Steroid regulation						
16125	Sterol metabolic process	73	44.1	80.8	2.997	7.3E−05
6694	Steroid biosynthetic process	75	46.3	72	3.237	5.7E−05
6695	Cholesterol biosynthetic process	25	55	80	2.846	4.1E−05
8203	Cholesterol metabolic process	67	43.4	79.1	2.729	6.0E−05
16126	Sterol biosynthetic process	31	50	83.9	2.67	7.4E−05
8202	Steroid metabolic process	145	36.5	79.3	2.358	9.8E−05
Cellular metabolism						
8152	Metabolic process	8113	28.8	76.1	4.835	1.3E−04
44238	Primary metabolic process	7338	29	75.1	4.83	1.3E−04
43170	Macromolecule metabolic process	6411	28.8	74.7	3.91	1.3E−04
44237	Cellular metabolic process	7311	28.9	75.4	4.651	1.3E−04
9059	Macromolecule biosynthetic process	899	34.8	65.5	4.476	1.5E−04
19538	Protein metabolic process	3404	29.4	74	3.263	1.3E−04
44249	Cellular biosynthetic process	646	33.1	74.9	3.156	1.4E−04
44255	Cellular lipid metabolic process	542	33.2	81.2	3.063	1.4E−04
19320	Hexose catabolic process	91	44.2	57.1	2.839	1.3E−04
46365	Monosaccharide catabolic process	91	44.2	57.1	2.839	1.3E−04
Developmental processes						
32502	Developmental process	3292	31.8	75.6	6.268	1.2E−04
32989	Cellular structure morphogenesis	485	39.4	77.9	5.61	7.9E−05
902	Cell morphogenesis	485	39.4	77.9	5.61	7.9E−05
9887	Organ morphogenesis	457	39.2	82.1	5.49	8.6E−05
9790	Embryonic development	362	39.2	94.5	5.229	6.1E−05
35295	Tube development	128	46.9	100	5.146	5.3E−05
48731	System development	1704	32.5	74.9	4.824	1.0E−04
35239	Tube morphogenesis	83	48.9	106	4.683	6.2E−05
48513	Organ development	1345	32.2	76.4	4.077	1.0E−04
50793	Regulation of developmental process	256	38.8	85.5	4.039	7.9E−05
Developmental signaling pathways						
8593	Regulation of Notch signaling pathway	5	100	80	3.304	2.0E−05
48013	Ephrin receptor signaling pathway	3	100	133.3	3.304	4.2E−05
30509	BMP signaling pathway	26	57.1	80.8	3.139	1.5E−04
7266	Rho protein signal transduction	102	41.5	80.4	3.002	1.6E−04
43405	Regulation of MAPK activity	77	44.4	70.1	2.929	9.7E−05
35023	Regulation of Rho protein signal transduction	72	42.2	88.9	2.781	1.8E−04
7265	Ras protein signal transduction	172	37.4	71.5	2.66	1.7E−04
9966	Regulation of signal transduction	411	32.1	78.8	2.171	1.6E−04
187	Activation of MAPK activity	48	43.8	66.7	2.164	9.9E−05
7219	Notch signaling pathway	51	40.9	86.3	2.113	5.6E−05
Mitotic cell cycle						
51301	Cell division	218	42.9	90.8	5.155	1.7E−04
278	Mitotic cell cycle	249	39.6	81.1	4.133	1.3E−04
7067	Mitosis	168	41.7	85.7	4.043	1.4E−04
87	M phase of mitotic cell cycle	170	41.5	86.5	4.039	1.4E−04
8283	Cell proliferation	648	35.3	63	3.925	9.8E−05
22402	Cell cycle process	646	34.9	69.2	3.923	1.4E−04
7049	Cell cycle	762	33.7	74.4	3.773	1.3E−04
6275	Regulation of DNA replication	19	70	52.6	3.083	1.5E−04
51726	Regulation of cell cycle	461	33.5	60.3	2.523	1.3E−04
74	Regulation of progression through cell cycle	459	33.5	59.9	2.509	1.3E−04

central role in guiding developmental processes [12,44]. Many of these signaling pathways are specifically implicated in guiding the processes of gonadal differentiation and testicular development [45] and germ cell differentiation [46]. In the current analysis, we capture the increased expression of genes involved in these signal transduction pathways throughout gestation and testicular maturation. These signaling pathways are then downregulated as testis tissue achieves maturation and begins the process of meiosis and spermatogenesis.

4.2. Pathway analysis can facilitate discovery of new roles for pathways dynamically changed in testicular development

In addition to accurately illustrating these well characterized developmental processes, quantitative pathway analysis is also a powerful tool for providing new insight into the stage-specific regulation of signaling pathways involved in the unique and complex processes of gonadal differentiation and spermatogenesis. The precise balance of these signaling pathways is hypothesized to play a key role in male development by initiating the differentiation of the fetal Leydig cells that produce masculinizing hormones [47]. The current analysis shows a wide range of signal transduction pathways that go through dramatic changes in expression in testicular tissue over the course of the first wave of spermatogenesis. Further investigation of the fluctuations in these signaling pathways over time could provide insight into their roles in the regulation and maintenance of spermatogenesis.

We also find that pathways related to cellular metabolism of lipids and proteins follow the same pattern of expression as steroidogenesis pathways during spermatogenesis. If these pathways are in fact linked, this is consistent with the hypothesis that cellular metabolism pathways are a target of androgen action, allowing androgens to modulate the testis environment to promote spermatogenesis [16]. These observations illustrate that our pathway-based approach can provide quantitative information on the dynamic changes seen in a range of signaling pathways over the course of development. Deeper exploration of this pathway analysis may reveal additional novel pathways that are important in reproductive development.

4.3. Benefits and drawbacks of the quantitative pathway-based approach

There are many benefits to applying a pathway-based approach to analyze temporal gene expression data. For example, we reduce the potential for overlooking key pathways whose subtle changes have large effects by considering the pathway as a whole as opposed to individual genes. We also reduce the amount of statistical noise, as entire gene networks are far less likely than individual genes to be significantly increased simply by chance. It is important to note that while average pathway expression provides an informative summary of pathway dynamics driving developmental processes, this method is not ideal for identification and characterization of single genes that serve as key developmental regulators. In addition, the quality of a pathway-based analysis is limited by the quality of the curation of each pathway. Indeed, there are several pathways enriched in our analysis (*e.g.* heart development and neural tube development) that are likely to be an artifact of genes that have simultaneous roles in multiple pathways or signal transduction that is highly conserved across a diverse range of developmental processes.

4.4. Applications for developmental toxicology: defining sensitive phases of development and generating complex, context dependent Adverse Outcome Pathways

In addition to providing insight into normal gonadal development and spermatogenesis, this quantitative gene ontology analysis offers a new lens through which to evaluate developmental pathology. This will be a particularly valuable tool for our field of developmental toxicology. Understanding the dynamics of signaling pathways can further define key phases of susceptibility to environmental factors. For example quantitative characterization of the pathway dynamics that drive steroid regulation can shed light on key windows of susceptibility of this process to endocrine disrupting chemicals [7,48].

The challenge of regulatory chemical testing requirements and the vast number of chemicals yet to be tested will require innovative computational toxicology methods [49,50]. Toxicity-related alterations in gene transcription and biological pathway dynamics have been proposed as valuable metrics for chemical risk assessment that could be generated by high-throughput methods [51,52]. To that end, a quantitative pathway-based approach can be applied to predict and measure the effects of chemical exposure on signaling pathway dynamics. This approach is a powerful way to quantify changes in the developmental dynamics of signaling pathways in response to genetic mutations and environmental factors. Changes in the peaks, slopes and duration of the dynamic expression patterns of signaling pathways in response to toxicant exposure will provide a sensitive measure of reproductive developmental toxicity and offer insight into mechanisms of toxicity. Quantitative pathway-based characterization of signaling pathway dynamics could also inform mathematical models for *in silico* simulation of the specific impacts of gene changes or environmental exposures on developmental outcomes. Furthermore, quantifying the perturbation of developmental pathway dynamics in response to toxicant exposures could facilitate articulation of “Adverse Outcome Pathways”, which are emerging as an increasingly valuable tool for translating toxicological data for risk assessment [53]. Therefore, in addition to shedding light on normal developmental dynamics, this quantitative pathway- based approach provides a framework for quantifying deviation from normal developmental processes.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

Transparency document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.11.008.

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