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Alterations in the Rat Serum Proteome Induced by Prepubertal Exposure to Bisphenol A and Genistein

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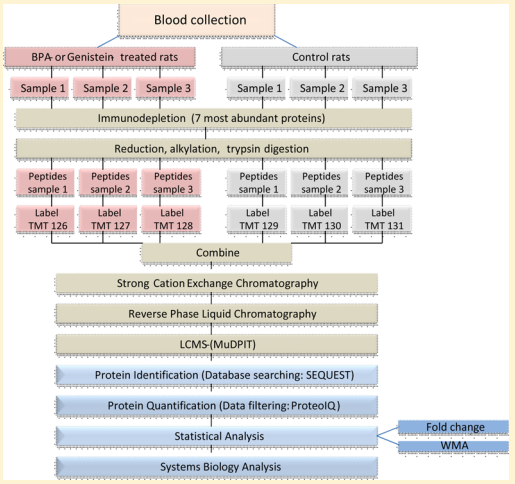
Supporting Information

ABSTRACT: Humans are exposed to an array of chemicals via the food, drink and air, including a significant number that can mimic endogenous hormones. One such chemical is Bisphenol A (BPA), a synthetic chemical that has been shown to cause developmental alterations and to predispose for mammary cancer in rodent models. In contrast, the phytochemical genistein has been reported to suppress chemically induced mammary cancer in rodents, and Asians ingesting a diet high in soy containing genistein have lower incidence of breast and prostate cancers. In this study, we sought to: (1) identify protein biomarkers of susceptibility from blood sera of rats exposed prepubertally to BPA or genistein using Isobaric Tandem Mass Tags quantitative mass spectrometry (TMT-MS) combined with MudPIT technology and, (2) explore the relevance of these proteins to carcinogenesis. Prepubertal exposures to BPA and genistein resulted in altered expression of 63 and 28 proteins in rat sera at postnatal day (PND) 21, and of 9 and 18 proteins in sera at PND35, respectively. This study demonstrates the value of using quantitative proteomic techniques to explore the effect of chemical exposure on the rat serum proteome and its potential for unraveling cellular targets altered by BPA and genistein involved in carcinogenesis.



KEYWORDS: *serum, proteomics, BPA, genistein, carcinogenesis*

INTRODUCTION

Studies suggest that breast cancer risk is influenced by endogenous hormones and exposure to environmental chemicals including synthetic compounds such as the plasticizer BPA and the soy phytochemical genistein. In the case of BPA, this hypothesis is supported by measurement of BPA in the urine of the human population,[1] the association of BPA with early onset of menarche in girls,[2] and the effects of BPA in mammary gland development and carcinogenesis in animal studies.[3−5] Using the estrogen receptor (ER)-positive MCF-7 breast cancer cell line, Krishnan et al. first showed that low concentrations of BPA (20−50 nM) increased cellular proliferation.[6] Also, treating both ER-positive and -negative cancer cell lines with low concentrations (0.1−100 nM) of BPA in combination with several commonly used chemotherapeutics was shown to increase proliferation and cell survival.[7] Hence, BPA is considered to be an endocrine disruptor, reported to alter normal estrogen, androgen, and thyroid hormone signaling. It causes adverse effects in breast, prostate, pancreatic, and liver cell lines.[8] This includes inducing proliferation, oxidative stress, and altering cell signaling pathways involved in carcinogenesis and glucose homeostasis. In rats, perinatal exposure to BPA through subcutaneously implanted osmotic pumps was observed to cause significant alterations in the mammary gland, including an increased number of mammary terminal end buds (TEBs), a decreased rate of apoptosis in the TEBs, increased percentage of cells expressing the progesterone

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receptor, and increased lateral branching in the mammary gland.[3] Durando et al. have shown that prenatal exposure to BPA coupled to a subcarcinogenic dose of *N*-nitroso-*N* methylurea resulted in an increased percentage of preneoplastic and neoplastic lesions in the mammary gland.[4] We have previously reported that mammary TEBs of 50 day old female rat offspring exposed prepubertally via lactating dams treated with 250 μg BPA/kg BW had increased cell proliferation.[5] Furthermore, female offspring exposed prepubertally to BPA and subsequently treated with the carcinogen dimethylbenzanthracene (DMBA) at postnatal day 50 (PND50) developed significantly more mammary adenocarcinomas than female offspring exposed prepubertally to sesame oil and at PND50 with DMBA.[5]

Epidemiological reports suggest that individuals consuming a diet high in soy have a reduced risk of developing breast cancer.[9,10] Although soy contains three major phytochemicals, its protective properties are often attributed to genistein (4,5,7-trihydroxyisoflavone). In vitro, genistein is an inhibitor of the epidermal growth factor receptor (IC50 = 2.6 uM),[11] topoisomerase I and II (IC50 = 110 μM),[12] and 5 alpha reductase (IC50 = 35 μM) [reviewed in ref 13]. Genistein has been shown to inhibit cell proliferation in both ER-positive and ER-negative breast cancer cell lines. It induces apoptosis and cell-cycle arrest and decreases cell motility. Genistein has also been shown to play a role in several signaling pathways indicated in carcinogenesis, reducing the expression or activation of several procarcinogenic proteins, such as cyclin D1, Akt, and erbB2, and increasing the expression of the tumor suppressor, phosphatase and tensin homologue (PTEN).[13] Given the similarities in molecular weight, chemical structure, and hydroxylation patterns between genistein and estradiol, it is not surprising that genistein has reported weak estrogen activities. While studies have found genistein capable of binding to both of the ERs, it has 20-fold increased affinity to ER beta as compared with ER alpha.[14] Because of the potential anticarcinogenic effects of genistein, we have investigated and demonstrated that perinatal exposure of rats to genistein suppresses DMBA-induced mammary cancer.[15] Genistein action early in postnatal life appears to impose an imprinting effect on the biochemical blue-print of the mammary gland that results in a long-term protective effect against carcinogenesis via reducing cell proliferation in mammary TEBs in adult rats.[16,17]

Of enigmatic interest are the reports that despite both chemicals possessing estrogenic properties, BPA and genistein regulate similar genes and proteins, albeit in different directions, and yield different health outcomes in the mammary gland.[5,13−16] For genistein, epidemiological studies also show that Asians eating a diet high in soy have reduced incidence of breast cancer.[9,10] A plausible explanation for the differential effects of these two compounds on breast cancer is that different targets are affected or, alternatively, that the same targets are affected in opposite directions. While it is important to understand mechanisms in the target tissue, it is also of great priority to identify biomarkers of exposure that can be easily tested. The blood provides a minimally invasive source of proteins that can serve as surrogate biomarkers of susceptibility.[18] Accordingly, the goal of this study was to identify proteins affected by lactational exposure to BPA and genistein in the rat serum proteome. It is plausible that these protein peptides can be differentially expressed in the serum and could serve as functional biomarkers of susceptibility to carcinogenesis. Some of these proteins have cellular functions linked to the pathophysiology of carcinogenesis or have been identified as BPA or genistein-targeted proteins in the mammary gland. Studies of this nature provide important information to uncover systemic changes induced by such chemicals and advance our understanding of the relationship among environmental chemical exposures, changes in the serum proteome, and carcinogenesis.

To the best of our knowledge, this is the first study conducted in rats to evaluate the effects in the rat serum proteome following exposure to environmentally relevant doses of two chemicals associated with cause and prevention of breast cancer. The timing of exposure, prepubertal, was selected from previous studies showing that lactational exposure of rats to BPA or genistein alters cell proliferation, apoptosis, growth factor signaling pathways, and susceptibility for the development of mammary tumors later in life.[5,15,16] Importantly, lactation is a plausible route for BPA and genistein exposure in humans.[19,20] The results of this study reveal that select serum proteins were differentially expressed by prepubertal BPA and genistein exposures on both PND21 and PND35. The significance of altered protein expressions at day 35 is that this is 15 days after the last BPA or genistein treatments, a time when the original effectors (BPA and genistein) are now absent through metabolism and disposition. Our previous studies with hormonally active chemical exposures during the neonatal or prepubetal periods of development have demonstrated that imprinting or programming effects can be manifested whereby permanent alterations to gene and protein expressions are established.[21,22] This results in changes to the "biochemical blue print" from which the host tissue responds, including activation/detoxication mechanisms and susceptibility for biochemical insult.

MATERIALS AND METHODS

Chemicals

Rabbit polyclonal antibodies to ATPase family AAA domain-containing protein 3A (ATAD3A; cat no. ab112572), hypermethylated in cancer 2 (HIC2, cat no. ab50905), and matrix metalloproteinase 3 (MMP3, cat no. ab53015) were purchased from Abcam (Cambridge, MA). The secondary antibody (goat anti-rabbit IgG HRP affinity purified PAb) was purchased from R&D Systems (Minneapolis, MN) and Chemilume was from Pierce Biotechnology (Rockford, IL). Genistein was provided by DSM Nutritional Products (Basel, Switzerland). BPA, sesame oil, and all other chemicals were from Sigma Chemical (St. Louis, MO).

Animals

Animal studies were conducted in accordance with the University of Alabama at Birmingham Guidelines for Animal Use and Care. Animals were treated humanely and with regard for alleviation of suffering. All animals were housed in a temperature-controlled facility with a 12 h light/dark cycle (lights on between 0800 and 2000 h). Female Sprague−Dawley CD rats (Charles River, Raleigh, NC) were bred with proven Sprague−Dawley CD studs and monitored for the presence of sperm. Sperm-positive females were individually housed in polypropylene cages with glass water bottles (both polycarbonate/BPA free), fed a phytoestrogen-free AIN-76A diet (Harlan Teklad Global Diets, Wilmington, DE), and randomly assigned to a treatment group. On the day of birth (designated as PND zero), offspring were sexed, and litters were culled to 10 offspring per lactating dam. For BPA treatment, lactating

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dams were gavaged intragastrically with 250 μg BPA/kg/day (Sigma Chemical, St. Louis, MO), while controls received an equivalent volume of the vehicle sesame oil beginning on postnatal day 2 (PND2) and continuing through PND20. The dosage of BPA is 200 times less than the lowest observed adverse effect level (LOAEL) of 50 mg/kg/day.[23] For genistein treatment, the lactating dams received 250 mg genistein/kg AIN-76A diet or AIN-76A diet only as controls from PND2 through PND20. The dosage of genistein was chosen based on previous studies from our laboratory, showing that this exposure resulted in serum concentrations in 21 day old rats of 726 pmol/mL,[15] which approximated levels found in Japanese populations eating a traditional diet high in soy.[24] Offspring were weaned on PND21 and continued on AIN-76A diet only.

On PND21 and PND35, female offspring were ketamine/xylazine-anesthetized, and blood was obtained by cardiac puncture prior to euthanasia. Blood was collected in red top, glass BD vacutainer tubes without additives, allowed to coagulate for 30 min at room temperature, and centrifuged at 15 000*g* for 15 min at 4 °C. Serum was collected, aliquoted, and stored at −80 °C for later analysis. Because lactational exposure via the dam results in a single exposure group per treatment, one offspring in each litter was randomly selected and treated as a single observation. A minimum of 10 litters per treatment group were used. For each chemical, two unique sets of control and treated animals were generated. One set was used for serum proteome TMT-MS analysis, and the other set was used for validation studies via Western blot analysis (8 rats/group).

Sample Preparation and Data Acquisition

In each experimental group (treated and control), nine individual serum samples were pooled into three samples (10 μL each). To improve proteomic coverage, we depleted blood serum of the seven most abundant proteins (serum albumin, IgG, fibrinogen, transferrin, IgM, haptoglobin, and alpha1-antitrypsin) using the SepproR IgY-R7 rat-specific spin column system (Sigma Chemical) following manufacturer's instructions. Immunodepletion using IgY columns has been previously shown to be highly reproducible.[25] The flow-through fractions were obtained after the immunodepletion step were collected, exchanged into 100 mM triethylammonium-bicarbonate (TEAB), and concentrated to approximately 20−30 μL using MWCO centrifugal filter devices (Amicon Ultra −0.5 mL 3k, Millipore, Billerica, MA). Protein concentrations of immunodepleted pooled serum samples were determined with the BCA protein assay (Pierce, Thermo Fisher Scientific). One hundred micrograms of protein from each group (composed of three animals per group) was labeled with amine-reactive tandem mass tag reagents (TMT 6 Label Reagents; Thermo Scientific, Lafayette, CO) according to the protocol supplied by the manufacturer as previously described.[26] In brief, protein was solubilized in 100 mM TEAB plus 0.1% SDS and reduced with 9.5 mM tris(2-carboxyethyl)phosphine for 1 h at 55 °C and then alkylated with 17 mM iodoacetamide for 30 min in the dark and digested with Trypsin Gold overnight at 37 °C (Promega, Madison WI). Each sample was incubated with a specific TMT tag reconstituted in 41 μL of acetonitrile (AcN) for 1 h at room temperature. The reaction was quenched by adding 8 μL of 5% hydroxylamine. Tagged samples were combined, and the labeled peptides were purified using a SCX Macrotrap (cat. no. TR1/25109/55, Michrom Bioresources, Auburn CA) and desalted using a Peptide Macrotrap (cat. no. TR1/25109/52, Michrom Bioresources). Sample volumes were reduced in a SpeedVac to near dryness and resuspended in 95% ddH_2O/5% ACN/0.1% formic acid to give a concentration of 2.5 μg/μL.

For each sample set, 25 μg of the combined TMT-labeled peptide digest was analyzed using an LTQ XL ion trap mass spectrometer equipped with a nanoelectrospray source and a Surveyor plus binary high-pressure liquid chromatography (HPLC) pump (Thermo Scientific, San Jose CA) using a split-flow configuration. Separations were carried out using a 14-fraction MudPIT approach, where the first column was a double-fritted 150 μm ID × 7 cm SCX (PolySULFOETHYL A 300 A, 5 μm, PolyLC) connected to a 150 μm × 13 cm pulled tip C-18 column (Jupiter C-18 300 A, 5 μm, Phenomenex, Torrance, CA). The HPLC was set up with two mobile phases that included solvent A (0.1% formic acid in ddH_2O), and solvent B (0.1% formic acid in 85% ddH_2O/15% ACN) and was programmed as follows: 15 min @ 0%B (2 μL/min, load), 65 min @ 0−50%B (∼0.5 nL/min, analyze), and 20 min @ 0% B (2 μL/min, equilibrate). This gradient was used for each step of the MudPIT analysis, in which the flow-through was first analyzed, followed by 13 additional fractions obtained by 35 μL injections of the following concentrations of ammonium acetate dissolved in ddH_2O: 25 mM, 32.5 mM, 40 mM, 50 mM, 75 mM, 100 mM,150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 1 M. The LTQ XL was operated in data-dependent triple play mode, with a survey scan range of 350−2000 m/z, followed by a zoom scan for charge-state determination and pulsed Q dissociation (PQD) scan for MS2, which were carried out with 2.0 Da isolation widths on the three topmost intense ions. MS data were collected in profile mode for all scan types. Charge-state screening and data-dependent dynamic exclusion were enabled, with exclusion of nonassigned peptides, a minimum signal intensity of 2000, a repeat count of 2, and an exclusion duration of 90s for ions ±1.5 m/z of the parent ion. The automatic gain control (AGC) settings were 3×10^4, 5×10^3, and 5×10^4 ions for survey, zoom, and PQD modes respectively. Scan times were set at 25, 50, and 250 ms for survey, zoom, and PQD modes, respectively. For PQD, the activation time, activation Q, and normalized collision energy were set at 0.1 ms, 0.7, and 35%, respectively. The spray voltage was set at 1.9 kV, with a capillary temperature of 170 °C. All MudPIT runs were carried out in duplicate.

Data Analysis

The XCalibur RAW files were centroided and converted to MzXML, and the mgf files were then created using both ReAdW and MzXML2Search, respectively (http://sourceforge.net/projects/sashimi/). The data were searched using SEQUEST (v.27 rev12, .dta files), set for two missed cleavages, a precursor mass window of 0.45 Da, tryptic enzyme, variable modification M @ 15.9949, and static modifications C @ 57.0293, and K and N-term@ 229.1629. Searches were performed with a rat subset of the UniRef100 database, which included common contaminants such as digestion enzymes and human keratins.

Identified peptides were filtered, grouped, and quantified using ProteoIQ (Premierbiosoft, Palo Alto, CA). Only peptides with charge state of ≥2+, a minimum peptide length of six amino acids, and nonzero quantities for all six mass tags were accepted for analysis. ProteoIQ incorporates the two most common methods for statistical validation of large proteome

Table 1. Differentially Regulated Serum Proteins Identified by TMT-MS at PND21 Following Prepubertal Exposure to BPA[a]

ProtKB/ID	protein names	group probability	no. unique peptides	fold change (Rx/C)[b]	SAM[c]
620833[d]	A kinase (PRKA) anchor protein 9 (AKAP9)	1.00	12	−1.69	1.32
D3ZIF5	adaptor-related protein complex 3, beta 1	1.00	10	+5.23	1.53
Q8VHE9	all-trans-retinol 13,14-reductase	0.98	5	+3.50	0.91
Q8R1Y7	amyloid beta (A4) precursor protein-binding, family B, member 1 (APBB1)	0.96	4	−1.72	1.44
D3Z822	ataxia telangiectasia and Rad3 related	0.98	6	+3.50	1.09
1305964[d]	**ATPase family AAA domain containing 3A (ATAD3A)**	0.97	4	+3.61	0.96
D4A7K5	**probable phospholipid-transporting ATPase IH (ATP11A)**	1.00	7	+1.57	1.27
D3ZAE9	ATP-binding cassette, subfamily A (ABC1), member 8	0.89	3	+1.51	0.85
A1L1L0	bobby sox homologue	0.94	3	+1.84	0.89
620734[d]	**catenin delta 2 (CTNND2)**	0.95	3	+1.97	1.58
O35112	CD166 antigen	0.99	6	+1.62	1.43
2317[d]	CD8b molecule	0.84	3	+1.63	1.65
D4A619	centriolin	0.94	3	−1.77	0.95
70878[d]	citron rho-interacting kinase (CIT)	1.00	15	+1.95	1.08
D3ZTC4	coiled-coil domain containing 88B	0.95	3	2.45	0.9
Q9R1E9	connective tissue growth factor 2 (CTGF)	0.98	4	−1.61	1.14
1305048[d]	**cytoplasmic FMR1 interacting protein 2 (CYFIP2)**	0.98	5	−2.26	0.96
Q63170	dynein heavy chain 7, axonema	0.94	3	+1.92	1.29
Q6XDA0	erythroid spectrin beta (SPTB)	1.00	6	+1.72	1.58
Q1JU68	**eukaryotic translation initiation factor 3 subunit A (EIF3A)**	1.00	4	+1.99	1.40
D3ZPU4	excision repair cross-complementing rodent repair deficiency	0.94	3	+1.92	1.14
Q8VI60	extracellular sulfatase Sulf-1	1.00	11	+1.63	0.93
D3ZAZ1	formin-like 1, isoform CRA_a	0.96	4	−3.05	1.05
D3ZRG9	FSHD region gene 1-like 1	0.99	6	+1.69	0.92
B1A2U8	growth factor inhibitor	0.99	6	+3.38	1.05
Q925G1	**hepatoma-derived growth factor-related protein 2 (HGDF)**	0.85	2	+1.62	1.87
D4A9X7	**hypermethylated in cancer 2 (HIC2)**	1.00	7	−3.73	0.98
P13599	IgG receptor FcRn large subunit p51	0.98	5	+1.75	1.33
D3ZUJ4	INO80 complex subunit	0.93	3	+2.11	0.95
C7E1V1	**inositol 1,4,5-trisphosphate receptor type 3 (ITPR3)**	1.00	3	+1.61	0.95
Q924W2	integrin alpha 6 (ITGA6)	1.00	6	−1.82	1.80
1311711[d]	**integrin alpha 11 (ITGA11)**	0.94	3	+1.61	0.81
P08934	kininogen-1	0.88	3	+1.55	1.03
O08984	lamin-B receptor (LBR)	0.94	4	+2.89	1.66
Q3MIF2	lethal malignant brain tumor-like protein 2	0.98	5	+1.58	0.83
D3ZJB5	MAX dimerization protein	0.91	3	+1.82	1.02
D3ZAR2	**microtubule associated serine/threonine kinase 2 (MAST2)**	0.98	4	+1.94	1.09
E9PTS4	**minichromosome maintenance deficient 5 (MCM5)**	0.94	2	+1.65	0.85
1306821[d]	myosin, heavy chain 14 (MYH14)	0.95	4	+4.17	1.37
Q3T1H0	non-SMC condensin II complex, subunit D3	1.00	8	+2.97	0.84
Q9R1A7	**nuclear receptor subfamily 1 group I member 2 (NR1I2)**	0.96	4	+2.18	1.23
D3ZAI8	polycystickidney disease 1-like 3	0.95	4	−4.55	1.57
D3ZW85	protein phosphatase 1 regulatory subunit 15A	1.00	6	+1.88	1.59
Q62770	protein unc-13 homologue C (Munc13−3)	1.00	12	+1.74	0.96
D5KUG4	**receptor tyrosine-protein kinase ERBB3 (ERBB3)**	0.90	3	+1.61	1.08
P49805	regulator of G-protein signaling 9 (RGS9)	0.94	4	+2.91	2.57
D3ZHD7	regulatory factor X, 5	0.95	3	+1.93	0.83
D3ZFE4	RGD1565058 (uncharacterized)	1.00	9	+2.39	1.36
D3ZKG8	ring finger and CCCH-type domains 1	0.88	2	+1.59	2.10
Q9QX72	selenocysteine insertion sequence-binding protein 2	0.99	5	+1.94	1.01
1309389[d]	slingshot protein phosphatase 2 (SSH2)	0.99	5	−1.91	1.09
Q5RJT4	sorting nexin 25	0.99	5	+1.80	1.44
P97690	structural maintenance of chromosomes protein 3 (SMC-3)	0.96	5	−1.56	1
Q03410	synaptonemal complex protein 1 (SCP-1)	0.84	2	+1.70	0.82
B5DF37	TAF6-like RNA polymerase II (TAF6L)	0.98	4	+1.82	1.55
D4A4U9	TBC1 domain family, member D1	0.84	3	+2.02	1.44
Q6U1J1	TBC1 domain family, member 22B	1.00	5	+1.65	0.92
D3ZIG1	transcription termination factor, RNA polymerase II	0.93	3	+1.76	1.30
D3ZZY2	UTP14, U3 small nucleolar ribonucleoprotein	0.99	6	+2.08	1.53
Q4G052	**wingless-type MMTV integration site family (WNT4)**	0.96	5	+2.36	1.24

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Table 1. continued

ProtKB/ID	protein names	group probability	no. unique peptides	fold change (Rx/C)[b]	SAM[c]
P22985	**xanthine dehydrogenase/oxidase (XDH/XO)**	0.95	3	−2.04	0.91
D3ZIN6	zinc finger protein 27	0.94	4	+1.83	0.86
D3ZNA1	zinc finger protein 608	0.97	4	−3.97	0.96

[a]Proteins in bold are carcinogenesis-related proteins. [b]Positive- and a negative-fold changes in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. [c]Significance analysis of microarray (SAM); cut off |±0.8|, calculated as described in the Materials and Methods. [d]Proteins identified using the Rat Genome DB ID.

Table 2. Differentially Regulated Serum Proteins Identified by TMT-MS at PND21 Following Prepubertal Exposure to Genistein[a]

ProtKB/ID	protein names	group probability	no. unique peptides	fold change (Rx/C)[b]	SAM[c]
69412[d]	A kinase (PRKA) anchor protein 6 (AKAP6)	0.97	4	+1.84	1.21
Q2I6B1	ATPase, H+ transporting, lysosomal V0 subunit A2	1.00	4	+1.84	1.26
B5DFK1	coatomer protein complex subunit alpha	0.99	2	+2.83	2.03
D3Z8X4	DNA polymerase	0.93	3	+1.52	1.12
D3ZYR1	FCH domain only protein 2	1.00	4	−3.69	2.35
P06866	haptoglobin	1.00	2	+2.75	2.08
Q6LD44	hemoglobin subunit beta-1	1.00	4	−1.65	1.29
P02091	hemoglobin subunit beta-1	1.00	4	−1.65	1.29
1309584[d]	interleukin-1 receptor-associated kinase 2 (IRAK2)	1.00	3	+1.5	1.38
P20156	**neurosecretory protein VGF 8a (VGF)**	0.90	2	−1.99	1.58
D4A6Y1	nuclear protein in the AT region	0.92	2	+1.83	1.02
D3ZLI9	PDZ domain containing 4	0.88	3	−2.13	0.86
O55035	peptidyl-prolyl cis−trans isomerase G	1.00	3	+1.74	1.59
Q9Z0W5	protein kinase C and casein kinase substrate in neurons protein 1 (PACSIN1)	0.80	2	+1.59	0.85
A5HV10	**protein tyrosine phosphatase receptor type K (PTPRK)**	1.00	2	+1.70	0.93
B1WC62	RGD1311188 protein (uncharacterized)	0.91	3	+2.61	1.39
3590[d]	**rho-associated coiled-coil containing protein kinase 2 (ROCK2)**	1.00	2	−2.99	1.11
Q24JW6	**serine (or cysteine) proteinase inhibitor, clade A (SERPINA1)**	0.94	2	−1.77	0.9
1305576[d]	**SET domain containing 2 (SETD2)**	0.90	4	+1.61	1.02
Q5U2T3	SPATS2-like protein	0.94	3	+1.59	0.85
A8C4G9	spinous and karyoplasmic protein	1.00	4	+1.56	1.71
P03957	**matrix metalloproteinase 3 (MMP3)**	0.90	2	−2.39	1.12
Q32PX5	structural maintenance of chromosomes 4	1.00	3	+3.01	0.95
D3ZDG1	syntaxin binding protein 5-like	1.00	3	+1.54	1.05
D4A3V9	**ubiquitin carboxyl-terminal hydrolase**	0.98	4	+1.54	2.36
1305414[d]	ubiquitin carboxyl-terminal hydrolase L5 (UCH1)	0.95	2	+1.69	0.81
O88461	VIP-receptor-gene repressor protein	1.00	7	+1.54	1.39
D4A0R0	zinc finger protein 59	0.89	3	+1.79	0.87

[a]Proteins in bold are carcinogenesis-related proteins. [b]Positive- and a negative-fold changes in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. [c]Significance analysis of microarray (SAM); cut off |± 0.8|, calculated as described in the Materials and Methods. [d]Proteins identified using the Rat Genome DB ID.

data sets, false discovery rate (FDR), and protein probability.[27−29] The FDR was set at <1% cutoff, with a total group probability of ≥0.7, with at least two peptides assigned per protein. Relative quantification was performed via spectral counting and spectral count abundances were normalized between samples.

Evaluation of TMT Labeling Efficiency

To determine the efficiency, reproducibility, and quantitative range of this approach, we conducted a preliminary experiment using a set of serum samples from untreated male rats that were immunodepleted, labeled with TMT, and analyzed as previously described. These samples were spiked in duplicate with bovine lactoferrin at 12, 60, and 300 ng per 100 μg of protein. Data from this experiment indicated a high level of reproducibility with an average relative standard deviation of <20% for the 15 proteins of varied concentration (Figure S.1A in the Supporting Information) and with a nearly linear quantitative range for the 25-fold range of the spiked equine lactoferrin that was used as an internal standard (Figure S.1A in the Supporting Information).

Western Blot Validation

Western blot analysis was performed to validate changes in protein expression detected by TMT-MS. Rat serum was immunodepleted using the SepproR IgY-R7 column system. Each sample was derived from only one rat randomly selected from separate litters per treatment group. Immunoblotting was performed as previously described[30] with modifications on eight biological samples per treatment group. In brief, depleted serum was diluted in RIPA lysis buffer (Pierce Biotechnology). Samples were centrifuged for 20 min at 16 000*g* at 4 °C. Equal protein content (20 μg) was loaded onto precast SDS Tris-HCL polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins

Table 3. Differentially Regulated Serum Proteins Identified by TMT-MS at PND35 Following Prepubertal Exposure to BPA[a]

ProtKB/ID	protein names	group probability	no. unique peptides	fold change (Rx/C)[b]	SAM[c]
P24090	**alpha-2-HS-glycoprotein (AHSG)**	1.00	5	+1.67	0.87
69293[d]	fetuin B	1.00	10	+1.52	1.43
P82995	**heat shock protein 90α (HSP-90α)**	0.71	3	+1.76	0.98
Q3KRF2	high density lipoprotein binding protein	0.85	3	+1.88	0.87
D3ZE56	phosphatidylinositol 4-phosphate 3-kinase C2 gamma (PIK3C2G)	1.00	2	+1.81	0.81
1311598[d]	**SIN3 transcription regulator family member A (SIN3A)**	0.98	2	+1.65	0.91
O08562	sodium channel protein type 9 subunit alpha	0.98	3	+1.66	0.97
Q08850	syntaxin-4 (STX4)	1.00	2	+2.41	0.96
D3ZG21	transcription factor 20	0.98	2	+1.70	1.8

[a]Proteins in bold are carcinogenesis-related proteins. [b]Positive- and a negative-fold changes in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. [c]Significance analysis of microarray (SAM); cut off |±0.8|, calculated as described in the Materials and Methods. [d]Proteins identified using the Rat Genome DB ID.

Table 4. Differentially Regulated Serum Proteins Identified by TMT-MS at PND35 Following Prepubertal Exposure to Genistein[a]

ProtKB/ID	protein names	group probability	unique peptides	fold change (Rx/C)[b]	SAM[c]
D4A7Z6	alpha-tectorin	0.82	3	−1.76	1.06
Q7TMA5	apolipoprotein B-100 (APOB)	1.00	10	+1.78	1.66
D3ZUU7	coiled-coil domain containing 88C	1.00	3	+1.61	1
D4A6E4	enhancer of polycomb homologue 1	1.00	3	−1.76	0.86
D3ZS47	excision repair cross-complementing rodent repair deficiency complementation group 6, (ERCC6)	0.87	3	+1.52	0.86
B2RYP7	integrator complex subunit 10	0.91	3	+1.82	1.26
1306378[d]	**lysine-specific demethylase 4A (KDM4A)**	0.92	3	−9.92	1.23
D4A7S4	nucleolar pre-ribosomal-associated protein 1	1.00	3	−2.61	0.92
P83900	Rap guanine nucleotide exchange factor 5	0.87	2	−1.94	0.81
Q6WDI9	**recombination activating gene 1 (RAG1)**	1.00	2	−2.27	1.14
D3ZS92	rotatin	1.00	4	+1.55	1.48
D3ZCH3	sorting nexin 13	1.00	3	+1.86	0.87
D3ZXT4	surfeit 6	0.94	2	−2.73	0.8
2324148[d]	**T-lymphoma invasion and metastasis-inducing protein 2 (TIAM2)**	0.94	2	−2.83	0.92
Q5XIB9	TRAF-interacting protein with FHA domain-containing protein A (TIFA)	0.95	2	−1.51	0.83
D3ZFE4	UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	0.94	2	+1.70	1.11
D3ZYV1	WD repeat-containing protein 60	0.81	4	−1.76	0.82
Q9QZ48	zinc finger and BTB domain-containing protein 7A	1.00	3	+3.14	0.89

[a]Proteins in bold are carcinogenesis-related proteins. [b]Positive- and a negative-fold change in protein expression indicate up- and down-regulation of protein expression relative to control, respectively. [c]Significance analysis of microarray (SAM); cut off |±0.8|, calculated as described in the Materials and Methods. [d]Proteins identified using the Rat Genome DB ID.

were wet-transferred to a nitrocellulose membrane overnight; then, the membrane was blocked at room temperature. Following blocking, the primary antibodies were added and incubated overnight at 4 °C. The secondary antibody and Chemilume were added, and protein expression was visualized using film exposure. Densitometry measurements were determined using ImageJ (http://rsb.info.nih.gov/ij/index.html). Kaleidoscope precision plus protein and prestained SDS-PAGE broad range standards (Bio-Rad) were used to validate the proteins of interest.

Statistical Analysis

For the proteomic data generated by TMT-MS, two separate nonparametric statistical analyses were performed between each comparison including control versus BPA and control versus genistein treatments. These nonparametric analyses include (1) the calculation of weight values by significance analysis of microarray[31,32] (SAM; cut off |0.8|) combined with (2) Wilcoxon (cut off of $p < 0.05$), which then were sorted according to the highest statistical relevance in each comparison. For SAM, whereby the weight value (W) is a statistically derived function that approaches significance as the distance between the means ($\mu 1 - \mu 2$) for each group increases, the SD ($\delta 1 - \delta 2$) decreases using the formula $W = (\mu 1 - \mu 2)/(\delta 1 - \delta 2)$. For protein abundance ratios determined with TMT-MS, we set a 1.5-fold change as the threshold for significance, determined empirically by analyzing the inner-quartile data from the control experiment previously indicated using ln−ln plots, where Pierson's correlation coefficient (R) was 0.98, and >99% of the normalized intensities fell between ±1.5 fold. In each case, any two of the three tests (SAM, Wilcoxon, or fold change) had to pass. Statistical analysis of Western blot analysis was performed by Fisher's exact test to determine significance ($P < 0.05$).

Bioinformatics and Systems Biology Analysis

Those proteins that were found to have significantly changed in the treated versus the control groups were further filtered for biological significance and also as a means of pseudovalidation by comparing key biological functions to cancer-related pathways. Protein classification was performed using PANTHER (Protein Analysis Through Evolutionary Relationships

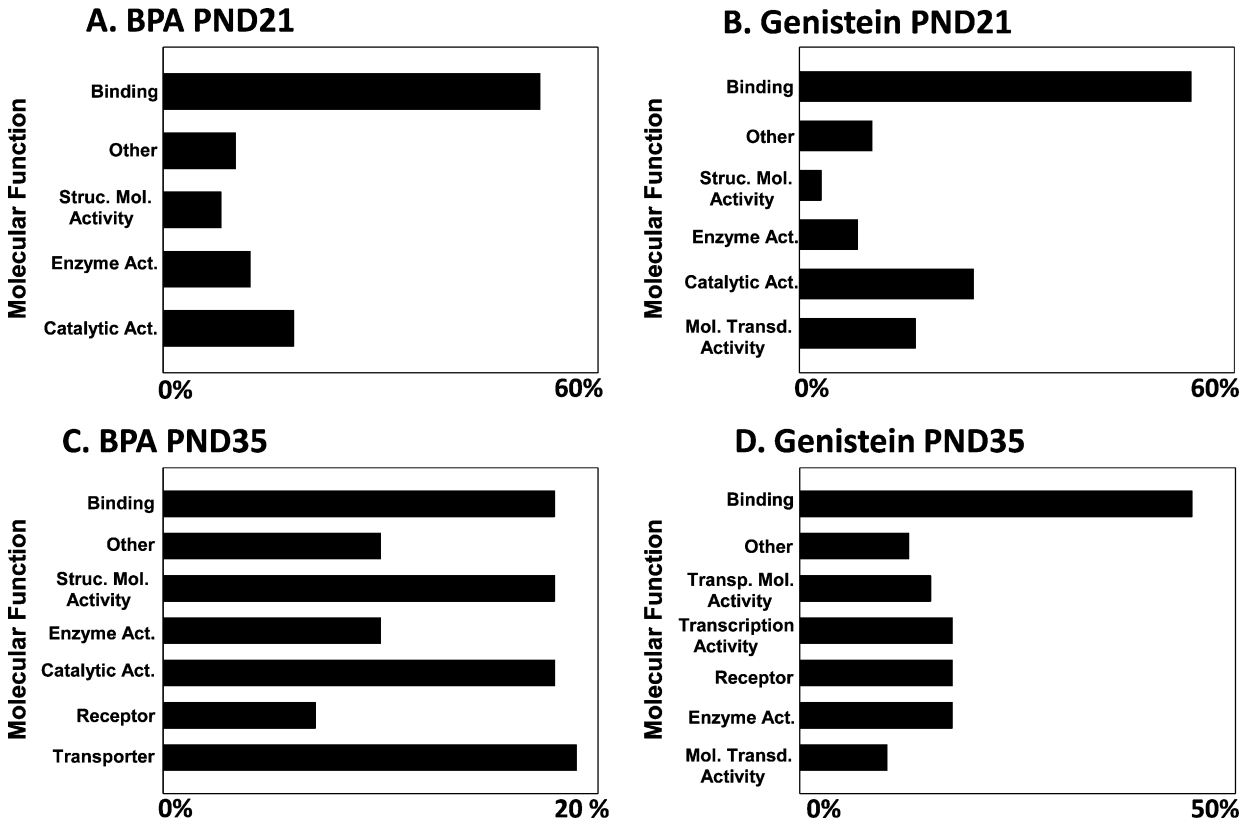

Figure 1. Bar chart representation of proteins differentially regulated by prepubertal exposure to BPA and classified according to molecular function on (A) PND21 and (C) PND35; and by prepubertal genistein exposure on (B) PND21 and (D) PND35. Classification by molecular function was performed using PANTHER (Protein Analysis Through Evolutionary Relationships System) (http://www.pantherdb.org).

System) (http://www.pantherdb.org). Protein classification led to the summation of total proteins known to be associated with each molecular function. Expression data were analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com) (IPA, Redwood City, CA). The IPA Canonical Pathways Analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant in our data set. Proteins from the data set that met the criteria for differential expression and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis.

RESULTS

Protein Identification and Quantification by TMT-MS

To identify targets altered by prepubertal exposure to BPA or genistein, we investigated proteins differentially regulated in the sera of PND21 and PND35 rats exposed via lactation using TMT-MS. Using this approach, we identified 63 and 28 proteins differentially expressed in the sera of PND21 rats exposed prepubertally to BPA and genistein, respectively (Tables 1 and 2). Interestingly, on PND35 (15 days after the last exposure), we identified only 9 and 18 proteins differentially expressed in the sera of rats exposed prepubertally to BPA and genistein, respectively (Tables 3 and 4).

Systems Biology Analysis

Proteins were classified according to their molecular function and biological processes. The main molecular functions affected by BPA and genistein at both PND21 and PND35 were (1) binding, (2) catalytic activity, (3) enzyme regulatory activity, and (4) structural molecular activity (Figure 1A–D). Pathway analysis showed that proteins altered by BPA or genistein exposure were involved in multiple canonical pathways. These pathways and associated proteins involved in the pathophysiology of cancer altered by BPA or genistein on PND21 and PND35 are listed in Supplementary Tables 1–4 in the Supporting Information.

Proteins that were differentially expressed by BPA or genistein exposures were further analyzed based on the pathophysiology of carcinogenesis. Out of 63 proteins differentially regulated in the sera of PND21 rats exposed prepubertally to BPA, 15 were associated with carcinogenesis (Table 1). Out of 28 proteins differentially regulated in the sera of PND21 rats exposed prepubertally to genistein, 7 were associated with carcinogenesis (Table 2). Some of the proteins altered by BPA in sera of PND21 rats and involved in carcinogenesis included ATPase family AAA domain containing protein 3A (ATAD3A), hypermethylated in cancer 2 (HIC2), wingless-type MMTV integration site family (WNT4), receptor tyrosine-protein kinase (ERBB3), cytoplasmic FMR1 interacting protein 2 (CYFIP2), probable phospholipid-transporting ATPase IH (ATP11A), integrin alpha 11 (ITGA11), and microtubule associated serine/threonine kinase 2 (MAST2) (Table 1). On PND35, 15 days after exposure ceased, we found several proteins altered from prepubertal BPA exposure that can be associated with carcinogenesis including: heat shock protein-90α (HSP-90α), alpha-2-HS-glycoprotein (AHSG), and SIN3 transcription regulator family member A (SIN3A) (Table 3).

Among the sera proteins found to be altered on PND21 by genistein were those with cellular functions associated with

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chemoprevention. These proteins included MMP3, rho-associated, coiled-coil containing protein kinase 2 (ROCK2), neurosecretory protein VGF 8a (VGF), serine (or cysteine) proteinase inhibitor, clade A (SERPINA1), ubiquitin carboxyl-terminal hydrolase L5 (UCH1), SET domain containing 2 (SETD2), and protein tyrosine phosphatase receptor type K (PTPRK) (Table 2). Sera proteins altered from prepubertal genistein exposure in PND35 rats (15 days after exposure ceased) with functions associated with decreased carcinogenesis included recombination activating gene 1 (RAG1), T-lymphoma invasion and metastasis-inducing protein 2 (TIAM2), and lysine-specific demethylase 4A (KDM4A) (Table 4).

Validation of Differentially Expressed Proteins by Western Blot Analysis

The use of specific antibodies provides an independent method of protein identification and quantification from that of MS. For this, we used Western blot analysis to validate changes in the expression levels of ATAD3A, HIC2, and MMP3 in the sera of rats exposed prepubertally to BPA and genistein. These proteins were selected based on their potential impact to predispose or prevent cancer and the availability of commercially prepared antibodies. Consistent with the results obtained with TMT-MS, Western blot analysis showed that ATAD3A was significantly up-regulated in the sera of prepubertal rats exposed to BPA when compared with the control group ($p = 0.0325$) (Figure 2A). Of special interest was that in contrast with BPA, genistein tended to decrease the expression of ATAD3A when compared with the control group ($p = 0.091$) (Figure 2B). Western blot analysis also confirmed that HIC2 expression in rat sera was down-regulated in rats treated prepubertally with BPA ($p = 0.0097$) (Figure 2A). In addition, we validated by Western blot analysis the decreased expression of MMP3 induced by genistein exposure in the sera of PND21 rats ($p = 0.0031$) (Figure 2B).

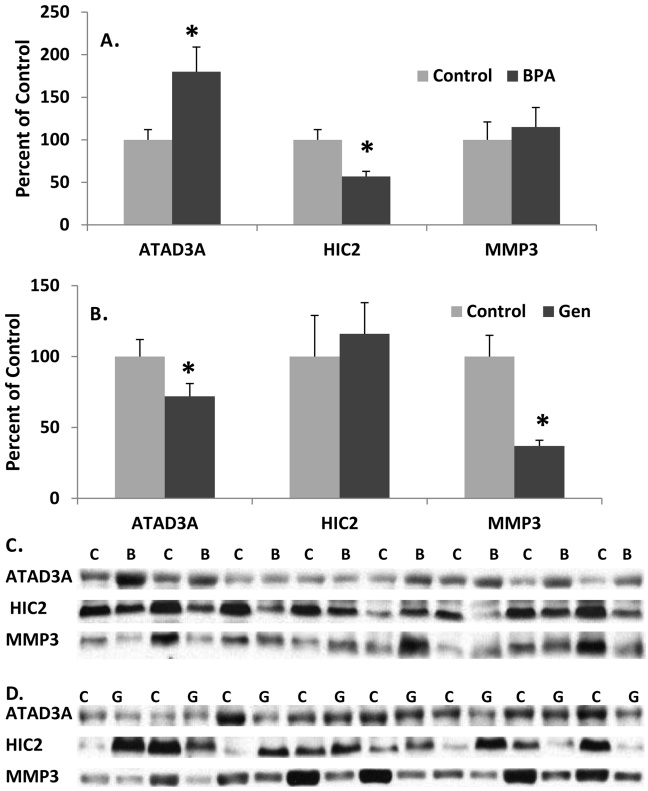

Figure 2. Western blot analysis of ATAD3A, HIC2, and MMP3 in the sera of PND21 rats exposed prepubertally via lactation to (A) BPA or (B) genistein. Quantification is reported as the percent of control with densitometry values for controls set to 100. Values represent mean density ± SEM as a percent of the control group ($n = 8$ per group). Asterisk (*) indicates statistically significant difference in detected protein abundance compared to control ($p < 0.05$). Panels C and D depict immunoblots of 40 μg of serum protein from the control and BPA- or genistein-treated groups, respectively. (C = control, B = BPA, G = genistein).

DISCUSSION

Breast cancer is one of the two most common cancers among women in the United States and one of the leading causes of cancer death among women of all races.[33] One risk factor associated with breast cancer in women includes longer exposure to endogenous estrogen because of a younger age at menarche. Furthermore, epidemiology studies suggest that heredity accounts for only 15% of all diagnosed breast cancer cases.[34] Therefore, we hypothesized that environmental exposure to chemicals such as BPA and genistein could affect breast cancer risk. In the case of BPA, this hypothesis is supported by the extensive human exposure to BPA,[1] the association of BPA with early onset of menarche in girls,[2] and the effects of BPA in mammary gland development and carcinogenesis in animal studies.[5,35,36] Some of these studies showed that exposure of female rats to BPA via lactation prior to puberty resulted in increased development of chemically induced mammary cancer[5,35] and lifetime exposure of erbB2/neu transgenic mice to BPA increased multiplicity and decreased latency of developing mammary tumors.[36] These increases in cancer were ascribed to increased cell proliferation and decreased apoptosis and correlated well with alteration of multiple proteins involved in carcinogenesis.[5,35,37] In regard to genistein, epidemiological reports suggest that individuals consuming a diet high in soy containing genistein, especially in preadolescence, have a reduced risk of developing breast cancer.[9,10] A multitude of in vitro and in vivo studies demonstrate chemopreventive properties of genistein.[11−17]

Although analysis of the mammary gland is feasible in animal studies, it is not a tissue readily available for study in humans. Therefore, we investigated changes affected by BPA or genistein exposure in the serum proteome using TMT-MS for biomarkers of susceptibility. This approach allowed us to survey global changes to the more abundant proteins associated with the rat serum proteome and the associated pathways that could be involved in the differential effects of BPA and genistein in carcinogenesis. Because 21 proteins comprise 90% of blood proteins and albumin represents one-third of this total,[38] we employed IgY-R7 affinity spin columns to remove highly abundant proteins. While it is possible that all of the blood proteins could be physiologically important as biomarkers or have interesting proteins bound, the IgY-R7 bound proteins were not further investigated. The removal of such "contaminants" allowed for the enrichment of low abundance proteins (chemokines, cytokines, growth factors, etc.) that would traditionally be "masked" by high abundance proteins. These depleted and concentrated samples were subjected to TMT isobaric mass tagging, which enables quantitative labeling and subsequent analysis by mass spectrometry.

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MS analysis was carried out on an LTQ XL instrument set up in triple play mode, with zoom scans (maximum resolution of ~20 K) carried out on the most intense three parent ions for each survey scan. While this approach allows for charge-state discrimination for up to MH^{4+} ions, it would have been optimal to have utilized a high-resolution instrument, such as an Orbitrap.[39] Part of the reason for this point relates to the extreme complexity of the serum proteome but also includes challenges related to coeluting isobaric peptides, which can affect TMT-based quantification.[40] With that, we feel that the topmost statistically significant and biologically relevant proteins presented here are reasonably robust, and we plan to move forward with similar work in a human cohort while utilizing a high-resolution instrument.

Using online automated 14 fraction nano-LC−ESI−MS (SCX/RP) MuDPIT, we identified 63 and 28 proteins differentially expressed on PND21 and 9 and 18 proteins differentially expressed on PND35 in the sera of rats exposed prepubertally to BPA and genistein, respectively. This illustrates that BPA and genistein exposures during the prepubertal period can exert direct effects on serum protein expressions that are reversible once the treatment is discontinued, as evidenced by not being expressed at PND35 as was at PND21.

On PND21, BPA increased the expression of several proteins in rat serum including ATAD3A, ERBB3, and WNT (Table 1). ATAD3A is a mitochondrial membrane protein that contributes to the stabilization of large mitochondrial DNA (mtDNA)−protein complexes or nucleoids.[41] Its protein expression is associated with lymphovascular invasion and is suggested to have an antiapoptotic role in lung adenocarcinoma.[42] The source of increased expression of ATAD3A in the serum by BPA is unknown at this time, however, it is plausible to hypothesize that increased serum ATAD3A could reflect the abundance of this protein in tissues that express this protein, including the mammary gland. Because prepubertal BPA exposure results in increased ATAD3A in the sera of PND21 rats and increased ATAD3A is associated with antiapoptotic actions,[42] we speculate that a decrease in apoptosis induced by overexpression of ATAD3A could favor DNA damaged cells and carcinogenesis. Consistent with this hypothesis are reports that apoptosis is decreased by BPA in the rodent mammary gland.[5,43]

In previous studies, ERBB3 and WNT were increased by BPA in the rodent mammary gland, and their increases were associated with increased carcinogenesis and proliferation of TEBs, respectively.[5,43] We previously reported that ERBB3 was increased in the mammary gland of rats exposed to BPA during lactation using this same treatment paradigm.[5] In humans, ERBB3 functions as an essential partner of ERBB2 to drive the proliferation of erbB2-overexpressing breast tumor cells.[44] The importance of ERBB3 in carcinogenesis is derived from its ability to directly recruit and activate PI-3K,[45,46] which is involved in the induction of cellular transformation.[47] In the case of WNT, previous studies showed that BPA increased the expression of members of the Wnt gene family such as Wnt4 in the mammary gland of mice.[43] Wnt4 is an important mediator of lateral branching in the mammary gland, and overexpression may be associated with abnormal proliferation of human breast tissue.[48]

Other cancer-related proteins increased by BPA in the sera of rats on PND21 were integrin alpha 11 (ITGA11), hepatome-derived growth factor-related protein 2 (HGDF), minichromosome maintenance deficient 5 (MCM5), microtubule associated serine/threonine kinase 2 (MAST2), catenin delta 2 (CTNND2), nuclear receptor subfamily 1 group 1 member 2 (NR1I2), eukaryotic transition initiation factor 3 subunit A (EIF3A), and inositol 1,4,5-triphosphate receptor type 3 (ITPR3) (Table 1). Increased expression of these proteins is also associated with cellular processes involved in carcinogenesis or overexpressed in diverse types of human cancers.[49−56]

Among the proteins down-regulated by BPA and validated by Western blot analysis in PND21 rat serum was HIC2. This protein is suggested to play a role as a tumor suppressor.[57] It interacts with HIC1, which significantly decreases the clonogenic survival of various cancer cell lines. HIC1 and HIC2 regulate the expression of several genes that participate in multiple cell functions including cell proliferation, apoptosis, differentiation, and neoplastic transformation.[57,58] The significance of decreased HIC2 in the sera of PND21 rats following prepubertal BPA exposure for mammary gland carcinogenesis requires further investigation. Nevertheless, increased expression of proteins that potentially favor carcinogenesis and decreased expression of proteins with tumor suppressor activity by BPA may provide more information on possible mechanisms in the increased mammary gland carcinogenesis induced by BPA in rats.

Prepubertal exposure to BPA also decreased the expression of CYFIP2 in PND21 serum (Table 1). CYFIP2 is involved in T-cell adhesion and p53/TP53-dependent induction of apoptosis. Inducible expression of CYFIP2 is sufficient for caspase activation and apoptosis.[59] Because CYFIP2 is down-regulated in the mammary gland by BPA exposure, it is possible that decreased CYFIP2 could be involved in the decreased apoptosis previously reported in rats lactationally exposed to BPA.[59] We speculate that down-regulation of CYFIP2 by BPA could decrease or block apoptosis and contribute to the carcinogenic phenotype.

A very interesting discovery of this study was altered expression of sera proteins that are reported to play a role in carcinogenesis in PND35 rats exposed prepubertally to BPA and genistein. For BPA, these proteins were HSP-90α, SIN3A, and AHSG (Table 3). HSP-90α is a chaperone protein that can influence cytoskeletal rearrangement, migration, and invasion of breast cancer cells.[60] Its overexpression correlates with poor prognosis in breast cancer.[61] Akt, HIF-1α, and Raf-1 are cellular targets of HSP-90α.[62] Interestingly, previous studies showed that prenatal and prepubertal BPA increased the levels of Akt in the rat mammary gland.[5,63] Therefore, it is possible that the effects of BPA on Akt identified in previous studies could be mediated by HSP-90α. SIN3A is expressed in breast cancer cells, and studies show that loss of SIN3A inhibits breast cancer cell growth.[64] Finally, AHSG is a serum biomarker for breast cancer diagnosis,[65] and the lack of AHSG in animal models decreases mammary tumor incidence.[66] Further studies are needed to investigate the relationship of increased HSP-90α, SIN3A, and AHSG levels in sera of BPA-treated rats and increased mammary gland tumorigenesis. These proteins hold potential as serum biomarkers altered by prepubertal BPA exposure.

Proteins found to be decreased in sera of PND 21 rats following prepubertal genistein exposure included MMP3, ROCK2, VGF, and SERPINA1, for which increased expressions are associated with carcinogenesis or overexpressed in diverse types of human cancers (Table 2). MMP3 plays a crucial role in the development of tissue architecture and is involved in

multiple physiological and pathological processes including tumor growth. In the mammary gland, MMP3 regulates branching morphogenesis, and ectopic expression of MMP3 in mouse mammary epithelia triggers supernumerary lateral branching and eventually, tumors.[67] MMP3 induces reactive oxygen species in normal mammary epithelial cells, causing DNA damage, genomic instability, and epithelial-to-mesenchymal transition (EMT).[68] Therefore, it is possible that decreased expression of MMP3 observed in our study would be reflective of a protective mechanism triggered by genistein to decrease ROS and EMT, resulting in decreased tumor formation. Also, genistein down-regulated ROCK2, VGF, and SERPINA1 for which increased expression is associated with carcinogenesis or overexpressed in diverse types of human cancers.[69−71] Other proteins altered by genistein on PND21 included the up-regulation of three tumor suppressor proteins including UCH1, SETD2, and PTPRK.[72−74] The decreased expression of proteins that potentially induce carcinogenesis and increased expression of proteins with tumor suppressor properties by genistein exposure further support the protective action of genistein.

On PND35, a different set of proteins reported to play a role in carcinogenesis were expressed in sera of rats exposed prepubertally to genistein. These proteins included RAG1, TIAM2, and KDM4A (Table 4). Inhibition of genistein on RAG1 has been reported previously in MCF-7 cells, and this effect was associated with the chemopreventive effects (growth arrest) of genistein in this cell line.[75] TIAM2 is overexpressed in human hepatocellular carcinoma,[76] and KDM4A is suggested to play a role in breast tumor formation by stimulating ER-alpha action.[77]

To demonstrate that TMT-MS was reliable in identification of differentially expressed proteins in rat serum, we utilized specific antibodies and Western blot analysis to valid this technology. For this, three proteins whose functions are associated with carcinogenesis and for which we could obtain antibodies were selected. Consistent with the TMT-MS results, we found: up- and down-regulation on PND21 by BPA of ATAD3A and HIC2, respectively, and down regulation on PND21 by genistein of MMP3. These results show that quantitative proteomics via TMT-MS is a reliable technique for identification of proteins in the serum.

In summary, we describe quantitative and reliable sample preparation for TMT labeling of serum proteins following immunodepletion. Using online automated 14 fraction nano-LC−ESI-MS (SCX/RP) MuDPIT, we identified 63 and 28 proteins differentially expressed on PND21 and 9 and 18 proteins differentially expressed on PND35 in the sera of rats exposed prepubertally to BPA and genistein, respectively. This illustrates that BPA and genistein exposures during the prepubertal period can exert direct effects on serum protein expression that are reversible once the treatment is discontinued, as evidenced by not being expressed at day PND35 as was at day PND21. Also important are the programming effects on protein expression at PND35 that are permanent manifestations as evidenced in the absence of the original chemical effectors. It is also telling that of the 108 sera proteins found to be differentially regulated by BPA and genistein at the two time periods, there was no duplication in the four age/treatment groups. This demonstrates chemical and developmental specificity. Using antibodies for a selected set of TMT-MS-identified proteins, we were able to validate and confirm the differential expression of these identified proteins. Although further studies are necessary to investigate the altered expression of these proteins in the mammary gland, these results show that quantitative proteomics via TMT-MS is a useful technique for the identification of molecular targets and their use as biomarkers of susceptibility. Likewise, the identification of these proteins should aid in the understanding of the mechanisms of action involved in the carcinogenic and chemoprotective actions of BPA and genistein, respectively. For the future, studying the rat serum proteome modulated by environmental chemicals is important because this technology and information could be used as a reference point for comparison with future human studies in a population at risk.

■ ASSOCIATED CONTENT

Supporting Information

Bar graph representation of normalized pseudospectral counts of highly abundant proteins in control rat serum spiked with 12, 60, and 300 ng bovine lactoferrin per 100 μg of protein, labeled with TMTs, and analyzed by an online automated 14 Fraction nano-LC-ESI-MS (SCX/RP) MuDPIT (PQD-LTQXL ThermoFinnigan). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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