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CROSS-SPECIES COMPARISON OF ESTROGENIC ENDOCRINE DISRUPTOR-INDUCED, UTEROTROPHIC GENE EXPRESSION IN THE RODENT

By

Joshua Caleb Kwekel

A DISSERTATION

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ABSTRACT

CROSS-SPECIES COMPARISON OF ESTROGENIC ENDOCRINE DISRUPTOR-INDUCED UTEROTROPHIC GENE EXPRESSION IN THE RODENT

By

Joshua Caleb Kwekel

Estrogenic endocrine disrupting chemicals (EEDCs) are a growing matter of concern in the etiology of several developmental, reproductive and general health related adverse health effects. These include breast cancer, decreasing fertility rates, and birth defects. These chemicals are structurally diverse and arise from a broad range of sources including natural products, pesticides, food processing and packaging materials, and pharmaceuticals. These chemicals mediate most of their effects through estrogen receptors (ERs) which are ligand-dependent transcription factors. Thus the main mechanism of estrogen signaling involves the regulation of expression of target genes. A major consideration in ER signaling is selective estrogen receptor modulator (SERM) activity, which consists of differential activation of the ER depending upon ligand-specific conformational changes and subsequent transcriptional activities of the activated ER. Thus understanding the activity of SERMs is an essential part of our assessment of EEDCs.

A fundamental aspect of toxicology and pharmacology are cross-species comparisons of data between surrogate models and humans. Thus understanding how different surrogates respond to the same stimulus is essential in resolving uncertainties in assessing safety and efficacy information between models as well as extrapolating information from surrogates to humans. Two prevalent rodent models common to toxicology and basic research are the rat and mouse. Furthermore, the enhanced rodent uterotrophic assay is a classic measure of estrogen activity *in vivo* and was thus utilized to characterize the estrogenicity of three estrogen ligands. Dose response and time course studies were conducted to examine the global gene expression profiles accompanying the uterotrophic response. Ethynylestradiol, a potent orally active and positive control estrogen; tamoxifen, a breast cancer drug and classic SERM; and o,p'-DDT, the estrogenic isomer of the legacy pesticide DDT and well known EEDC were evaluated in the mouse and rat during uterotrophy. Comparative analysis of the temporal gene expression of these three ER ligands was performed and results reveal a high degree of overlap in differentially expressed genes between ligand and species. Furthermore, ligand- and species-specific responses were also characterized and phenotypically linked. Carbonic anhydrase 2, a gene with multiple endocrine-related effecter roles was identified as a notable candidate biomarker gene exhibiting divergent regulation between species and ligand.

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LIST OF ABBREVIATIONS

4WVD	4-way Venn diagram
AF-1	activation function 1
AF-2	activation function 2
ANOVA	analysis of variance
AP-1	activating protein 1
AR	androgen receptor
bl2seq	BLAST two sequences alignment
BrdU	5-bromo-2-deoxyuridine
Ca2 or Car2	carbonic anhydrase 2
CAD	coactive divergent direction
cAMP	cyclic adenosine monophosphate
CAR	constituitive androstane receptor
CAS	coactive similar direction
СВР	CREB binding protein
cDNA	complentary deoxyribonucleic acid
chIP	chromatin immunoprecipitation
cRE	cis regulatory element
Ct	threshold cycle
DAD	displaced active divergent direction
DAS	displaced active similar direction
DDD	dichloro-diphenyl-dichloroethane
DDE	dichloro-diphenyl-dichloroethylene

DDT	dichloro-diphenyl-trichloroethane
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
E2	17β-estradiol
EDC	endocrine disrupting chemical
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE	ethynylestradiol
EEDC	estrogenic endocrine disrupting chemical
ER	estrogen receptor
ERE	estrogen response element
EST	expressed sequence tag
FQPA	Food Quality Protection Act
GEO	Gene Expression Omnibus
GO	Gene Ontology
H&E	hematoxylin and eosin
HAT	histone acetyltransferase
HSD	hydroxysteroid dehydrogenase
HSD	honestly significant difference
HSP	heat shock protein
i.p.	intraperitoneal
IMAGE	Integrated Molecular Analysis of Genomes and their Expression
LE	luminal epithelium

LEC	luminal epithelial cell
LECH	luminal epithelial cell height
МАРК	mitogen activated phosphokinase
MIAME	Minimal Information About a Microarray Experiment
Mm	Mus musculus
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NBF	neutral buffered formalin
NCBI	National Center for Biological Information
NTC	no template control
O,p'-DDT	2, 4'-dichloro-diphenyl-trichloroethane
pCAF	p300/CBP associated factor
PCR	polymerase chain reaction
PD	pharmacodynamic
РК	pharmacokinetic
PWM	position weight matrix
PXR	pregnane X receptor
QA/QC	quality assurance, quality control
QRT-PCR	quantitative real time polymerase chain reaction
Redox	Reducing/Oxidizing
Rn	Rattus norvegicus
RNA	ribonucleic acid
SAGE	serial analysis of gene expression

- SDWA Safe Drinking Water Act
- SEM standard error of the mean
- SERM selective estrogen receptor modulator
- SRC steroid receptor cofactor
- TAM tamoxifen
- TCA tricarboxylic acid
- TCDD 2,3,7,8 tetrachloro-dibenzo-p-dioxin
- TR thyroid receptor
- TSS transcriptional start site
- UCSC University of California, Santa Cruz
- UTR untranslated region
- UWC uterine water content
- UWW uterine wet weight
- VEH vehicle

CHAPTER ONE

INTRODUCTION: ESTROGENIC ENDOCRINE DISRUPTORS AND CROSS-SPECIES COMPARISONS OF RODENT UTEROTROPHY

ESTROGEN SIGNALING

Estrogen is a potent physiological chemical. It is the principle female steroid hormone circulating in the blood and it regulates growth, development, and reproductive health, in addition to a broad spectrum of other cellular processes. There are few people who have not felt the impact of this chemical in their lives personally or through a loved one. Whether through infertility, pregnancy complications, menopause or breast cancer, estrogen plays an important physiological role that affects people in profound ways.

Estrogen's importance and potency is due in large part to the varying levels and distributions of the receptor that mediates its activities. The estrogen receptor is a member of the nuclear receptor superfamily which acts as ligand-dependent transcription factors. The N-terminal domain is a variable region containing the constitutively active activation function-1 (AF-1) domain which interacts with and recruits transcriptional coactivators/corepressors [1]. The DNA binding domain binds to the major groove of DNA to estrogen response elements (EREs). After the hinge region and dimerization surface is the ligand binding domain that overlaps with the ligand-dependent activation function-2 (AF-2) region which also interacts with coactivators/repressors. Steroid hormones passively diffuse into the cell and nucleus; bind the receptor, releasing chaperone proteins, such as heat shock protein (HSP) 90, which sequester the receptor in the cytoplasm. Upon ligand binding, the activated receptor is able to form homo or

heterodimers which then can bind DNA via direct or indirect mechanisms to modulate gene expression. The classic direct binding mechanism involves ER's binding an ERE, which consist of an inverted palindrome of the consensus sequence (A)GGTCC separated by three nucleotides. Transcriptional activation is then facilitated by ER's recruitment of coactivators including members of the steroid receptor coactivator (SRC/p160) family which interact with histone acetyltransferase (HAT)-containing coactivators (p300, CBP, pCAF). These proteins then function by a variety of mechanisms to modify and remodel chromatin and facilitate further recruitment of basal transcriptional machinery and initiate transcription. ERs can also function via indirect genomic actions through a tethering interaction with transcription factors such as Fos/Jun at AP-1 sites, Sp1 at GC-rich sites, cAMP-responsive elements, or NfkB. Activated ER can also signal through non-genomic mechanisms via MAPK kinase cascades, and membrane bound ERs have been characterized.

The estrogen receptor is present in two, unique gene product, isoforms, ER α , which is the more widely distributed and potent ER, and ER β which was discovered in 1996 [2]. ER α presents with full length protein while the ER β isoform has a shorter N-terminal domain and thus modified, non-constitutively active, AF-1 function. ER α is widely distributed in many tissues, with notable expression in the uterus, prostate, ovary, testes, epididymis, bone, breast, liver, various parts of the brain and adipose tissue. ER β is found in colon, prostate, testes, ovary, bone marrow, salivary gland, vascular endothelium, lung and various parts of the brain.

Endogenous estrogens are produced in a variety of tissues including the placenta, testis, brain, bone, adipose tissue and most notably the ovaries [3]. 17β -estradiol (E₂) is

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the most potent estrogen followed by the significantly weaker estrone, and estriol, oxidized and hydroxylated forms of E_2 , respectively. Estrogens are secreted into the bloodstream where they are transported to target tissues either as free molecules or via carrier proteins such as albumin or sex steroid binding proteins. Estrogens are thus in equilibrium with these carrier proteins at target cells where the hydrophobic hormone can passively diffuse into cells and nuclei and bind resident ERs. Estrogens which pass through the liver may be metabolized by hydroxysteroid dehydrogenase 17 β (HSD17 β) enzymes followed by sulfation or glucuronidation conjugation reactions [4] and subsequent elimination in the urine.

ENDOCRINE DISRUPTION

Concerns have been raised about exogenous chemicals which can cause adverse health effects in intact organisms or their progeny as a result of a change in endocrine function. Such chemicals have been termed endocrine disrupting chemicals (EDCs) and mediate their disruptive activities by three major modes of action: 1) inappropriate agonist or antagonist activity of mimetic compounds, 2) perturbation of normal hormone biosynthesis and metabolism, or 3) inhibition of hormone receptor levels or function [5]. These chemicals have been shown to be most disruptive in androgen, estrogen and thyroid signaling [6]. The necessity to develop and improve programs and assays to assess and characterize EDCs was mandated in the U.S. by Congressional legislation of the Safe Drinking Water Act (SDWA) and Food Quality Protection Act (FQPA) in 1996. These policies addressed the need for the Environmental Protection Agency (EPA) to evaluate and broaden current screening programs for EDCs. This resulted in the formation of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). EDSTAC identified in their initial report [7] that disruption of estrogen and androgen signaling is mediated primarily through mimetic compounds via their respective receptors (ER and AR). EDSTAC also proposed a tiered approach for screening and identifying EDCs, namely tier 1 studies would utilize structure/activity data to screen for potential ECDs. Tier 2 would develop and implement in vitro assays for the screening of potential EDCs by measuring their ability to bind to and activate hormone receptors as well as solicit changes in estrogen sensitive genes. Tier 3 studies would then develop and implement in vivo assays and programs to characterize the EDC's disruptive effects upon hormone signaling activities using developmental and reproductive toxicity models. Subsequently, screening assays have focused on ER and AR binding and activation and uterotrophic responses, while characterization methods seek to elucidate downstream harmful effects and toxicity due to EDC modulation of hormone receptor activities.

Many compounds are now being screened for selective estrogen receptor modulator (SERM) activity which exhibit tissue specific activities dependent upon cellular context. Tamoxifen (TAM), a first generation SERM, targets ER-positive breast cancers where it antagonizes the activities of endogenous estrogens to slow the growth of or prevent re-occurrences of tumors. However, TAM also exhibits agonistic activities in the uterus where it contributes to an increased risk for endometrial cancer and uterine sarcomas. It is becoming increasingly evident that a variety of environmental compounds, industrial contaminants, and pesticides interfere with human and animal endocrine systems [8]. DDT is one of the most well known organochlorine pesticides despite its ban from the United States in 1972. DDT is comprised of ~85% p,p'-DDT and 15% o,p'-DDT, with trace amounts of o,o'-DDT and metabolites DDE and DDD. Focus is given to o,p'-DDT due to its xenoestrogen properties of binding to and activating estrogen receptors. It is further reported that the *levo*-enantiomer of o,p'-DDT and not the *dextro*- preferentially binds the estrogen receptor and blocks endogenous estrogens from binding [9]. However, given the mixture of enantiomers present in commercially available and the exorbitant cost of chiral separation, the racemic mixture is often utilized in testing.

THE ENHANCED UTEROTROPHIC ASSAY

The rodent uterotrophic assay is a tier 1 level *in vivo* bioassay for assessing the estrogenicity of candidate chemicals. The classic endpoint of uterine wet weight is often accompanied by measurements of luminal epithelial cell height [10] and water imbibition. Estrogenic responses can therefore be monitored across a range of physiological, cellular, and molecular endpoints. To properly elucidate the molecular targets of EDC's disruptive effects in estrogen signaling, a comprehensive understanding of the temporal gene expression responses to endogenous estrogens must be elucidated. While it is difficult to perform mechanistic studies *in vivo*, insights can be gained by correlating gene expression changes with phenotypic endpoints [11]. The evaluation and comparison of the molecular, cellular and physiological endpoints of estradiol and EDCs in the rodent uterus facilitate a better understanding of estrogenic responses and their disruption by EDCs.

The combined physiologic, morphologic and molecular assessments of estrogenicity have been termed the enhanced uterotrophic assay [12], and includes measures of sensitive uterine parameters previously described (uterine wet weight, epithelial cell height and changes in gene expression of well characterized estrogenic genes). Advances in genomic technologies provide researchers with high-throughput tools such as microarray technology which allow the simultaneous measure of relative gene expression changes across thousands of genes at a time. This technology is well suited to addressing studies of estrogenicity because estrogens mediate their responses through ligand-dependent transcription factors which are responsible for changes in gene expression.

SPECIES-COMPARISONS

It is understood that most biological information is gained for the benefit of humans, thus biological data in the form of genetic functional annotations regarding the molecular mechanisms, cellular locations and biological pathways involved in each biological problem we research will be transferred to apply to the human case or related applications. This is especially relevant in the areas of toxicology and pharmacology where the molecular mechanisms and causal relationships that apply to safety and efficacy assessments are understood in the context of the model system or surrogate species.

The application of genomics data requires *a priori* knowledge of genome annotation and orthologous relationships between the species of interest. Public resources such as HomoloGene and Ensembl provide comprehensive orthology mappings

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between all species with sufficient data available for mapping. An understanding of the criteria utilized in making orthology designations are required before adopting, whether that be genome sequence similarity, synteny, phylogenetic tree matching or functional complemenarity. However, once a set of orthology designations are determined, all future comparisons must of course utilize that resource in order for legitimate comparisons to be made.

The identification metric used to map between two species adds a layer of confusion into the analysis if care is not taken to distinguish clones that are known to have unique genome locus, (as with an Entrez Gene identifier) as opposed to genes that are merely being designated as orthologous based upon some other criteria metric. Therefore, an understanding of the differences in numbers of genes and orthologs must always be considered to avoid unnecessary confusion. Furthermore, reporting of numbers of genes must be filtered back through the species specific databases which specify the most current annotation.

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CHAPTER TWO

STATEMENT OF PROBLEM, HYPOTHESIS AND SPECIFIC AIMS

STATEMENT OF PROBLEM

Endocrine disrupting chemicals (EDCs) are a class of exogenous chemicals that cause adverse health effects in intact organisms or their progeny as a result of change in endocrine function. EDCs arise from a variety of natural and man-made sources and have been shown to interact with wild-life to modulate endocrine function with adverse outcomes [1]. Constant, low-level exposure and high chemical stability, combined with the bioaccumulation in the fat of animals higher in the food chain and humans has the potential to achieve biologically significant exposures and adverse health effects over the course of a life time. Estrogen and estrogenic endocrine disrupting chemicals (EEDCs) elicit their effects through the ER, a ligand dependent transcription factor [2]. Thus, evaluating the ER's genomic activities in modulating gene expression is a robust method for assessing the mechanisms associated with an estrogenic endocrine disruptor's adverse health effects. However, full estrogen receptor activation is dependent upon ligand structure and interface in the ligand binding pocket. This phenomenon has been best characterized by a class of pharmaceuticals called selective estrogen receptor modulators (SERMs) which exhibit tissue-specific activities and gene expression based upon the conformational change induced by the ligand [3]. Currently, gene expression biomarkers of exposure are not clearly defined or robust across cell type, organ, sex or species. Cross-species extrapolations from surrogate species to humans are a consistent source of uncertainty in the application of safety or efficacy data to humans. This is no less true in

genomics where the relationships between *cis* and *trans* regulatory factors across species is not readily available for evaluating linkages between gene expression and phenotype.

Collectively, these factors of how endocrine disruptor's adverse health effects are predicted by gene expression changes, the bearing that ligand structure has on the transcriptional behavior of activated ER and the extrapolation of these findings across species have lead to the following hypothesis.

HYPOTHESIS

"Estrogenic endocrine disrupting compounds target a subset of estrogen responsive, uterotrophic genes which are conserved across diverse ligands and rodent models."

SPECIFIC AIMS

In order to test this hypothesis, the immature rodent uterotrophic assay, a sensitive *in vivo* model for evaluating estrogen exposure will be used, incorporating global gene expression profiles to accomplish these specific aims:

- Characterize rat uterine gene expression in response to ethynylestradiol (EE) in the context of the uterotrophic assay.
 - a. Classic rodent bioassay involving three treatments of the potent oral estrogen, EE, over three days and sacrifice and necropsy 24 h following the final treatment.
 - b. Endpoints of uterine wet weight, water imbibition, luminal epithelial cell height, histopathological evaluations and global gene expression will be

monitored to encompass the full uterotrophic response. Custom rat cDNA microarrays will be produced and maintained in-house and utilized for expression profiling.

- c. Dose-response and time course studies will be performed to evaluate the potency and efficacy, as well as temporal dynamics of uterine response.
- d. QRT-PCR will be utilized following microarray experiments to more sensitively and specifically verify select gene responses.
- 2. Characterize the expression of TAM and o,p'-DDT in the rat
 - a. Identical study designs, endpoints, methods and analysis will be used to evaluate TAM and o,p'-DDT induced uterotrophy.
- Conduct cross-species comparisons between the mouse and rat for EE, TAM and o,p'-DDT
 - Mouse data for EE, TAM and o,p'-DDT will be generated through interlaboratory collaborations or personal experimentation using custom mouse cDNA microarrays.
 - b. Orthologous gene relationships between rat and mouse will be utilized to make appropriate comparisons across species.
 - c. An integrated analysis will be used to assess TAM and o,p'-DDT in the context of both species and the positive control estrogen, EE.

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CHAPTER THREE

Kwekel JC, Burgoon LD, Burt JW, Harkema JR, Zacharewski TR. A cross-species analysis of the rodent uterotrophic program: elucidation of conserved responses and targets of estrogen signaling. Physiol Genomics. 2005 Nov 17;23(3):327-42. Epub 2005 Sep 20.

CHAPTER THREE

CROSS-SPECIES ANALYSIS OF THE RODENT UTEROTROPHIC GENE EXPRESSION PROGRAM

ABSTRACT

Physiological, morphological and transcriptional alterations elicited by ethynylestradiol in the uteri of Sprague-Dawley rats and C57BL/6 mice were assessed using comparable study designs, microarray platforms and analysis methods in order to identify conserved estrogen signaling networks. Comparative analysis identified 153 orthologous gene pairs which were positively correlated, suggesting conserved transcriptional targets important in uterine proliferation. Functional annotation for these responses were associated with a number of functional categories including water and solute transport, cell cycle control, DNA replication and energetics. The identification of conserved temporal expression patterns of orthologs provides experimental support for the transfer of functional annotation from mouse orthologs to 44 previously unannotated rat ESTs based on their homology and co-expression patterns. The identification of comparable temporal phenotypic responses linked to related gene expression profiles demonstrates the ability of systematic comparative genomic assessments to elucidate important conserved mechanisms in rodent estrogen signaling during uterine proliferation.

INTRODUCTION

Renewed investigations into the modes of estrogen action have emerged in order to improve the efficacy of selective estrogen receptor modulators (SERMs), a structurally diverse group of drugs, natural products, industrial chemicals and environmental contaminants which elicit tissue specific agonist and antagonist responses. Concerns regarding the potential adverse health effects have also resulted in a comprehensive screening program to assess commerce chemicals and environmental contaminants for their potential to inappropriately activating or antagonizing normal ER function [1, 2]. SERMs typically induce a subset of responsive genes but produce little to no uterotrophic response, while others elicit a strong uterotrophic response but fail to induce the full gene expression spectrum in all target tissues [3-5]. This variability warrants further investigation into the tissue-specific transcriptional program of SERMs in relation to their cellular and physiological endpoints in order to assess the potential adverse effects of exogenous estrogenic compounds and to further elucidate the mechanisms involved in SERM activities. In this study the rodent uterine response to ethynyl estradiol (EE), a prototypic oral estrogen, is assessed to identify conserved responses and targets of estrogen signaling for further studies of estrogenic compounds.

The rodent uterotrophic assay is the gold standard *in vivo* assay for assessing estrogenicity [6-8]. While it provides a robust physiological endpoint, it lacks utility in further elucidating the mode of action of diverse estrogenic compounds. Estrogens primarily exert their effects via binding and activation of estrogen receptors (ERs), which function as ligand-dependent transcription factors. Activated ER complexes recruit coactivators or co-repressors to chromatin leading to the transcriptional modulation of responsive genes [9]. Studies have demonstrated that transcriptional responses can vary

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depending upon the ligand structure which confers differential receptor complex conformations and transactivation activities [10, 11].

The enhanced uterotrophic assay [8, 12], incorporates histological and transcriptional evaluations to complement the uterotrophic endpoint. The current study extends this approach by comparatively examining global gene expression, histological and morphological responses in Sprague Dawley rats and C57BL/6 mice in a comprehensive time course to identify the conserved transcriptional targets critical to the observed molecular and physiological responses to EE. Temporally conserved and divergent transcriptional responses that are phenotypically anchored to histological and morphometric endpoints were identified providing new insights into the conserved modes of action involved in rodent uterine hypertrophy and hyperplasia induced.

MATERIALS AND METHODS

Husbandry - Female Sprague-Dawley rats and C57BL/6 mice, ovariectomized on post natal day (PND) 20 and all within 10% of the average body weight were obtained from Charles River Laboratories (Raleigh, NC) on PND 25. Animals were housed in polycarbonate cages containing cellulose fiber chip bedding (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) and maintained at 40-60% humidity and 23°C on a 12hr dark/light cycle (7am-7pm). Animals were fed de-ionized water and Harlan Teklad 22/5 Rodent Diet 8640 *ad libitum* (Madison, WI), and acclimatized for 4 days prior to treatment. Immature rodent uteri have been used since it is more sensitive to estrogen exposure when compared to the adult ovariectomized animals [13]. *Treatments* - Animals were treated once or once daily for three days via oral gavage with 100 μ g/kg b.w. 17 α -ethynylestradiol (EE) in 0.1 mL of sesame oil vehicle (Sigma Chemical, St Louis, MO) (Figure 3.1). This dose was empirically derived as it elicits a maximal uterotrophic response through the oral route while showing no acute toxic effects. Animals (n=5) receiving a single dose of vehicle or EE were sacrificed 2, 4, 8, 12, 18, or 24 hrs after treatment. Animals receiving one daily dose on three consecutive days were sacrificed 72 hrs after initial dosing, as per the uterotrophic assay. Doses of EE were calculated based on average weights of the animals prior to dosing.

Independent histopathology studies using the same study design (n=4) was performed for each species, with the exception that animals received an intraperitoneal (i.p.) injection of 50 mg/kg b.w. 5-bromo-2-deoxyuridine (BrdU) (Sigma Chemical, St Louis, MO) 2 hrs prior to sacrifice. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Necropsy - Animals were sacrificed by cervical dislocation and animal body weights were recorded. The uterine body was dissected at the border of the cervix and whole uteri were harvested and stripped of extraneous connective tissue and fat. Whole uterine weights were recorded before (wet) and after (blotted) being blotted under pressure with absorbent tissue and were subsequently snap-frozen in liquid nitrogen and stored at - 80°C. Weight due to water was calculated as the difference between the wet and blotted weights. Necropsy for the histopathology study was performed identically to the gene expression time course study with the exception that tissues were not blotted and were placed in tubes containing 1 mL of 10% neutral buffered formalin (NBF) (VWR, West Chester, PA) and stored at room temperature for at least 24 hrs prior to further


Time Course Experimental Design. A comprehensive *in vivo* time course study was performed in which immature, ovariectomized Sprague Dawley rats and C57BL/6 mice were administered an oral doses of 100 μ g/kg b.w. EE or sesame oil vehicle followed by sacrifice and tissue harvest at 2, 4, 8, 12, 18, and 24 hrs after dosing. Another group of animals received a single dose, once per day on three consecutive days followed by sacrifice and tissue harvest 72 hrs after the initial dose per the uterotrophic assay.

processing. Statistical analysis of wet weight and water content were conducted using a two-way ANOVA with a Tukey's Honestly Significant Difference (HSD) post hoc test, n=5, p<0.05 (SAS 9.1, Cary, NC).

RNA Isolation - Total RNA was isolated from whole uteri (~20 mg/uteri) using Trizol Reagent (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. Uteri were removed from -80°C storage and immediately homogenized in 1 mL Trizol Reagent using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was resuspended in The RNA Storage Solution (Ambion, Austin, TX). RNA concentrations were calculated by spectrophotometric methods (A₂₆₀) and purity assessed by the A₂₆₀:A₂₈₀ ratio and by visual inspection of 1 μ g on a denaturing gel.

Histological Processing - Uteri fixed for 24 hrs in NBF were dissected and 5-7 mm midhorn sections were embedded in paraffin according to standard histological techniques. Five µm thick cross-sections containing both uterine horns were cut and mounted on glass slides and stained with hematoxylin and eosin. Additionally, a serial section was cut, mounted and stained with anti-BrdU antibody (BD Biosciences, Palo Alto, CA; Vector Substrate Kit 1 (Vector Red) Vector Laboratories; Burlingame, CA) and counterstained with hematoxylin. All embedding, mounting and staining of tissues were performed at the Histology/Immunohistochemistry Laboratory, Michigan State University (http://humanpathology.msu.edu/histology/index.html).

Histopathological and Morphometric Assessment - Histological slides were evaluated according to standardized National Toxicology Program (NTP) pathology codes. Morphometric analyses were performed on mid-horn cross sections of both uterine horns for each animal using image analysis software (Scion Image, Scioncorp, Frederick,

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Maryland) and standard morphometric techniques. The length of basal lamina underlying the luminal epithelium (LE) and corresponding areas of LE, stroma and myometrium were quantified for multiple representative sectors of each section. Total luminal and glandular circumference were also quantified. Anti-BrdU labeling indices were quantified for LE cell height and stromal compartments on a per-cell, per-area (mm²) basis, respectively. Morphometric analyses for the mouse were limited to LE cell height, stromal thickness and LE BrdU labeling indices as these parameters capture the most sensitive histological endpoints in the uterotrophic assay. Statistical analyses on all morphometry data were performed using a two-way ANOVA with a Tukey's HSD post hoc test, n=4, p < 0.05 (SAS 9.1).

Quantitative Real-Time PCR (QRT-PCR) Analysis - For each sample, 1.0 μ g of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0 μ I) was used as the template in a 30 μ I PCR reaction containing 0.1 μ M each of forward and reverse gene-specific primers designed using Primer3 [14], 3 mM MgCl₂, 1.0 mM dNTPs, 0.025 IU AmpliTaq Gold and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Gene names, accession numbers, forward and reverse primer sequences and amplicon sizes are listed in Supplementary Table 1 which can be found at <u>http://www.bch.msu.edu/</u> \sim zacharet/publications/supplementary/index.html. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems) on an Applied Biosystems PRISM 7000 Sequence Detection System using the following conditions: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was performed to assess the

specificity of the primers and the uniformity of the PCR generated products. Each plate contained duplicate standards of purified PCR products of known template concentration covering six orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number versus threshold cycle (Ct). No template controls (NTC) were also included on each plate. Samples with a Ct value within 2 SD of the mean Ct values for the NTCs were considered below the limits of detection. The copy number of each unknown sample for each gene was standardized to the geometric mean of two house-keeping genes (IA and Rpl7) to control for differences in RNA loading, quality and cDNA synthesis. Statistical significance of differentially expressed genes was determined using two-way ANOVA followed by t-test for vehicle treatment comparisons (SAS 9.1). For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one. Correlations of microarray to real time data was performed using a Pearson's correlation of fold changes relative to VEHs (R Statistical Package 1.9.1) on a per time point basis.

Array Platform - Spotted rat cDNA microarrays were produced in-house from the LION Bioscience's Rat cDNA library (LION Bioscience, Heidelberg Germany) consisting of 8,567 clones representing 3,022 unique genes (Unigene Build #48) which were selected based on their level of annotation as well as sequence similarity to well annotated human and mouse genes. Mouse cDNA arrays of the same platform and construction, consisted of 13,361 features, representing 7,948 unique genes (Unigene Build #48) containing clones from multiple sources including the National Institute on Aging (NIA), Environmental Protection Agency (EPA), IMAGE Consortium and Lion Biosciences. Detailed protocols for microarray construction, labeling of the cDNA probe, sample hybridization and slide washing can be found at <u>http://dbzach.fst.msu.edu/interfaces/</u><u>microarray.html</u>. Briefly, PCR amplified DNA was robotically arrayed onto epoxy coated glass slides (Nexterion, previously Quantifoil, Jena, Germany), using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 32 (8 x 4) Chipmaker 2 pins (Telechem) at the Genomics Technology Support Facility at Michigan State University (<u>http://www.genomics.msu.edu</u>).

Array Experimental Design and Protocols - Temporal changes in gene expression of EE treated rat and mouse uteri were assessed using an independent reference design in which samples from estrogen treated animals are co-hybridized with VEHs. Comparisons were performed on 3 biological replicates x 2 independent labelings of each sample (incorporating a dye swap) for each time point. For the rat study, total RNA (15 μ g) was reverse transcribed in the presence of Cy3- or Cy5-labeled dUTP (Amersham, Piscataway, NJ) to create fluor-labeled cDNA, which was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). Cy3- and Cy5-labeled samples were mixed, vacuum dried and resuspended in 32 μ l of hybridization buffer (40% formamide, 4x SSC, 1% SDS) with 20 µg polydA and 20 µg of mouse COT-1 DNA (Invitrogen, Carlsbad, CA) as a competitor. This probe mixture was heated at 95°C for 2 min and was then hybridized to the array under a 22 x 40 mm LifterSlip[™] coverslip (Erie Scientific, Portsmouth, NH) in a light protected and humidified hybridization chamber (Corning, Corning, NY). Due to the limited amount of RNA isolated from mouse uteri (~8 µg/mouse), a 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1 µg of total RNA, according to manufacturer's specifications, was used for probe labeling in the mouse microarray experiments. Samples were hybridized for 18– 24 hrs at 42°C in a water bath. Slides were then washed, dried by centrifugation and scanned at 635 (Cy5) and 532 nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were analyzed for feature and background intensities using GenePix Pro 3.0 (Axon Instruments Inc., Union City, CA). It is well documented that replicate arrays of identical samples within the same array-labeling protocol or platform (i.e. direct label) render correlation coefficients of approximately 0.85 to 0.95 We are therefore confident in the comparison of array data utilizing direct labeling to those following the Genisphere protocol due to correlations between the two platforms which fall in this same range (http://www.genisphere.com/array_detection_900.html).

Array Data Normalization and Statistical Analysis - Data were normalized using a semiparametric approach [15]. Model-based t-values were calculated from normalized data, comparing treated from vehicle responses per time-point. Empirical Bayes analysis was used to calculate posterior probabilities of activity (P1(t)-value) on a per gene and timepoint basis using the model-based t-value [15]. Gene lists were filtered for activity based on the P1(t)-value which indicates increasing activity as the value approaches 1.0. A conservative P1(t) cutoff of 0.999999 was used to filter the expression data and to define active gene lists for both the rat and mouse data sets. All arrays in both studies were subjected to quality control assessment to ensure assay performance and data consistency through the study (14), and stored within dbZach (http://dbzach.fst.msu.edu), a MIAME supportive relational database that ensures proper data management and facilitates data analysis. Complete data sets with annotation and P1(t) values are available in Supplementary Table 2 and 3 at the website above. *Gene Annotation, Clustering and Interpretation* - Features refer to cDNA clones spotted on the array and are assigned a GenBank accession number, gene name, symbol, and LocusLink ID where annotation is available. Gene names of well-annotated human or mouse orthologs were adopted for rat clones which have limited or no annotation and where sequence similarity (bl2seq) according to a given E-value cutoff ($1x10^{-30}$) indicates orthology. For brevity and consistency, genes will be referenced by their official gene symbol as defined by NCBI's Entrez Gene. A full listing of all abbreviated genes with their full names and Locus link identifiers can be found in Supplemental Tables 2 and 3. Expression data meeting the initial P1(*t*) cutoff were grouped using a k-means cluster algorithm in GeneSpring 7.1 (Silicon Genetics, Redwood City, CA).

RESULTS

As previous gene expression studies [5, 16-18] have focused on murine responses to estrogen, the focus for this study was placed on the rat.

Uterine Wet Weights and Water Content - A 6-fold induction in rat uterine wet weight relative to body weight was observed at 72 hrs (Figure 3.2A), consistent with previous reports [19]. A 10-fold increase in water content accounted for approximately 31% of total uterine wet weight at 72 hrs. This response was preceded by early increases in wet weight (3.3-fold) due to water imbibition [20, 21] between 4 and 12 hrs which subsided by 18 hrs (Figure 3.2B). Eotaxin is the E_2 sensitive chemotactic factor responsible for recruiting eosinophils to the uterine epithelia and stroma where they mediate the edematous response at 6 hrs [22]. The water imbibition response provides an endpoint for estrogen treatment which is not correlated with downstream uterotrophic effects [23] Figure 3.2. Increases in Uterine Wet Weight and Water Content due to EE. Changes elicited in uterine wet weight (A) and water content (B) after oral exposure to 100 ug/kg b.w EE. Changes in uterine wet and blotted weights were recorded at each time point for EE and VEH (n = 5) animals. Water content calculated as the difference between the wet and blotted weights was calculated at each time point for EE and vehicle treated animals. Tissue weights were normalized to body weight for each animal and average weights per group are indicated at each time point. Error bars represent the SEM for the average fold change. * p < 0.05.



FIGURE 3.2

and can therefore serve as a sensitive marker of immediate early gene regulation not causative of proliferation.

A comparable uterotrophic response was observed in the mouse with a 7-fold induction in uterine wet weight. However, the water imbibition response was masked or temporally shifted as increases in weight due to water was not significantly different in EE treated tissues until 12 hrs (Figure 3.2). Some water evaporation from mouse uteri during tissue harvest under the dissection scope may have contributed to a temporal shift in this response due to their small size relative to the rat uterus.

Histopathology - Several histological parameters were modulated by EE at multiple time points (Figures 3.3A-C) in both species. Indications of stromal edema (4-18 hrs) and eosinophil infiltration (8, 12, 24, 72 hrs) were evident in both species consistent with previous reports linking eosinophilia with edema 6 hrs after treatment in the rat uterus [24-26]. Evidence of both luminal epithelial (LE) cell hypertrophy (increases in cell height) at 24 and 72 hrs and stromal and LE cell hyperplasia (numbers of mitotic bodies) at 18 and 24 hrs in EE treated slides indicate diverse and large scale cell type-specific proliferation which culminate in the uterotrophic response at 72 hrs. The marked presence of apoptotic bodies were also observed in stromal, glandular epithelial (GE) cell and LE cell compartments (72 hrs) consistent with the marked decrease in uterine weights in rats after 72 hrs (data not shown). The mouse and rat exhibited similar uterine histology and temporal severity with the exception that endometrial invaginations or ruffling were more pronounced in the mouse at the 72 hr time point as compared to the rat.

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Alterations in Rat Uterine Histopathology due to EE. Rat uterine mid-horn cross sections (5µm) were mounted and stained with hematoxylin and eosin. Stromal edema was evident between 4 and 12 hrs in EE treated samples. Normal and edematous stromal compartments of vehicle and EE treated samples respectively are depicted with arrows (A) from the 8 hr time point. A second section of each block was also cut and immunohistochemically stained with arti-BrdU (red nuclear stain) and hematoxylin. Samples from the 18 and 24 hr (B) EE treated group show marked Brdu staining in the luminal epithelium (LE), stroma, and glandular epithelium (GE) compared to VEH samples. Luminal epithelial cell height, a sensitive morphological marker of estrogen exposure, exhibited a 2.7-fold induction in EE treated samples (C) compared to VEHs at 72 hrs. Bar = 100µm.

Morphometry - Luminal epithelial cell height (LECH) is a classical marker of estrogen exposure [27, 28] and has been used to demonstrate the estrogenicity of a number of structurally diverse ligands [27-30]. LECH was significantly induced at 24 and 72 hrs in both rat (1.6 and 2.7-fold) and mouse (1.4 and 2.1-fold) EE treated samples (Figure 3.4A). Stromal and myometrial thickening are necessary structural modifications and may prove to be equally informative and indicative of estrogen exposure [28]. The response of the stromal compartment (Figure 3.4B) showed moderate increases in overall thickness at early and late time points compared to VEHs. Changes in total luminal circumference (data not shown) loosely paralleled wet weight and LECH changes. In contrast, changes in total glandular circumference (data not shown) were not observed until 72. Glandular epithelia are responsible for secretions produced and deposited into the lumen during normal uterine cycling, thus preparing the uterus for pregnancy after implantation [31, 32]. Increases in uterine gland circumference at 72 hrs therefore indicate a developmental and homeostatic role of estrogen in facilitating this response.

The luminal epithelial hypertrophic response observed at 24 and 72 hrs (Figure 3.4A) is consistent with the hyperplastic growth at 18 and 24 hrs as demonstrated by increased anti-BrdU labeling (Figure 3.4C). BrdU labeling in VEH luminal epithelium exhibited basal level staining of approximately 4% labeled nuclei per total nuclei in rats and was increased in treated samples to 15% and 34% at 18 and 24 hrs, respectively. Minimal, non-significant, staining was observed at all other time points. BrdU labeling in the stromal compartment (data not shown) was strongly correlated with LEC staining (data not shown), and exhibited a 6- and 15-fold induction in BrdU labeled cells per area at 18 and 24 hrs respectively.



Morphometric Analysis of EE Induced Changes in Uterine Cellular and Compartmental Structures. Morphometric methods were used to quantify morphological and immunohistochemical indices of EE response in rat uterine mid-horn histological cross sections. Average luminal epithelial cell height (A) and stromal thickness (B) were calculated for each animal. Averages for each treatment group and time point are indicated for EE and vehicle samples. Immunohistochemical staining for BrdU incorporation into luminal epithelial (C) was performed on EE and vehicle treated samples at each time point and average percentages of labeled nuclei were calculated. Error bars represent the SEM for the average fold change. * p < 0.05.

Mouse morphometric parameters of LE cell height, stromal thickness, and LE BrdU incorporation showed similar responses compared to the rat (Figure 3.4). LE cell height showed significant induction of approximately 1.35-fold at 18 and 24 hrs, and 1.85-fold at 72 hrs respectively, while stromal thickness showed less reproducible [33-35] inductions at multiple time points. LE cells exhibited 26% and 24% labeling with BrdU at 18 and 24 hrs respectively while minimal staining was observed at other time points relative to baseline vehicle levels.

Microarray Data Filtering and Clustering - Empirical Bayes analysis identified 2,652 features representing 1,116 unique genes, which were active at one or more time points in the rat study. Approximately 51% of the active features (~570 genes) were active between 8 and 24 hrs (Figure 3.5) indicating the greatest transcriptional activity begins 12 to 16 hrs prior to peak mitotic activity as indicated in the histopathology and BrdU staining (Figure 3.4E-F). Following the 8 to 24 hrs time points, 4 hrs was the next most active time point with approximately 409 significant changes in expression. In the mouse study, 3,102 features representing 2,313 unique genes were found to comprise the active set. The 1,116 active rat genes obtained from Empirical Bayes analysis were used for clustering and for the elucidation of functional pathways affected by EE. K-means clustering revealed six distinct clusters which distinguished up from down, early from late, and transient from sustained changes in expression (Figure 3.6). The mouse active gene set was clustered in the same manner resulting in 6 comparable clusters (data not shown).

QRT-PCR verification of Microarray Results - Twenty active rat genes identified in the microarray analysis were selected for verification by QRT-PCR analysis (Figure 3.7).



Number of Active Genes per Time Point. The distribution of active genes (P1(t) > 0.999999) per time point for rat and mouse indicate the most transcriptional activity between 8 and 24 hrs.



K-means Clustering of the Rat Active Gene List. Gene expression data from the 2,844 features comprising the rat active gene list (P1(t) > 0.99999) were best represented by 6 k-means clusters consisting of up immediate-early (A), up middle (B), down early (C), up sustained (D), up late (E) and down sustained (F) patterns of expression. Time and fold change are indicated on the x- and y-axis respectively. The number of features in each cluster is indicated. A black pseudo-line representing the general pattern of expression has been superimposed on each cluster.

Quantitative Real Time PCR Verification of Microarray Results. Quantitative real time PCR was used to verify the temporal gene expression profiles of individual genes selected from microarray data representative of each k-means cluster as well as specific functional categories. Bars (left axis) and lines (right axis where present) represent data obtained by QRT-PCR and microarray analysis, respectively, while the x-axis represents time (hr). The average fold change relative to time matched vehicle controls for 3 animals per group is shown. Genes are indicated by their official gene symbols. Error bars represent the SEM for the average fold change. *p < 0.05 for QRT-PCR.



FIGURE 3.7A



FIGURE 3.7 (continued)

Genes were selected to represent a majority of both the temporal patterns identified by kmeans clustering and their respective functional categories in addition to their robust response. Patterns of temporal expression between the array and ORT-PCR data were correlated using a Pearson's correlation on the fold changes relative to VEHs. An average correlation of 0.83 (range: 0.57-0.96, Stat3 correlation of 0.059 excluded) indicated good agreement between the two methods. Stat3 had a weaker correlation with maximal up-regulation of 3.6-fold at 2 hrs in the microarray data, while the ORT-PCR data indicated maximal induction of 7-fold at 4 hrs. While the reasons for this disparity between the methods are unclear, both are consistent with the role Stat3 plays in immediate early expression in response to growth stimulus in proliferating tissues [36]. Compression of microarray expression data compared to other, more sensitive measures of mRNA expression such as QRT-PCR has been previously documented [37], and was observed with C3, Cabl3, Aqp8 and Tk1. Two classically estrogen induced and repressed genes, Egr1 and Clu, respectively, [12, 38-40] which were not represented on the array were also analyzed by ORT-PCR. Egr1 was significantly up-regulated 7- and 21-fold at 2 and 4 hrs respectively (data not shown). Clu was repressed 14-fold at 72 hrs.

Functional Annotation of Rat transcriptional responses - Further analysis and interpretation of the gene expression data was facilitated by the identification and population of functional gene categories with active genes from the microarray data set. Phenotypically anchored changes in expression were tentatively assigned functions based on associations with the histopathological assessment, manual annotation obtained from the reported literature, and computationally extracted, over-represented Gene Ontology (http://www.geneontology.org) associated functional annotations. Categories of response

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Functional Category	Accession	Link	Symbol	Regulation	Fold change	Time Points (hrs)	Cluster
Proliferation	NM 021835	24516	Jun	•	5	2	• ۲
	XM_234422	314322	Fos	◄	4-40	2,4,8	۲.
	NM_012747	25125	Stat3	◄	1.5-3.5	2,4,8,12	۲.
	XM ²³⁷⁹⁹⁹	291005	Gadd45g	4	3-19	2,4,8,12,18	< 4
	NM_019296	54237	Cdc2a	4	4	24	Ξ
	NM_133571	171102	Cdc25a	4	7	12,18	en a
	NM ⁰⁰³⁸⁷		Tkl	4	2.3-4.5	18,24	Ξî
	NM_024403	79255	ATF-4	4	2-3	2,4,8,12,18	n B
	NM_178866	24482	Igfl	4	2-7	2,4,8,12,18,24,72	Ξų
	NM_171992	58919	Ccnd1	▲ & ▼	1.7 & 1.6	8,12,18 & 72	B i
	NM_053593	94201	Cdk4	4	1.8	8,12,18,24	n u
	NM_017282	29672	Pmsa5	◀	7	8,12,18,24	n u
	NM_138548	191575	Nmel	4	3.3	8,12,18,24	n (
	AF305713		Tgfbi	•	2-6	2,4,8,12,18,24,72,96	с I
	NM 053743	114562	Cdc37	•	3-5 2	12,18,24,72,84	ا بنا
	NM_012588	24484	Igfbp3		2-5	ALL	ц
Water/ion	NM 019158	29172	Aqp8		2.5	8,12	8
Movement	NM_031648	58971	Fxydl		2-7	24,72,84,96,120,144	U I
	XM_343137	25480	Mip	•	2-8	2,4,8,12,18,24,72,84,96,120	ום
	NM_012779	25241	Aqp5		24	4,12,18,24,72,84,96	<u>ت</u> (
	NM_022388	64190	Fxyd4		2-4	4,8,12,18,24	Ľ.
Angiogenesis	NM 031836	83785	Vegf		2.5	2,4	A
mmune	NM 053412	84472	EIII		7	8,12,18	£ A
Response	NM_031051	81683	Mif	4	1.7-2.5	12,18,24	2 1 1
	XM_346863	360452	ប	•	4-17	24,72,84,96,120	ш I
	NM 001002805	406161	C4-2	∀& ▲	2.7 & 3	24 & 72,84,96,120	ш
Fatty Acid	NM 145878	140868	Fabp5		2.5-4.6	8,12,18,24	Ξî
Metabolism	NM_053607	94340	Fac15	4	1.5-2	8,12,18,24,72,96	æ í
	NM_053445	84575	Fads1	◄	2.7	24	n u
	NM_017332	50671	Fasn	4	6	12	n u
	NM_130826	170670	Hadha	4	1.5-2.2	12,18,24	Ξ ú
	NM_057186	113965	Hadhsc		6	8,12	מי
	NM_053407	84431	Asah	</th <th>1.5-2.3</th> <th>2,4,8,18,24</th> <th>מנ</th>	1.5-2.3	2,4,8,18,24	מנ
	NM_012598	24539	I ⁻ PI		ы. Р. С	18 12_72_84_96	ייי נ
	1700CO MN	73710	10102	v sd mehiolo ocen			

Table 3.1A Functional Categories of Response

a Fold change of EE treated samples relative to time-matched vehicle controls. b Time points at which $p(1) \ge 0.999999$. c K-means clusters as shown in Figure 5.

Functional		Locus	Gene		Ec.14	4	ບ
Category	Accession	Link	Symbol	Regulation	change	Time Points (hrs)	Cluster
Cholesterol	NM_022389	64191	Dhcr7	•	2-4	8,12,18,24	B
Regulation	NM 019238	29580	Fdfl	4	m	8,12,18,24	B
	NM_053539	89784	Idil	•	2-6.8	2,4,8,12,18,24,72	B
	NM_031062	81726	рлМ	•	2-3.4	12,18,24	B
	NM_017136	29230	Sqle	•	3-5	8,12,18,24	B
	NM 080886	140910	Sc4mol		2-3	8,12,18,24	В
Redox	NM 053800	116484	Txn		1.7-2	8,12,18,24	B
Related	NM 031614	58819	Txnrd1	•	2.5-4	4,8,12,18,24	B
	NM 032614	58815	Txnl2	•	7	8,12,18,24	B
	NM 012844	25315	Ephx1	•	2-4.5	4,8,12,18,24	ပ
	NM 057114	117254	Prdx1	•	1.3-2	4,8,12,18,24,72,84,96	D
	NM 183403	29326	Gpx2	•	2-12	4,8,12,18,24,72,84,96	۵
Metabolism	NM 012571	24401	Gotl	•	2.2	8	В
Enzymes	NM_138515	171522	Cyp2d18	►	7	12,18	с С
	NM_153318	266689	Cyp4f6		2-3	8,12,18,24	U
	NM 178847	301517	Cyp27a1	►	2-3	4,8,12,18,24	ပ
	NM_057101	24298	Cyp21a1	►	2-3	8,12,18,24	с С
	NM_172038	64352	Gstm5		2-4	4,8,12,18,24,72	с С
	NM 134349	171341	Mgstl	►	7	4,8,12,18,24	с С
	NM 016999	24307	Cyp4b1	•	2.5-5	12,72,84,96,120,144,168	ц
	NM 031834	83783	Sultlal		2-7	4,8,12,18,24,72,84,96	F
Apoptosis	NM_053713	114505	Klf4	•	2-4	2,4,8,12,18,24,72	A
	NM 053720	114512	Aatf	4	2-3	4,8,12,18	B
	NM_031356	83533	Pdcd8	◄	ы	12,18,24	B
	NM_172322	282817	Pycard	•	2.3	72,84,96	Е
a Fold change of I	E treated samples	relative to 1	time-matche	d vehicle contro	ols.		

Table 3.1B Functional Categories of Response

b Time points at which p(1)t>0.999999. c K-means clusters as shown in Figure 5.

evident from histopathology include edema, hyperplasia, hypertrophy, immune cell response, and apoptosis. Those identified through Gene Ontology, and manual annotation include proliferation (e.g. signaling proteins, protein biosynthesis and turnover, cell cycle control, replication), angiogenesis, fatty acid metabolism, cholesterol biosynthesis, redox control, and xenobiotic metabolism (Table 1).

Comparison of EE Elicited Rat and Mouse Uterine Responses - The development, growth and regulation of the uterus are critical for reproduction and thus the regulatory events affecting these processes are expected to be highly conserved between species. Comparative analysis of gene expression serves to verify genome-wide approaches to assay conserved transcriptional responses to a common stimulus. Rat and mouse orthologous gene pairs were derived from Ensembl and HomoloGene. However, these databases are often incomplete or overly-conservative in identifying orthologous gene pairs. Therefore, additional orthologous relationships (17% of those reported) were manually derived based on mRNA sequence similarity as determined by Bl2Seq analysis using an E-score cut-off of 1×10^{-30} when the rat and mouse gene also shared a common official gene symbol. Determinations of estrogen responsiveness, orthology, and unique gene annotation were collated resulting in a list of 211 reciprocally active rat and mouse orthologs, (Figure 3.8).

Orthologous genes that are reciprocally active do not necessarily display similar directional or temporal patterns of expression. Therefore, to determine if the orthologous gene pairs had comparable temporal expression patterns a Pearson correlation analysis was performed according to temporal fold-change on a gene by gene basis (Figure 3.9). This analysis identified 153 positively- (1.0 to 0.1) (Table 2), 12 non- (0.1 to -0.1), and

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Species Comparison of Array Composition, Gene Activity and Orthology. Array features and represented unique genes as determined by LocusLink ID on each array are shown. The number of orthologous relationships between the rat and mouse array as defined by Ensembl and Bl2Seq comparison of RefSeq mRNAs with similar gene symbol and E-value < 10^{-30} . Active genes determined by a P1(t) > 0.999999 at any time point for both species.



Temporal Correlation of Reciprocally Active, Orthologous Genes. Reciprocally active, orthologous genes were temporally correlated according to fold change of EE treated samples relative to time matched vehicle controls using a Pearson's correlation. Orthologs with a correlation of -1 to -0.1, -0.1 to 0.1 and 0.1 to 1 were designated as negatively-, un- and positively correlated respectively. The cDNA array probe sequences for orthologs not positively correlated were subsequently compared using Bl2Seq analysis; sequence alignments having an E-score of 10⁻³⁰ or less were considered to be overlapping probe pairs. 46 negatively- (-0.1 to -1.0) (Table 3) correlated orthologous gene pairs. Agglomerative hierarchical clustering (GeneSpring v7.1) of the 153 positively correlated genes indicates that gene expression patterns between 8 and 24 hrs display similar patterns of expression within species while gene expression at early (2-4 hrs) and late (72 hrs) time points clustered by time point, rather than species (Figure 3.10). Of the 153 positively correlated, 95 showed a common pattern of up-regulation between 8 and 24 hrs indicating a high proportion of conserved gene expression changes, and suggesting that the mechanisms of regulation are conserved. A number of these genes have been previously reported in microarray studies [5, 16, 17, 20, 23, 33, 41-45] to be responsive to estrogen treatment in the rodent uterus (Table 2).

cDNA probe sequences for the 54 orthologs not positively correlated were subsequently compared to determine if they queried comparable regions of the gene using Bl2Seq analysis. Sequence alignments with E-score of 10⁻³⁰ were considered to be overlapping probe sets. Thirty of the 58 non-positively correlated ortholog pairs were found to have overlapping sequences indicating that the same region of the gene was queried, and therefore differentially regulated.

DISCUSSION

The enhanced rodent uterotrophic assay was performed using EE as a prototypic estrogen in order to identify and phenotypically anchor conserved gene expression profiles important for uterotrophy. The analysis of these conserved targets provides baseline transcriptional program information involved in the uterotrophic response through the elucidation of the functional significance of these gene expression changes



Hierarchical Clustering of Positively Correlated Rat-Mouse Orthologous Gene Expression Profiles. The 153 orthologous pairs were hierarchically clustered by gene and time. Rat and mouse data are indicated in red and black text respectively and time points (hrs) are labeled. Orthologs for 2, 4, and 72 hrs cluster by time between species while data points from 8 to 24 hrs cluster by species indicating a high level of temporal conservation between species in early and late responses while data points between 8 and 24 hrs are not distinguishable between species. and their putative functions as reported within Gene Ontology annotations (www.geneontology.org) and the published literature.

Increases in uterine wet weight were preceded by conserved and coordinated gene alterations in multiple functional categories including cell cycle control, redox control, DNA replication, protein synthesis and transport, pro- and anti-apoptotic genes, xenobiotic metabolism, cell-cell communication and angiogenesis. Several gene expression profiles populate each of these categories and collectively serve as the basis for the development of a uterine molecular finger print of estrogen exposure. Furthermore, these conserved targets of estrogen signaling in the rodent uterus may also support extrapolations to human uterine responses.

Previous rat uterine gene expression studies have reported similar gene expression profiles in response to estrogen [3, 41]. However, the study designs used limit the interpretation of the functional significance of the coordinated responses. For example, Naciff, et al. [41] utilized pooled uterine and ovarian tissues from rats treated subcutaneously with EE for dose-dependent gene expression studies which confound the elucidation of tissue-specific responses due to the differential distributions of ER α and ER β [46, 47] in the uterus and ovaries. Moreover, an important consideration in modeling estrogenicity is the fact that the primary route of exposure to diverse estrogenic compounds in human populations is oral. Diel, et al. [8] supplemented the uterotrophic assay by evaluating the expression of a select number of known estrogen responsive genes at 72 hrs in conjunction with standard gravimetric and histological evaluations [8]. However, using a small number of transcriptional markers to determine estrogenicity limits the ability to phenotypically anchor gene expression changes [48].

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		Rat			Mouse	
Functional	C		b			b
Categories	Gene	a Time Point	Fold	Direction	Time Point	Fold
Ű	Symbol	Time I out	Change		I me I ome	Change
Signaling	Fos	2,4,8	4.4 to 40.2		2,4,12	2.9 to 5.9
00	Gjal	8,12,18,72	2.3		4,8,12,18	1.8 to 2.7
	Igfl	2,4,8,12,18,24,72	3.7 to 7.1		8,12,18,24	3.1 to 4.4
	Jun	2	2.6		2,18,24,72	0.3 to 1.6
	Map2k2	8,12,18,24	2		8,12	2
	Mapk3	4,8	0.5	▼	4,8,12	0.4 to 0.6
	Mdk	72	0.4	▼	72	0 to 0.4
Transcription	Atf4	2,4,8,12,18,24	1.3 to 3.1		2,4,8,12,18,24	1.7 to 4.4
	Bcap37	4,8,12,18,24	1.4 to 2		8,12,18,24	1.8 to 2.4
	Idb1	4,18,72	2.1 to 2.5		2,4,24,72	0.5 to 3.1
	Rere	4,8,12,18	0.4 to 0.5	▼	4,8,12,24,72	0.4 to 0.5
	Irf7	4,24,72	1.9 to 3.6		24,72	2.1
RNA Processing	Bopl	2,4,8,12,18,24	1.4 to 3.2		4,8,12,18,24	2 to 2.7
	Hnrpab	2,4,8,12,18,24	1.6 to 3.1		4,8,12,18,24	2.3 to 3.3
	Ppmlg	4,8,12,18,24	1.5 to 2.3		4,12,18	1.9 to 2.7
Amino Acid	Asns	4,8,12,18	1.7 to 2.9		4,8,12,18,24	2 to 5.2
Biosynthesis	Fah	12,18	0.3		12,18,24,72	0.3 to 0.5
	Phgdh	12,18,24	2.1 to 2.3		8	2.9
	Got1	2,4,8	2.5 to 2.8		4	3.6
Translation	Dnajc3	8,12,18,24	1.6 to 2.3		8,12	2.7
	Eif2s2	2,4,8,12,18,24	1.8 to 2.7		4,8,12,18,24	2.4 to 4.3
	Eif4e	4,8,12,18,24	1.6 to 2.1		4,12,18	1.9 to 2.7
	Eif5	4,8,12,18,24	2 to 3.3		4,8,12,18	1.5 to 1.9
	Gspt1	2,4,8,12,18,24	1.5 to 2.6		4,8,12,18,24	2.1 to 2.6
Protein Folding	Cct3	4,8,12,18,24	1.9 to 2.3		4,8,12,18,24	2.2 to 3.2
and Transport	Cct4	4,8,12,18	1.7 to 2.3		4,8,12,18	1.4 to 1.6
	Ran	4,8,12,18,24	3.6 to 5.2		4,8,12,18,24	2.5 to 3.2
	Timm23	2,4,8,12,18,24	1.3 to 2.2		4,8,12,18,24	2 to 2.5
	Icpl	4,8,12,18,24	1.7 to 2.4		4,8,12,18,24	1.6 to 2
Heat Shock	Hspa4	4,8,12,18,24	2.5 to 3		4,8,12,18,24,72	2 to 3.4
Proteins	Hspas	4,8,12,18,24	2.1 to 2.3		4,8,12,18,24,72	2.6 to 4.4
	Hspað	4	2.7		4,8,12	2.7 to 3.9
	Hspca	2,4,0,12,10,24	1.9 to 3.4		4,0,12,10	2.1 10 2.0
Durthin	Nodde	4,8,12,18,24	$\frac{2.0103.0}{1.8 \pm 0.10}$		0,12,10,24	$\frac{2.5 \text{ to } 3.0}{1.4 \text{ to } 1.0}$
Protein	Dema/	0,10,24	1.0 10 1.9		4,0,12,10,24	1.4 10 1.9
Turnover	Psma5	10,24	$\frac{2}{13 \text{ to } 10}$		12,10	2.2 to 2.4
	Psillas Dembs	4,0,12,10,24 9 10 19 01	1.5 to 1.9		4,0,12,10,24	1.5 to 2.5
Call Culas	Cond1	8 12 18 72	1.5 to 1.7		18	2.1 10 5.1
Central	Cdc22	0,12,10,72 7A	42		18 24 72	33 to 66
Control	Ndro?	48121824	0.2 to 0.5		8 12 18 24	0.4 to 0.5
	Phh	48171874	1.5 to 7.4	Å	8 12 18	1.7 to 2
	Sfrn1	8 12 18 24 72	0.3 to 0.5	T T	8 18 24 72	0.4 to 0.4
DNA	Fen1	24	27		18.24	2.3
Benlication	Hmgh?	24	2.7		18.24	2
Tepication	Nme1	8,12,18,24	3.1 to 3.6		4,8,12,18,24	2.5 to 6.2

Table 3.2 Conserved and Coordinated Estrogen Responsive Genes in the rodent uterus

a Time points at which p(1)t>0.999999.

b Fold change of EE treated samples relative to time-matched vehicle controls

		Rat				Mouse	
Functional Categories	Gene	a Time Point	Fold	Dire	ction	a Time ^a Point	Fold
	Syllinoi		Change				Change
Energetics	Atp5g1	8,12,18,24	2.2 to 2.5		•	4,8,12,18,24	1.7 to 3.4
	Ckb	2,4,8,12,18,24	2 to 3.6		•	4	2.7
	Ckmt1	72	8.4		•	72	5.3
	Cycs	2,4,8,12,18,24	2 to 3.7		•	4,8,12,18,24	2.8 to 4.6
	G6pdx	8,12,18,24,72	1.7 to 2		•	4,8,12,18,24,72	2.3 to 3.8
	Pgk1	8,12,18,24	2.8 to 3.2		•	8,12,18,24,72	1.9 to 3.7
	Slc25a4	8,12,18,24	1.4 to 1.9		•	8,12,18,24	1.6 to 2
	Slc25a5	4,8,12,18,24	2.2 to 3.1			4,8,12,18,24,72	3.5 to 5.3
Fatt Acid/	Acadm	12,18	0.6 to 0.7	1	/	8,12,18,24,72	0.5 to 0.6
Cholesterol	Sc4mol	8,18,24	2.7 to 3.2		•	4,8,12,18	1.6 to 2.1
Biosynthesis	Sqle	8,12,18,24	3.3 to 4.9			8	2.4
	Lpl	18	0.3	1	•	18	0.2
	Арос	8,12,18,24	0.3 to 0.4		/	18	0.4
Redox	Seppl	4,8,12,18,24,	0.2 to 0.4	1	•	4,8,12,18,24	0.4 to 0.5
Regulation	Txnl2	8,12,18,24,	1.6 to 2.3		•	4,8,12,18	1.5 to 1.9
	Txnrd1	2,4,8,12,18,24,	1.7 to 3.9		A	4,8,12,18,24,72	1.7 to 2.1
Vesicle	Arf2	2,4,8,12,18,24,	1.4 to 2		•	4,8,12,18	1.4 to 1.7
Transport	Arf4	4,8,12,18,24,	1.4 to 1.9		•	4,8,12,18	1.7 to 2
	Hexa	8,12,18	0.4 to 0.5	1	•	8,12,18,24	0.5 to 0.7
	Rabggtb	4,8,12,18,24	1.4 to 2.4		▲	4,8,12,18	1.9 to 2.7
	Ssr4	8,12,18,24,72	2 to 2.6		•	8,12,18,24,72	1.5 to 2.3
Xenobiotic	Cyp21a1	8,12,18,24	0.3 to 0.4		•	12,18,24	0.4 to 0.5
Metabolism	Cyp4b1	12,18,72	0.4 to 0.4		•	12,18	0.4 to 0.4
	Ephx1	4,8,12,18,24	0.2 to 0.4		•	4,8,12,18,24,72	0.2 to 0.5
	Mgst1	4,8,12,18,24	0.3 to 0.6		V	4,18,24	0.2 to 0.2
Cell Structure	Krt1-19	4,8,12,18,24,72	1.6 to 3.4		•	12,18,24,72	1.6 to 2.3
	Lmna	4,8,12,18,24	1.5 to 2		•	8,12,18,24	1.8 to 2.3
	Tubb5	8,12,18,24	2 to 2.9			12,18,24	2.4 to 3.1
Water/Ion	Atp1b3	2,4,8,12,18,24,72	1.6 to 4.2		•	4,12,18,24,72	1.5 to 2.1
Channel	Aqp8	8,12	2.2		•	4	2.3 to 2.9
	Fxyd4	24,72	0.3 to 0.3			72	0 to 0.5
	Slc9a3r2	72	0 to 0.5		/	72	0 to 0.4
Hematopoiesis	Gata2	8,12,18,24,72	0.5 to 0.6		•	24,72	0.4 to 0.5
	Hba-al	24,72	0.3 to 0.3			18	0.3 to 0.3
	Hbb-b2	24,72	0.4 to 0.4		V	12,18,72	0.2 to 0.4
Uncategorized	Armet	2,4,8,12,18,24	1.5 to 3.6		•	4,8,12,18,24,72	3.9 to 9.7
or Other	C3	18,24,72	4.1 to 9.2		A	72	0 to 3.6
,	Calr	4,12	2.3 to 2.7			8,12,18,24	2.5 to 4.6
	Сре	4,8,12,18,24,72	0.2 to 0.6			8,12,18,24,72	0.2 to 0.4
	Enpp2	12,18,24,72	0.2 to 0.3			8,12,18,24,72	0.3 to 0.4
	Odc1	2,4,8,12,18,24	1.7 to 4.8		A	4,8,12,18,24	1.9 to 3.4
	Penk-rs	18,24,72	0.4 to 0.5	'		4,8,12,18,24,72	0.2 to 0.4
	Serpinhl	8,12,18,24	2.3 to 2.8	4		4,8,12,18,24	1.9 to 3.7
	Xpnpep1	8,12,18,24	1.7 to 2.1			8,12,18,24,72	2 to 2.4

Table 3.2 (continued) Conserved and Coordinated Estrogen Responsive Genes

a Time points at which p(1)t>0.999999.

b Fold change of EE treated samples relative to time-matched vehicle controls

Global assessments facilitate a more comprehensive determination of transcriptional targets in the physiological context as well as identifying mechanistically-based biomarkers. Nevertheless, there is good agreement between these studies which currently can only be appreciated on a qualitative basis due to the limited reporting of the data required to fully correlate their expression profiles.

EE induction of uterine wet weight is a well conserved phenotypic response. The strong conservation of this response is due to the exemplary manner in which the immature, ovariectomized rodent uterotrophic assay exhibits synchronized cell cycle progression and subsequent proliferation in response to novel estrogen exposure [49]. The wave of uterine cell growth in response to estrogen administration has been previously characterized at the gene expression level in mice [5, 16] while responses in the rat and comparative rodent studies have not been reported. In this study, rat and mouse data sets were normalized and statistically analyzed, utilizing identical array platforms to facilitate comparisons. Orthologous gene pairs exhibiting a positive correlation were assigned to functional gene categories (Table 2) in order to annotate their mechanistic roles and facilitate phenotypic anchoring.

Immediate-early genes and signaling molecules are the first line of EE responsive, conserved responders which initiate down stream cascades of transcription and translation. Many genes are regulated through AP-1 [50], a major mitotic effecter and transcription factor composed of c-Jun and Fos which are both immediate-early genes induced by estrogen in the rodent uterus. Igf1, another major estrogen mediator which binds its membrane bound receptor and activates Mapk signaling cascades [51], was also up-regulated between 4 and 24 hrs. Two members of this Mapk cascade were regulated

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			Rat					Mouse	
Functional Category	Gene Symbol	Locus Link	Active Time Points	Fold Change	Direc	tion	Locus Link	Active Time Points	Fold Change
Chromatin Remodeling	Smarcd2	83833	4,8,12,18,	0.4 to 0.6	•	•	83796	4,8,12,18,24,72	2.5 to 3.7
RNA Processing	Cugbp2	29428	8,12,18,24,	0.5 to 0.6	•	•	14007	12,18,24,72	1.8 to 2
Cell Cycle Control	Cdc37	114562	12,18,24,72	0.2 to 0.2	•	•	12539	4,8,12,18,24,	1.6 to 2.9
Matabalian	Dlat	81654	12,18,24,	1.6 to 2.2	•		235339	4,8,12,18,24,72	0.1 to 0.4
MCGADOIISII	Tcn2	64365	8,12,18,24,	0.4 to 0.5	•	•	21452	4,12,24,72	3.1 to 9.3
Energetics	Ca2	54231	8,12,18,24,	0.4 to 0.5		•	12349	12,18,24,72	4.5 to 6
Lipid Transport	Apoe	25728	8,12,18,24,	1.8 to 2.1	•	•	11816	8,12,18,24,72	0.2 to 0.4
Protein Turnover	Cstb	25308	8,12,72	0.5 to 1.5	•	•	13014	4,8,12,	2 to 2.7
a Time points a	at which p(1)t>0.999	.666	200	1	1			

TTA 5 . C 5 L Later Tolland Die b Fold change of EE treated samples relative to time-matched vehicle controls

by EE with Map2k2 up-regulated and Mapk3 was down-regulated. Mdk, a growth factor with undetermined function was down regulated at 72 hrs. Its absence could possibly play a role in halting growth at peak uterine size. Gja1, while not a signaling molecule itself, encodes a gap junction protein crucial for cell to cell communication during concerted tissue responses to mitotic stimuli [52] and exhibited conserved up-regulation in both species between 8 and 18 hrs.

Conserved gene expression changes involved in transcription included Atf4, Bcap37, Gata2, Id1, Rere, and Irf7. Rere acts as a transcriptional co-repressor through interactions with HDAC1 and also plays a role in pro-apoptotic signaling [53, 54], consistent with its sustained down-regulation at mid time points in rats and mice. Conversely, Bcap37, referred to as REA for repressor of estrogen receptor activity, interacts with HDAC1 to repress nuclear receptor mediated transcription [55] and was up-regulated for several intermediate time points in both species. There was also conserved induction of Bop1, Hnrpab, and Ppm1g, which are involved in mRNA processing. Hnrpab, a heterogeneous nuclear ribonucleoprotein involved in mRNA splicing and processing has been shown to be a methylation target of Hrmt112 [56], a protein-arginine methyltransferase which was also up-regulated by EE in the rat. The Ppmg1 gene encodes a phosphatase required for spliceosome assembly [57].

Following transcriptional activation and mRNA processing there was induction of protein synthesis, processing, and turnover. Conserved responses included the amino acid biosynthesis genes: Asns, Got1, Fah, Phgdh; translation related components: Eif2s2, Eif4e, Eif5, Gspt1, Dnajc3; protein folding and transport: Cct3, Cct4, Tcp1, RAN; Heat shock proteins: Hspa4, Hspa5, Hspa8, Hspca, Hspe1; and protein degradation related

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genes: 26S Proteasome complex members (Psma4, Psma5, Psma6, and Psmb5), and Nedd8. Gspt1 functions as the eukaryotic releasing factor in mediating translational termination via interaction with poly-A binding proteins [58] and was up regulated ~ 2.5 fold between 4 and 24 hrs. Tcp1, a component of Cct3 and Cct4 (chaperonin containing Tcp1 subunits 3 and 4) were all coordinately up-regulated. These genes encode chaperone proteins involved in stabilization of un- or mis-folded proteins and are important in cyclin E stabilization [59] which is important in cell cycle progression. In both rats and mice, Phgdh, an important enzyme in L-serine biosynthesis, was induced at mid time points while Fah, the rate limiting and final enzyme step in tyrosine synthesis, was down-regulated, suggesting that it may have other yet unknown conserved functions. Specific signals controlling and inducing cell cycle progression (Ccnd1, Cdc2a, Phb, Ndrg2, Sfrp1) and DNA replication (Fen1, Hmgb2, Nme1) were also conserved. Estrogen responsive Cend1 and Cdc2a, key regulators of G1/S-phase, and G2/M-phase transition respectively [34, 60] were both up-regulated, suggesting concerted uterine cell cycle transitions induced by EE in the ovariectomized model.

Subsequent to increases in translational activity and protein content are conserved induction of protein degradation and turnover genes [46, 61] such as those involved in the 26S proteasome complex (e.g., Psma4, Psma5, Psma6, and Psmb5). Nedd8 is involved in proteasome mediated degradation of ER α [33]and was up regulated at mid time points. APP-binding protein 1 (App-bp1)/Uba3, the catalytic subunit of the Nedd8-activating enzyme was up-regulated at similar time points in the mouse. Furthermore, Appbp1 is a binding partner of amyloid precursor protein (App) which has been implicated in Alzheimer's disease [36]. App expression was down regulated in both species. Itm2b which has similar behavior and function as APP in diverse brain lesions associated with dementia [62], was also down-regulated in parallel with App. This is possibly due to the down regulation of Tgfbi seen in the rat study. Tgfbi has been shown to positively regulate App [36]. It is unclear what role App plays in regulating the Nedd8 degradation pathway but the network of interactions is suggestive of a regulatory mechanism involved in ER degradation via the 26S proteasome.

Increased energy demands were reflected in both species by the induction of genes involved in mitochondrial (Cycs, Atp5g1, Ckmt1, Slc25a4, Slc25a5) as well as cytosolic (Ckb, G6pd Pgk1) energy production and homeostasis. Slc25a5, an ADP/ATP translocase, catalyzes the exchange of cytosolic ADP for mitochondrial ATP and has been implicated as a marker of cell growth and proliferation due to its key role in regulating cytosolic energy needs [63]. G6pd on the other hand functions mainly in generating the other energy currency of the cell, NADPH, which is used in many cell processes including fatty acid and cholesterol biosynthesis via the pentose phosphate pathway [64]. Several genes involved in the biosynthesis or transport of membrane precursors were also regulated by EE including Acadm, Apoc1, Lpl, Sqle, and Sc4mol. Acadm and Lpl, enzymes in the fatty acid β -oxidation pathway, were down regulated. In contrast, Sc4mol which catalyzes the final C-4 methyloxidation step in cholesterol formation [65], was up regulated, consistent with the need for building blocks for new membranes demanded by rapid cell division [35].

Despite the overlaps in gene expression profiles, several divergent patterns were identified. Apoe, a known regulator of lipid transport and uptake in the liver, was induced in the rat while repressed in the mouse at intermediate time points. Carbonic anhydrase 2, which catalyzes the reversible hydration of carbon dioxide to carbonate, has recently been shown to be crucial for adenogenesis (gland development) in the uterus [46]. The fact that Ca2 expression was repressed in rat while induced in the mouse could explain increased glandular invaginations observed in mouse but not rat at 72 hrs. Cdc37, a regulator of Hsp90 phosphorylation, as well as Cstb, Cugbp2, Dlat, Smarcd2, and Tcn2 were also differentially regulated at similar time points. Bl2Seq sequence comparisons of these differentially regulated orthologous pairs indicated that several of these cDNA probes overlapped comparable transcript regions (Table 3). Therefore, their differential expression can not solely be attributed to probes querying different transcript regions. Consequently, EE regulation of these gene expression responses are not conserved suggesting that their role is not critical for the uterotrophic response.

This comparative study has comprehensively assessed the physiological, morphological and transcriptional programs elicited by EE in the rat and mouse uterus using comparable study designs, assay platforms and analysis methods. 153 reciprocally active and positively correlated gene expression profiles were identified, suggesting theses are important responses that share a conserved mode of action. Moreover, comparable temporal expression patterns provide further evidence that orthologous genes are functionally related, supporting the transfer of mouse functional annotation to unknown rat ESTs with tentative annotation. For example, of the 153 reciprocally active and positively correlated gene expression profiles, 36 rat sequences had no official name but a gene symbol, 1 had a name but no symbol, and 7 had neither a name or symbol. Many of these tentative assignments were based only on sequence homology, but the coexpression data described here provides empirical support that the genes are orthologous
and functionally related. Comparable approaches have recently been used to identify functionally related genes across more divergent species (e.g. extrapolations between human, worm, fly and yeast [66, 67]; extrapolating bacteria, yeast, and fly annotation to the worm [66, 67]). Additional analyses including dose response studies, promoter response element comparisons, and cell-type specific assessments will not only further elucidate the importance of these conserved and divergent EE-induced uterine proliferation responses, but will also provide additional evidence regarding the conservation of estrogen response between rodent and human orthologous gene pairs.

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CHAPTER FOUR

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CHAPTER FOUR

TAMOXIFEN ELICITED UTEROTROPHY: CROSS-SPECIES AND CROSS-LIGAND ANALYSIS OF GENE EXPRESSION

ABSTRACT

Tamoxifen (TAM) is a well characterized breast cancer drug and selective estrogen receptor modulator (SERM). TAM's partial agonist activation of estrogen receptor has been characterized for specific gene promoters but not at the genomic level in vivo. Furthermore, reducing uncertainties associated with cross-species extrapolations of pharmaco- and toxicogenomic data remains a formidable challenge. A comparative ligand and species analysis approach was conducted to systematically assess the physiological, morphological and uterine gene expression alterations elicited across time by TAM and ethynylestradiol (EE) in immature ovariectomized Sprague-Dawley rats and C57BL/6 mice. Differential gene expression was evaluated using custom cDNA microarrays, and the data was compared to identify conserved and divergent responses. 902 genes were differentially regulated in all four studies, 398 of which exhibit identical temporal expression patterns. Comparative analysis of EE and TAM differentially expressed gene lists suggest TAM regulates no unique uterine genes that are conserved in the rat and mouse. This demonstrates that the partial agonist activities of TAM extend to molecular targets in regulating only a subset of EE- responsive genes. Ligand-conserved, species-divergent expression of carbonic anhydrase 2 was observed in the microarray data and confirmed by real time PCR. The identification of comparable temporal phenotypic responses linked to related gene expression profiles demonstrates that

systematic comparative genomic assessments can elucidate important conserved and divergent mechanisms in rodent estrogen signaling during uterine proliferation.

INTRODUCTION

The estrogen receptor (ER) is a master transcriptional regulator involved in the proliferation and differentiation of many tissues, most notably the female reproductive tract. It functions as a ligand-dependent transcription factor with two activation functions (AF-1 and AF-2) which interact with cofactors (SRC-1, GRIP1, CBP/p300) to modulate transcription using different mechanisms [1, 2]. The genomic activities of ER are mediated via direct DNA binding at estrogen response elements (EREs) or through indirect tethering mechanisms involving AP-1, Sp1, or Nf- κ B [3]. It also has been shown to use non-genomic mechanisms via membrane associated ERs which activate various protein kinase cascades [4]. Furthermore, two distinct ER isoforms exist which have divergent functionality as well as tissue- and cell type-specific expression. For example, whereas mammary tissue expresses both ERa and ER β [5] at comparable levels in different cells, the uterus predominantly expresses ERa.

TAM is a well characterized breast cancer drug and prophylactic that is a selective estrogen receptor modulator (SERM). SERMs are structurally diverse compounds that bind the ER and elicit ligand- and tissue-specific effects [6], [7], such as inhibiting the proliferative effects of estrogen in ER-positive breast cancers, while maintaining partial agonistic activity in other tissues [8]. SERM binding causes a unique conformation in the ER ligand binding domain which alters the apposition of ER helix 12 and function of AF-2 relative to 17β -estradiol (E2), while still allowing for the ligand-independent functionality of AF-1 [9].

TAM-induced ER-mediated gene expression has been characterized on a promoter/gene specific basis [10, 11]. However, the effect of TAM on global uterine gene expression has not been comprehensively examined. Whether or not the modulation of helix 12/AF-2 by TAM results in merely decreased efficacy or specificity for typical ER gene targets or potentiates unique cofactor interactions and thus novel genomic targets has not been fully examined. Therefore, elucidating the genomic targets of TAM is important to the understanding of SERM-ER proliferative activities, especially due to its association with uterine cancer following continuous treatment [12], which is currently understood to be a function of its partial agonist activity.

The classic rodent uterotrophic assay to assess the estrogenic potential of a chemical examines both the physiological and histomorphological endpoints in the uterus [13]. Uterine wet weight, water imbibition and luminal epithelial cell height induction are typically evaluated after three consecutive daily treatments [14]. This assay can be extended by including temporal gene expression analysis, which can be anchored to these more apical endpoints in order to provide a more comprehensive assessment of the molecular and physiological effects of treatment [15, 16].

Furthermore, this enriched assessment can also be used to evaluate the ability of surrogate models to accurately predict human responses to drugs, industrial chemicals, natural products and environmental contaminants. Elucidating species-specific differences in either gene function or regulation is an important factor for reducing uncertainties associated with cross-species extrapolation of data in risk assessment. Therefore, a cross-species comparative method was employed to examine temporal gene

expression in rodents during TAM and ethynylestradiol (EE) induced uterotrophy. MATERIALS AND METHODS

Husbandry - Experimental designs and methods for the rat TAM data paralleled previously published rat and mouse EE and mouse TAM studies [17, 18]. Briefly, female Sprague-Dawley rats and C57BL/6 mice, ovariectomized on PND 20 and all within 10% of the average body weight were obtained from Charles River Laboratories (Raleigh, NC) on day 25. Animals were housed in polycarbonate cages containing cellulose fibre chip bedding (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) and maintained at 40-60% humidity and 23°C in a room with a 12hrs dark/light cycle (7am-7pm). Animals were allowed free access to de-ionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for 4 days prior to dosing.

Treatments - In dose response studies, animals received three consecutive, daily oral treatments of EE (0.01 to 300 μ g/kg) or TAM (3 to 3000 μ g/kg b.w.). Animals were sacrificed 72 hrs after the initial dose. In time course studies animals were treated once or once daily for three consecutive days via oral gavage with 100 μ g/kg b.w. EE or TAM in 0.1 mL of sesame oil vehicle (Sigma Chemical, St Louis, MO), (EE time course treatment in mouse, MmEE; EE time course treatment in rat, RnEE; EE in mouse, MmEE; TAM in mouse, MmTAM;). This oral dose was empirically derived and chosen because it elicited a maximal uterotrophic response in both species while showing no acute toxic effects. Animals receiving a single dose were sacrificed 2, 4, 8, 12, 18, or 24 hrs after treatment. Animals receiving three consecutive, daily doses were sacrificed 72 hrs post initial dose. An equal number of time-matched vehicle control (VEH) animals were treated in the same manner, (n=8 for MmEE, n=5 for all others). Doses of EE and TAM were calculated based on average weights of each treatment and VEH group prior to dosing. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Necropsy - Animals were sacrificed by cervical dislocation and animal body weights were recorded. The uterine body was dissected at the border of the cervix and whole uteri were harvested and stripped of extraneous connective tissue and fat. Whole uterine weights were recorded before (wet) and after (blotted) being blotted under pressure with absorbent tissues and were subsequently snap-frozen in liquid nitrogen and stored at -80°C. Weight due to water was calculated as the difference between the wet and blotted weights. A small (~5 mm) section of the right, distal uterine horn was placed in 10% neutral buffered formalin (NBF; VWR, West Chester, PA) and stored at RT for at least 24 hrs prior to further processing. Statistical analysis of wet weight and water content were conducted using a two-way ANOVA with a Tukey's Honestly Significant Difference post hoc test, p<0.05 (SAS 9.1, SAS Institute Inc. Cary, NC).

RNA Isolation - Total RNA was isolated from whole uteri (~20 mg/rat, ~3 mg/mouse) using Trizol® Reagent (Invitrogen, Carlsbad, CA). Uteri were removed from -80°C storage and immediately homogenized in 1 mL Trizol® Reagent using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to manufacturer's protocol and resuspended in The RNA Storage Solution (Ambion, Austin, TX). Concentration was calculated by spectrophotometric methods (A₂₆₀) and purity assessed by the A₂₆₀:A₂₈₀ ratio and by visual inspection of 1 μ g on a denaturing gel.

Histological Processing - NBF-fixed uterine sections were routinely processed and embedded in paraffin according to standard histological techniques. Five µm crosssections were mounted on glass slides and stained with hematoxylin and eosin. All embedding, mounting and staining of tissues were performed at the Histology/Immunohistochemistry Laboratory, located in the Department of Physiology, of Michigan State University.

Histopathological and Morphometric Assessment - Histological slides were scored according to standardized National Toxicology Program (NTP) pathology codes. Morphometric analyses were performed on cross sections for each animal using image analysis software (Scion Image, Scion Corporation, Frederick, MD) and standard morphometric techniques. Briefly, the contour length of basal lamina underlying the luminal epithelium (LE) and corresponding areas of LE was quantified for multiple, representative sectors of each section. Statistical analyses on all morphometry data were performed using a two-way ANOVA with a Tukey's Honestly Significant Difference post hoc test, n=5, p<0.05 (SAS 9.1, SAS Institute Inc.).

Microarray Platform - Rat cDNA arrays were produced in-house using a LION Bioscience's Rat cDNA library (LION Bioscience, Heidelberg Germany) consisting of 8,567 clones representing 5,684 unique genes (Unigene Build #48). Clones were selected based on their level of annotation as well as sequence similarity to well annotated human and mouse genes. Detailed protocols for microarray construction, labeling of the cDNA probe, sample hybridization and slide washing can be found at <u>http://dbzach.fst.msu.edu/</u> <u>interfaces/microarray.html</u>. Briefly, PCR amplified DNA was robotically arrayed onto epoxy coated glass slides (SCHOTT Louisville, KY), using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 32 (8 x 4) or 48 (12 x 4) Chipmaker 2 pins (Telechem, Atlanta, GA) for the rat and mouse arrays, respectively, at the Genomics Technology Support Facility at Michigan State University [http://www.genomics.msu.edu].

Array Experimental Design - Temporal changes in gene expression were assessed using an independent reference design in which samples from EE and TAM treated animals were co-hybridized with VEH. Comparisons were performed on 3 biological replicates x 2 independent labelings of each sample (incorporating a dye swap) for each time point. Total RNA (15 µg) was reverse transcribed in the presence of Cv3- or Cv5labeled dUTP (Amersham, Piscataway, NJ) to create fluor-labeled cDNA, which was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). In contrast, note that mouse array experiments used a 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1 ug of total RNA, according to manufacturer's specifications, for probe labeling. Cy3- and Cy5-labeled samples were mixed, vacuum concentrated (~1-2 µl) and resuspended in 32 µl of hybridization buffer (40% formamide, 4x SSC, 1% SDS) with 20 µg polydA and 20 µg of mouse COT-1 DNA (Invitrogen) as a competitor. This probe mixture was heated at 95°C for 2 min and was then hybridized to the array under a 22 x 40 mm lifterslip (Erie Scientific Company, Portsmouth, NH) in a light protected and humidified hybridization chamber (Corning Inc., Corning, NY). Samples were hybridized for 18–24 h at 42°C in a water bath. Slides were then washed, dried by centrifugation and scanned at 635 (Cy5) and 532 nm (Cy3) on a GenePix 4000B microarray scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 (Molecular Devices).

Array Data Normalization and Statistical Analysis - Data sets for rat and mouse EE and mouse TAM have been previously published [17, 18] and have been integrated

into the current comparative analysis with rat TAM data. As previously described with these data sets, rat TAM microarray data were first examined using a quality assurance protocol prior to further analysis to ensure consistent, high quality data throughout the dose-response and time course studies prior to normalization and further analysis [19]. Data were normalized using a semi-parametric approach [19]. Model-based t-values were calculated from normalized data, comparing treated from VEH responses per timepoint. Empirical Bayes analysis was used to calculate posterior probabilities of activity (P1(t)-value) on a per gene and time-point basis using the model-based t-value [20]. Genes were filtered for differential expression based on the P1(t)-value which indicates increasing activity as the value approaches 1.0. A two-tiered set of criteria including a statistical P1(t) > 0.999 and |fold-change| > 1.5 was used as an initial selection filter of the expression data and defined initial differentially expressed gene lists in both the rat and mouse. All data was deposited into dbZach (http://dbzach.fst.msu.edu), a MIAME compliant relational database that ensures proper data management and facilitates data analysis. Complete data sets with annotation and P1(t) values are available as Supplementary Tables 1-4 at [URL].

Expression Data Annotation and Coactivity Analysis - Features refer to unique cDNA clones spotted on the array and are assigned a GenBank accession number, gene name, symbol, and Entrez Gene ID where annotation is available. For the sake of brevity and consistency, genes are referenced by their official gene symbol as defined by NCBI. Rat and mouse orthologous gene pairs were derived from the publicly available Ensembl and HomoloGene databases. Comparing gene expression between species or ligand treatment involved examining the time, direction, and statistical significance of the

change in expression on a gene by gene basis. Similarities and differences in gene expression patterns were designated as coactive-similar direction (CAS), coactivedivergent direction (CAD), displaced active-similar direction (DAS), and displaced active-divergent direction (DAD). Comparative analysis was conducted using a multivariate correlation-based visualization application developed in-house. Additional analysis was performed using the 4-way Venn Diagram Generator (4VDG, [http://www.pangloss.com/seidel/Protocols/venn4.cgi], which involved applying an initial relaxed filtering criteria (|fold change| > 1.3 and P1(t)>0.99) to each data set, then entering respective Entrez Gene IDs (mouse IDs were used for rat where HomoloGene indicated orthology) into the 4VDG. The output categories were then filtered for only those genes which met more stringent criteria (|fold change| > 1.5 and P1(t)>0.999) at any one time point. Gene lists for each 4-way Venn category are available in Supplementary Table 5.

QRT-PCR (Real Time) Analysis – For each sample, 1.0 μ g of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0 μ l) was used as the template in a 30 μ l PCR reaction containing 0.1 μ M each of forward and reverse gene-specific primers designed using Primer3 (15), 3 mM MgCl₂, 1.0 mM dNTPs, 0.025 IU AmpliTaq Gold and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Gene names, accession numbers, forward and reverse primer sequences and amplicon sizes are listed in Supplementary Table 6. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems) on an Applied Biosystems PRISM 7500 Sequence Detection System using the following conditions: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was performed to assess the specificity of the primers and the uniformity of the PCR generated products. Each plate contained duplicate standards of purified PCR products of known template concentration covering six orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number versus threshold cycle (Ct). No template controls (NTC) were also included on each plate. Samples with a Ct value within 2 SD of the mean Ct values for the NTCs were considered below the limits of detection. The copy number of each unknown sample for each gene was standardized to the geometric mean of house-keeping gene, Rpl7 to control for differences in RNA loading, quality and cDNA synthesis. Statistical significance of induced or repressed genes was determined using two-way ANOVA followed by t-test for VEH treatment comparisons (SAS 9.1, SAS Institute Inc.). For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

RESULTS

Uterine Wet Weight (UWW) and Water Content – Increases in UWW were used to evaluate the responsiveness to EE and TAM. Dose response studies were performed using 0.01, 0.1, 1, 10, 100, 300 μ g/kg b.w. EE, and 3, 10, 30, 100, 300, 1000, 3000 μ g/kg b.w. TAM. In each case, 100 μ g/kg approached the maximum uterotrophic response and this dose was subsequently used for the time course studies (Figure 4.1). Whereas TAM was equipotent to EE in eliciting uterotrophy in C57BL/6 mice and Sprague-Dawley rats, it was 43% less efficacious in both species eliciting only 4- and 5-fold induction (in the rat and mouse, respectively) compared to the ~9-fold increase induced by EE. In the time



Dose Response Uterine Wet Weights (UWW) UWW was measured across several EE and TAM doses in the mouse and rat. A plot of the fold change increase in wet weight is plotted. A dose response curve was fit to the data (GraphPad 4.0) to estimate EC_{50} values. 100 µg/kg b.w. approximates the maximum response in all four cases and was used in subsequent time course studies. ED_{50} values were comparable between ligands in the rat while exhibiting only a two fold difference in the mouse, indicating conservation of sensitivity to EE and TAM.

course studies, 100 µg/kg of EE or TAM was orally administered once daily for three consecutive days. UWW and water content changes were measured at each time point (Tables 1 and 2). EE induced UWW increases only at 24 and 72 hrs in the mouse, while in the rat the classic water imbibition response occurred between 4 and 12 hrs followed by maximum induction at 72 hrs. TAM induction of water imbibition was delayed approximately 8 hrs in the rat and subdued in both (45% and 65% of EE in rat and mouse, respectively). The increases in uterine water content suggest that early increases in UWW are due at least in part to this water imbibition. As in the wet weight, the changes in water content after TAM treatment temporally lagged behind EE and were notably less efficacious. Therefore, the large difference in wet weight between EE and TAM at 72 hrs is possibly due to early differences in gene expression responsible for water imbibition.

Histomorphology – Induction of luminal epithelial height (LEH) is a classic estrogen response [21, 22] in the rodent uterus. No increases in LEH were observed in any group between 2 and 12 hrs (Figure 4.2). However, EE induced LEH as early as 18 hrs in the mouse and 24 hrs in the rat. While EE and TAM produced comparable levels of LEH induction (~2.6-fold) in the mouse at 72 hrs, TAM treatment in the rat elicited a significantly greater LEH increase (p<0.05, 4.4-fold for TAM and 2.6-fold for EE). EE induced moderate to marked stromal edema beginning as early as 4 and 8 hrs while TAM induced severe stromal and myometrial edema at 12 hrs. Proliferative indices (number of mitotic bodies) were noted in EE treated uteri at 18 and 24 hrs but not detected in TAM treated samples. Moderate to severe hyperplasia and hypertrophy in the stroma and epithelium were present at 72 hrs in all studies. There was mild apoptotic cell death in the

		Mouse			Rat	
Time (h)	VEH	EEa	TAM ^b	VEH	EEa	TAM ^b
7	5.54 ± 1.59	4.31 ± 1.02	6.31 ± 2.83	22.46 ± 3.67	21.92 ± 5.12	24.03 ± 5.18
4	5.67 ± 1.14	6.22 ± 1.29	7.00 ± 1.02	21.14 ± 4.18	32.32 ± 5.89*	25.43 ± 4.49
8	5.66 ± 1.45	6.97 ± 0.73	8.16 ± 3.42	22.25 ± 2.97	37.64 ± 4.90*	29.75 ± 5.95
12	4.61 ± 1.32	7.48 ± 1.47	8.18 ± 2.50	22.33 ± 3.96	36.97 ± 9.80*	35.58 ± 3.28
18	7.43 ± 1.94	12.44 ± 3.42	8.68 ± 1.05*	22.39 ± 4.45	31.59 ± 6.48	38.87 ± 9.92*
24	4.53 ± 0.67	11.48 ± 2.58*	9.87 ± 2.15*	18.99 ± 2.63	$41.20 \pm 6.90^{*}$	34.15 ± 3.31*
72	5.01 ± 1.18	35.16 ± 8.51*	$40.99 \pm 11.65^*$	17.78 ± 2.26	95.49 ± 21.86*	62.32 ± 7.93*
Average w	eight (mg) ± sta	ndard deviation. a	n=8 compared to V	EH of n=8. b n=5	compared to VEH	of n=5

Table 4.1 Uterine Relative Wet Weight

		Mouse			Rat	
Time (h)	VEH	EEa	TAM ^b	VEH	EEa	TAM ^b
2	1.34 ± 0.93	0.86 ± 0.60	1.27 ± 0.50	4 .66 ± 2.32	5.27 ± 1.06	7.34 ± 1.95
4	1.23 ± 0.69	1.38 ± 0.51	1.13 ± 0.49	4.74 ± 1.84	$10.85 \pm 1.81^*$	7.38 ± 1.15
80	1.23 ± 0.82	1.97 ± 0.58	1.26 ± 0.58	4 .56 ± 1.89	12.98 ± 1.92*	6.81 ± 2.12
12	0.70 ± 0.53	2.86 ± 1.03*	1.49 ± 1.51*	4.19 ± 1.69	9.90 ± 3.68*	9.38 ± 2.52*
18	1.55 ± 0.65	3.82 ± 1.27*	2.08 ± 0.26	4.51 ± 1.83	6.40 ± 1.18	11.15 ± 2.56*
24	0.95 ± 0.45	2.64 ± 0.81	2.18 ± 1.04*	4 .37 ± 2.18	7.50 ± 1.26	9.73 ± 1.23
72	1.29 ± 0.93	11.41 ± 6.08*	7.43 ± 4.22*	3.97 ± 1.31	30.16 ± 13.77 *	13.25 ± 3.46*
Average w of n=5* p	/ater weight (mg) < 0.05, 2-way AN	± standard deviati 4OVA, Tukey's pos	on. a n=8 compar st hoc test	ed to VEH of n=8	3. b n=5 compare	d to VEH

Content	
Water	
Relative	
: Uterine	
able 4.2	
F	



LEH induction was temporally delayed in response to TAM treatment in both species. Mouse induction was comparable between b TAM Temporal Changes in Luminal Epithelial Height (LEH). LEH was quantified for each treatment group and compared to VEH controls. ligands while TAM-induced LEH was significantly (p<0.05) greater than EE in rats. a treatment different than VEH. treatment different than time matched EE treatment. epithelium in TAM studies at 72 hrs while mild to marked apoptosis was noted in EE samples. The species difference in uterine architecture (luminal invaginations seen in the mouse but not rat) previously observed [18] at 72 hrs was not as pronounced in TAM treated samples.

Comparative Species and Ligand Analysis of Gene Expression – Global gene expression changes with respect to VEH controls were measured and compared across time for MmEE, MmTAM, RnEE, and RnTAM treatments. Pair-wise comparisons between compounds per species and between species per compound were made to investigate conserved responses. A two-tiered, bipartite (P1(t) and fold change) approach was used to screen for conserved differentially expressed genes (Figure 4.3).

In the mouse, 3,663 and 2,821 genes were differentially regulated by EE and TAM respectively, with 2,631 common differentially expressed genes across all time points (Figure 4.3B). These 2,631 common, differentially expressed genes were then further examined to assess the similarity of their EE- and TAM-elicited temporal expression profiles by comparing their expression profiles in order to assess similarity between the treatments (Figure 4.3C). This comparison is comparable to a correlation analysis in assessing the similarity of statistically significant differential gene expression relative to a control elicited by EE and TAM. Each differentially expressed gene was designated as either CAS, CAD, DAS, or DAD based on the relationship between the time and direction of differential regulation, and the significance (P1(t)) of the expression profile relative to the VEH control. For example, Igf1 is designated as CAS in the rat because it was up regulated in a similar temporal pattern by both EE and TAM between 12-24 hrs, while Junb was designated DAS because although it was up regulated by both

Comparative Analysis of Species-Conserved, Ligand-Specific Gene Expression (A) cDNA microarrays were used containing 13,361 mouse clones representing 8,734 unique genes and 8,507 rat clones representing 5,684 unique genes. (B) Differentially expressed genes regulated by each ligand were identified using relaxed criteria to minimize the likelihood of false-negatives that marginally failed to meet the selection criteria. Differentially expressed genes elicited by both EE and TAM were analyzed for similarity in their temporal profiles by comparative analysis. Genes were designated as either CoActive-Similar direction (CAS), CoActive-Divergent direction (CAD), Displaced Active Similar direction (DAS), or Displaced Active Divergent direction (DAD) based on the relationship between the time and direction of differential regulation, and the significance (P1(t)) of the expression profile relative to the VEH control. (C) The comparative results were plotted on a coordinate correlation graph. A majority of genes show positive correlation between ligands for both fold change and P1(t) value. (D) Cross species analysis of ligand-divergent (CAD) expression profiles indicate no conservation.

FIGURE 4.3



compounds, EE induction was at 4 hrs but TAM treatment temporally shifted the induction to 12 hrs. These designations assist in developing gene lists and provide a preliminary assessment of the similarity in gene expression profile on a qualitative level that takes into account the statistical difference from VEH which typically is not considered in correlation analyses. Using this approach, >85% of EE and TAM induced genes were designated as CAS (2255 genes). Only a few were categorized as CAD or "divergently regulated" (28 genes, 1% of the overlap).

Similarly in the rat, 2,284 and 2,087 genes were differentially expressed by EE and TAM, respectively, resulting in 1,950 commonly regulated genes (Figure 4.3B). In both species, roughly 93% of TAM-regulated genes overlapped with EE. Of the 1,950 genes differentially expressed by both EE and TAM in the rat, 86% (1,686 genes) were designated as CAS while only 23 were CAD. Comparison of the 28 mouse CAD genes to the 23 rat CAD genes found no overlapping orthologs (Figure 4.3D), suggesting that there are no conserved differentially regulated genes for which EE and TAM elicit divergent uterine responses in the rat and mouse.

Comparison of Orthologous Gene Expression – A comparable approach was used to examine the cross-species differential gene expression effects of EE and TAM on orthologous rat and mouse genes. 3,417 unique orthologous genes were represented on the rat and mouse array platforms as determined by NCBI's HomoloGene database (Figure 4.4A). In response to EE treatment, 2,095 and 2,181 of the 3,417 orthologous genes were differentially expressed in the mouse and rat, respectively, with 1,634 orthologs differentially expressed in both species (~75%) (Figure 4.4B). These 1,634 differentially expressed orthologs were analyzed for coactivity of which 1,116 genes

Comparative Analysis of Ligand-Conserved, Species-Specific Gene Expression. (A) cDNA microarrays were used containing 13,361 mouse clones representing 8,734 unique genes and 8,507 rat clones representing 5,684 unique genes. There were 3,417 orthologous genes represented on the mouse and rat cDNA microarrays as determined by HomoloGene. (B) Differentially expressed genes were assessed for similar expression patterns using relaxed criteria (|fold change| > 1.3; P1(t)>0.99) to minimize the likelihood of false-negatives that marginally failed to meet the selection criteria. (C) Common differentially expressed orthologous genes were examined for comparable expression patterns, and designated according to the coactivity categories (CAS, CAD, DAS, DAD) as described in Figure 4.3. The results were plotted on a coordinate correlation graph. A high proportion of differentially expressed orthologs exhibited a positive correlation when considering both fold-change and P1(t). (D) Comparisons of species-divergent (CAD) expression profiles identified 35 genes that were differentially expressed in the mouse and P1(t). (D) Expression platernially expressed in the mouse and the rat but exhibited putative divergent regulation elicited by EE and TAM.

FIGURE 4.4



(68%) were designated as CAS and 206 CAD. Similarly, 1,252 mouse and 1,441 rat genes were differentially expressed following TAM treatment with 891 orthologs differentially expressed in both species. 705 (79%) of these were designated as CAS genes while 63 genes were designated CAD. A comparison of the EE and TAM CAD orthologs identified 35 genes that are divergently regulated between the mouse and rat in response to both EE and TAM. Of these 35 genes, 9 were represented by only a single feature on the microarray for both species, 12 were represented by 2 or more features in at least one species that had poor internal correlation, and 14 were represented by 2 or more features in at least one species that were internally consistent. Thus, a strength of evidence approach was taken for pursuing genes exhibiting putative divergent regulation with quantitative real time PCR.

QRT-PCR Verification of Microarray Data – The expression profiles of 25 genes (including known estrogen responsive genes: Fos, C3, Calb3, Igf1, Sult1a1, Aqp5, and Vegf) accounting for the spectrum of temporal patterns and functional categories were verified by QRT-PCR in either species or compound. In general there was a good correlation between the QRT-PCR and microarray results (data not shown). Fourteen orthologs that exhibited putative divergent regulation in the mouse compared to the rat following EE and TAM treatment were further investigated. However, QRT-PCR indicated that these genes exhibited either a CAS relationship between species or were inconclusive, except for carbonic anhydrase 2 (designated Car2 in the mouse and Ca2 in the rat and human, hereafter referred to as Ca2 to represent all orthologs). QRT-PCR analysis of Ca2 confirmed the microarray data (correlation coefficient r=0.95, 0.98, 0.82, and 0.78 for MmEE, MmTAM, RnEE and RnTAM, respectively; Figure 4.5). These



Quantitative Real Time PCR Confirmation of Species-Specific ER Regulation of Ca2. The temporal gene expression of carbonic anhydrase 2 (designated Car2 in the mouse and Ca2 in the rat and human, hereafter referred to as Ca2 to represent all orthologs) was further validated by species-conserved and -unique primer sets. The QRT-PCR results confirm the microarray results indicating that Ca2 is differentially regulated in the mouse when compared to the rat. This differential regulation was elicited by both EE and TAM. Mouse Ca2 expression is significantly (p<0.05) induced relative to VEH (y = 1) while rat expression is significantly (p<0.05) repressed (n=5, Tukey's HSD post hoc test), at multiple time points as indicated by the asterisk (*).

results were confirmed by two different sets of primers, with one set querying species specific regions of the mouse and rat mRNA, and the second set designed to amplify the same region in both species using the same primers.

Four-Way Venn Analysis – Following pair-wise comparisons by chemical and species, an integrated comparison of all four data sets was performed where differentially expressed orthologous genes (NCBI, HomoloGene) for each data set were entered into the 4-way Venn Diagram Generator. The number and identity of unique genes populating the overlaps of each data set was determined for common orthologs (Figure 4.6). The comparison of all four data sets resulted in 902 genes being commonly regulated at any time point (Venn subset I). Of the 902 overlapping genes, 398 exhibited a similar temporal profile (CAS) across all four data sets, suggesting comparable modes of regulation which we refer to as "orthologous expression". These genes represented functional categories associated with cellular proliferation and differentiation; hallmarks of estrogen induced uterotrophy. Selected conserved examples exhibiting orthologous expression in terms of either fold-change alone (Fos, C3, Igf1, Ca3, Sepp1) or large increase in copy number, regardless of fold change (Cd24a, Slc25a5, Krt13, Dcn, Itm2b) are provided in Table 3.

Of interest are the 128 and 12 genes populating subsets B and K, respectively. Subset B represents putative species-conserved EE-specific responses, of which, less than 60% exhibited similar direction of regulation (CAS or DAS designation) by TAM. Most of these genes were regulated by TAM but did not meet the fold change or P1(t) selection criteria, suggesting a potency issue rather than unique regulation. For example S100a8, a calcium binding protein, was induced 1.5-fold (Mm) and 3.5-fold (Rn) by EE while TAM



Four Way Venn Analysis of Active Genes. Each data set was converted into ortholog space and processed for activity using relaxed criteria prior to analysis using the 4-way Venn Diagram Generator (<u>http://www.pangloss.com/seidel/Protocols/venn4.cgi</u>). The Venn set was subsequently filtered for genes which met the initial, high-stringency criteria to ensure robust comparisons.

only induced a 1.3-fold change in the mouse that did not meet the statistical cutoff while in the rat the statistical cutoff was achieved but induction was only 1.2-fold. Likewise, further analysis of the 12 species-conserved TAM-specific responses revealed that only Myh6 (myosin, heavy chain 6), a component of muscle fibre that is likely expressed in the myometrium, exhibited an overlap in temporal expression between species. However, comparable expression is only observed at 72 hrs where secondary and tertiary effects are likely to overlap. In addition, there is insufficient data to exclude the possibility that Myh6 is regulated by TAM via an ER-independent mechanism.

Collectively, under the current experimental conditions and the available microarray platform, these results suggest there are no species-conserved, TAM-specific or -divergent genes which are regulated in a separate manner when compared to EE in the rodent uterus. Approximately 400 genes exhibited highly conserved and correlated expression across all four data sets, defining a robust gene set that is predictable and integral to ER-mediated uterotrophy in the rodent. However, species-specific gene expression profiles are difficult to assess and confirm given the complex orthologous relationships between the species specific array probe sets. Many of the putative differences suggested may be attributed to differing probe sensitivities or representations within respective genes. The lone identified exception is the species-divergent regulation of Ca2 that is conserved in response to both ER ligands and confirmed by homologous and species-specific QRT-PCR primer pairs.

DISCUSSION

The uterotrophic response has been used as an acute assay to assess the estrogenicity of a compound using both physiological and cytoarchitectural endpoints. Our extended

Functional Category	Gene Symbol	Entrez Gene	Direction of Regulation	Time ^a
Energetics	Aldoa	11674	Up	Sustained
	Atp5b	11947	Up	Sustained
	Atp5a1	11951	Up	Sustained
	Ckb	12709	Un	Mid
	Cox7h	20463	Un	Sustained
	Cycs	13063	Un	Sustained
	Ldha	16828	Un	Sustained
	Pak1	18655	Un	Sustained
	SIc25a4	11730	Up	Mid
	SIc25a5	11740	Up	Sustained
	Typi2	E991E	Up	Mid/Sustained
Chaparona &	Cot7	12469	Up	Sustained
Brotoin Folding	Hanologa	12400	Up	Sustaineu
Frotein Folding	Hene	14000	Up	Sustained
	Hspas	14828	Up	Sustained
	Hspal	15510	Op	Mid
0.0.0	Hspei	15528	Up	MIG
Cell Structure	Dynin	56455	Up	Sustained
	Krt13	16663	Up	Sustained
	Krt8	16691	Up	Sustained
	Serpinh1	12406	Up	Mid
	Tubb2c	227613	Up	Mid
Cell Signaling	App	11820	Down	Sustained
	Cd24a	12484	Up	Late
	Dcn	13179	Down	Sustained
	lgf1	16000	Up	Sustained
and the second second	Sepp1	20363	Down	Mid
Transcription &	Atf4	11911	Up	Sustained
RNA Processing	Cnot4	53621	Up	Sustained
	Fos	14281	Up	Early
	Hnrpab	15384	Up	Sustained
	Hnrpu	51810	Up	Sustained
Translation &	Eif1	20918	Up	Mid
Protein Turnover	Eif2s2	67204	Up	Sustained
	Psma5	26442	Up	Mid
	Psmb5	19173	Up	Mid
Uncategorized	Armet	315989	Up	Sustained
or Other	C3	24232	Up	Late
	Car3	12350	Down	Late
	Cst3	13010	Down	Sustained
	Hba-a1	15122	Down	Late
	ltm2b	16432	Down	Mid
	LV6e	17069	Down/Lin	Early/Late
	Man	17313	Down	Sustained
	Rap	10294	Un	Mid
	rkan Tura	19384	Up	DIN
	1 x 1 1	22166	Up	MIC

Table 4.3 Species and Compound Conserved Genes

a Early = 2, 4 or 8 hrs, Mid = 8, 12, 18 or 24 hrs, Late = 72 hrs, Sustained =

3 or more consecutive timepoints.
design incorporates early monitoring of global gene expression (2-24 hrs) to capture the ER-mediated effects as well as subsequent secondary and tertiary responses. Early gene expression was also examined for conserved responses across ligand and species. The highly parallel nature of the EE and TAM study designs and subsequent analyses facilitated more robust comparisons, thus increasing confidence in the identification of conserved uterine responses between species and ligands.

EE and TAM doses were selected based on UWW dose response studies which identified 100 μ g/kg as the most efficacious dose for each compound. Although differing pharmacokinetics may account for discrepancies between rodents and humans, 100 μ g/kg TAM is below the pharmacological dose of Nolvadex® (tamoxifen citrate, 20-40 mg/day) prescribed to women (300-400 μ g/kg), assuming an average weight of 70 kg. Moreover, the ovariectomized model provides increased sensitivity to estrogen action in the immature rodent and thus allows for a more comprehensive assessment of the partialagonistic activity of TAM in the uterus.

The physiological effects were comparable between species and ligand with EE eliciting the classic uterotrophic responses while TAM showed decreased efficacy with only partial agonist activity, as previously reported [23]. However, the more pronounced induction of luminal epithelial cell height in the rat compared to the mouse was unexpected. Previous rat studies have not observed increased efficacy of TAM in inducing luminal epithelial cell height relative to estrogen [24, 25], which may be due to differences in animal age, ovariectomy status or other study design issues. However, a comparable increase in TAM LEH induction in rats relative to estrogen was previously reported as not statistically different [26]. Furthermore, the uterotrophic effects of EE

and TAM in ovariectomized Cynomolgus Macaque monkeys [27] showed a noticeable increase in TAM induced LEH relative to EE that was not statistically significant. The similarity in LEH response to higher order mammals/primates suggests that the rat may be a better model of the human response, but further study is required.

The partial agonist effects of TAM with regard to its ER-complex conformation and differential interactions at specific canonical response elements (including EREs and AP-1 sites) as well as gene specific promoters (Vitellogenin, Complement component 3, prolactin) have been well studied. However, the targets of TAM-ER complex have not been comprehensively elucidated in the rodent uterus. Although direct primary ligand-ER effects can not be deduced from expression profiling alone, our comparative approach provides evidence of several conserved responses elicited by two structurally diverse ligands. As primary effects give way to secondary responses, gene expression cascades are sequentially propagated across time. If the two ligand-receptor complexes elicit different behaviors at the primary response genes, it is likely that subsequent differences in secondary and tertiary responses would also be propagated over time. However, this is not the case as EE and TAM elicited comparable expression profiles that are maintained throughout the time course in both species. This suggests that TAM elicits parallel uterine gene expression behavior when compared EE, despite the temporal shift of expression due to pharmacokinetic differences [28].

An inclusive, multi-step approach to identifying differentially expressed genes was used to ensure conservation of gene expression between ligand or species could not be attributed to strict cut offs applied during screening. Comparison of gene expression profiles revealed highly similar sets of genes between species and compounds (Figure 4.3

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and 4). When profiles were aligned and compared on a quantitative scale, TAM typically elicited a lower fold change when compared to EE, consistent with its partial agonist activity. However, four notable species-conserved orthologs (Hsp90aa1, Slc9a3r1, Acsbg1, Col5a1) were identified where TAM induction exceeded that of EE. For example, Slc9a3r1, a cytoskeletal-membrane protein binding-protein that functions to maintain epithelial cell structure and polarity [29], was induced 4.1-fold (Rn) and 3.6-fold (Mm) by TAM but only 2.7-fold (Rn) and 3-fold (Mm) by EE, respectively. Few genes exhibited unique differential gene expression suggesting that TAM elicits uterotrophic affects through the same target genes affected by EE (Figure 4.6). This is significant, as little information is known about how conformational changes in the SERM-ER complex affect the number or types of uterine genes modulated after treatment.

A large number of regulated genes were differentially expressed across all four data sets, of which, 398 exhibited comparable patterns of expression (Supplementary Tables 1-4), and likely represent conserved ER-mediated uterine responses. They were associated with several functional categories including cellular energetics, chaperone and protein folding, cell structure, cell signaling, transcription and RNA processing, and translation and protein turnover (Table 3). Although most genes were up regulated, those associated with the cell cycle and mitogenic activity were down regulated, consistent with an overall proliferative response [30, 31]. Several of these conserved early (e.g., Fos and Vegf), mid (e.g., Ccnd1) and late (e.g., C3) responders responses are known ER targets regulated via estrogen response elements (EREs), AP-1, and Sp1 sites. The temporal differences in expression (between 2 and 72 hrs after treatment) of genes known to be ER regulated affirms the bi- or multi-phasic activities of activated ER during uterotrophy [32]. Although the TAM profiles exhibited a temporal shift for early regulated genes (Fos and Vegf), genes differentially expressed at 18, 24 and 72 hrs (Ccnd1, Tk1) exhibited little temporal shift. This suggests a pharmacokinetic difference which is not applied equally to all responses or is masked by the sustained nature of mid to late gene responses.

TAM perturbs calcium homeostasis causing intracellular influx of Ca^{2+} ions which is related to cytotoxic, thrombotic and systemic effects [33, 34]. This is believed to be ER independent, and is suggestive of alternative mechanisms activated only by TAM. However, no differential gene expression was observed regarding genes involved in calcium homeostasis, including Ca^{2+} transporters Atp2a1, -2a2, -2b1, and -2b2, calcium transporters that are targets of the calcineurin/Crz1 signaling pathway which regulates intracellular Ca^{2+} homeostasis. Moreover, the increased LEH responsiveness in rat is not explained by our current data, although this may be due to differences in genome coverage represented on our mouse and rat cDNA microarrays. It is also likely that there are significant species-specific post-transcriptional differences that are not assessed using gene expression approaches.

In summary, our parallel study design afforded a robust comparative analysis of EE and TAM elicited responses in the rat and mouse uterus. TAM induced UWW and LEH in a manner consistent with its partial-agonist activity at a dose equipotent to EE. This suggests that EE and TAM elicit comparable uterine gene expression profiles despite conformation differences in the liganded ER complexes, and that the transcriptional activity via AF-1, which is activated by both EE and TAM, is sufficient to elicit uterine differential gene expression. This is consistent with mice studies with targeted disruption of the ER DNA binding domain [35], which did not exhibit acute uterotrophy but still elicited epithelial proliferation and increased LEH. It also supports the sufficiency of the AF-1 domain alone (via TAM binding) to mediate uterotrophy. Although differences in LEH induction were observed between EE and TAM, collectively our data suggests that TAM does not elicit a unique uterine gene expression profile when compared to EE. However, more comprehensive studies are warranted that would examine the differential expression of all orthologous rat and mouse genes.

CONCLUSIONS

In summary, our parallel study design afforded a robust comparative analysis of EE and TAM elicited responses in the rat and mouse uterus. TAM induced UWW and LEH in a manner consistent with its partial-agonist activity at a dose equipotent to EE. This suggests that EE and TAM elicit comparable uterine gene expression profiles despite conformation differences in the liganded ER complexes, and that the transcriptional activity via AF-1, which is activated by both EE and TAM, is sufficient to elicit uterine differential gene expression. This is consistent with mice studies with targeted disruption of the ER DNA binding domain [35], which did not exhibit acute uterotrophy but still elicited epithelial proliferation and increased LEH. It also supports the sufficiency of the AF-1 domain alone (via TAM binding) to mediate uterotrophy. Although differences in LEH induction were observed between EE and TAM, collectively our data suggests that TAM does not elicit a unique uterine gene expression profile when compared to EE. However, more comprehensive studies are warranted that would examine the differential expression of all orthologous rat and mouse genes.

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CHAPTER FIVE

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CHAPTER FIVE

COMPARATIVE TOXICOGENOMIC EVALUATION OF O,P'-DDT ELICITED UTEROTROPHY IN THE MOUSE AND RAT

ABSTRACT

1,1,1-trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane (o,p'-DDT) is a well known organochlorine pesticide and endocrine disruptor. Although o.p-DDT has been known to bind the estrogen receptor for several decades, its in vivo role in modulating global gene expression has not been characterized. Furthermore, reducing uncertainties associated with cross-species extrapolations of pharmacoand toxicogenomic data remains a formidable challenge. A comparative ligand and species analysis approach was conducted to systematically assess the uterine gene expression alterations elicited across time by $o_{,p}$ '-DDT and ethynylestradiol (EE) in immature ovariectomized C57BL/6 mice and Sprague-Dawley rats. Differential gene expression was evaluated using custom cDNA microarrays, and the data was compared to identify conserved and divergent responses. 1,256 genes were differentially regulated in all four studies, 559 of which exhibited identical temporal expression patterns. However, a putative set of 51 genes was identified which exhibited species-conserved, o,p'-DDTspecific regulation during uterotrophy. Comparative analysis of EE and $o_{,p}$ '-DDT differentially expressed gene lists suggest $o_{,p}$ '-DDT is a close estrogen mimic at high doses. Species- and ligand-divergent expression of carbonic anhydrase 2 was observed in the microarray data and confirmed by quantitative real-time PCR. The identification of comparable temporal phenotypic responses linked to gene expression profiles

demonstrate that systematic comparative genomic assessments are valuable for elucidating important conserved and divergent mechanisms in rodent estrogen signaling during uterine proliferation.

INTRODUCTION

Dichloro-diphenyl-trichloroethane (DDT) is a legacy organochlorine pesticide that was widely used until 1972 when it was banned in the United States. However, DDT continues to be used in developing countries as a malarial vector (mosquitoes) control agent [1]. Due to its lipophilic and bioaccumulative nature, DDT remains a ubiquitous environmental contaminant, achieving levels of biological significance in humans and animals high in the food chain [2] [3]. Technical grade DDT is composed of several congeners, primarily p,p'-DDT (~85% of total DDT), with o,p'-DDT accounting for the remaining percentage, with trace amounts of 0,0'-DDT, DDE and DDD also being present. P,p'-DDT and the prominent metabolite DDE elicit a broad range of toxic effects through multiple mechanisms. However, the o,p'-DDT congener binds and activates the estrogen receptor (ER), causing endocrine disruption [4].

Efforts to characterize estrogenic endocrine disruption have focused upon disruption of hormone synthesis or metabolism, repression or increase in turnover of receptor levels, or mimicking or modulating endogenous ligand-receptor interactions [5]. As o,p'-DDT functions as an ER ligand, characterization of the ER's activation and transcriptional activity in sensitive tissues provides a robust method for evaluating the scope of o,p'-DDT's potential disruptive activities. The uterotrophic bioassay was developed as one method to identify and prioritize estrogenic chemicals based upon

induction of uterine wet weight (UWW), water imbibition and luminal epithelial height (LEH) [6]. However, the uterotrophic assay is relatively insensitive and does not provide insight into mechanism of action. Thus, molecular endpoints of gene induction were included [7] and resulted in the refinement of the enhanced uterotrophic assay, assessing gene expression endpoints (Vegf, Calb9, C3, clusterin) as markers of exposure. In the present study, similarly to other published reports [8],[9],[10],[11], global gene expression has been incorporated into the enhanced uterotrophic assay, utilizing dose-response and time course study designs to assess the respective dynamics in ER-mediated gene expression.

Furthermore, cross-species extrapolation (between surrogates and humans) remains a consistent area of uncertainty for applying hazard identification and risk assessment data to humans. Thus, the C57BL/6 mouse and Sprague Dawley rat, two widely used model systems, were used in the current study to assess the estrogenicity of o,p'-DDT. The comparative design allowed for determination of conserved and divergent gene expression profiles between the two rodent models to assess the power of a given set of genes to be predictive of similar response in more distantly related species. This study reports the uterotrophic effects of o,p'-DDT in the mouse and rat, as well as evaluation of the conserved expression profiles between o,p'-DDT and a previously published EE data set [12]. Finally, a putative set of unique or divergent o,p'-DDT regulated genes in comparison to EE is also highlighted.

MATERIALS AND METHODS

Husbandry

Female Sprague-Dawley rats and C57BL/6 mice, ovariectomized on PND 20, and all within 10% of the average body weight were obtained from Charles River Laboratories (Raleigh, NC) on day 25. Animals were housed in polycarbonate cages containing cellulose fiber chip bedding (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) and maintained at 40-60% humidity and 23°C in a room with a 12 h dark/light cycle (7am-7pm). Animals were allowed free access to de-ionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for 4 days prior to dosing.

Treatments

In dose response studies, animals received treatment of 0, 1, 3, 10, 30, 100, or 300 mg/kg b.w. o,p'-DDT (Supelco, Bellefonte, PA) in 0.1 mL of sesame oil (Sigma Chemical, St Louis, MO) vehicle (Veh) by oral gavage once daily for three consecutive days (n=5). Animals were sacrificed 24 h after the last treatment (72 h after the initial dose). Time course study animals were treated once or once daily for three consecutive days in the same manner with 300 mg/kg b.w. o,p'-DDT. This oral dose was empirically derived and chosen because it elicited a maximal uterotrophic response in both species while showing no acute toxic effects. Animals receiving a single dose were sacrificed 2, 4, 8, 12, 18, or 24 h after treatment (72 h). An equal number of time-matched vehicle control (Veh) animals were treated in the same manner, (n=5). In order to confirm that o,p'-DDT was eliciting its uterotrophic effects via the estrogen receptor, the uterotrophic response of o,p'-DDT was assessed in the rat with and without co-treatment of the pure anti-estrogen ICI 182780 (Sigma) dissolved in 1X PBS. Rats were treated with 30 mg/kg b.w. o,p'-

DDT by gavage and 50 mg/kg ICI 182780 b.w. via intraperitoneal injection once daily for three days and sacrificed 24 h after the last treatment (72 h). A Veh + ICI 182780 control group was also included. Doses of o,p'-DDT were calculated based on average weights of each treatment and Veh group prior to dosing. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Necropsy

Animals were sacrificed by cervical dislocation and animal body weights were recorded. The uterine body was dissected at the border of the cervix and whole uteri were harvested and stripped of extraneous connective tissue and fat, taking care not to lose any luminal fluid. Whole uterine weights were recorded before (wet) and after (blotted) being blotted under pressure with absorbent tissue and were subsequently snap-frozen in liquid nitrogen and stored at -80°C. Weight due to water content was calculated as the difference between the wet and blotted weights. A small (~5 mm) section of the right, distal uterine horn was placed in 10% neutral buffered formalin (NBF; VWR, West Chester, PA) and stored at RT for at least 24 h prior to further processing. Statistical analysis of wet weight and water content were conducted using a two-way ANOVA with a Tukey's Honestly Significant Difference post hoc test, p<0.05 (SAS 9.1, SAS Institute Inc. Cary, NC).

RNA Isolation

Total RNA was isolated from rat uteri (~20 mg) using Trizol® Reagent (Invitrogen, Carlsbad, CA). Uteri were removed from -80°C storage and immediately homogenized in 1 mL Trizol® Reagent using a Mixer Mill 300 tissue homogenizer (Retsch, Germany).

Mouse uteri (~3 mg) were processed in a similar manner using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was isolated according to manufacturer's protocol and resuspended in The RNA Storage Solution (Ambion, Austin, TX). Concentration was calculated by spectrophotometric methods (A_{260}) and purity assessed by the A_{260} : A_{280} ratio and by visual inspection of 1 µg on a denaturing gel.

Histological Processing

NBF-fixed uterine sections were routinely processed and embedded in paraffin according to standard histological techniques. Five µm cross-sections were mounted on glass slides and stained with hematoxylin and eosin. All embedding, mounting and staining of tissues were performed at the Histology/Immunohistochemistry Laboratory, located in the Department of Physiology, of Michigan State University.

Histopathological and Morphometric Assessment

Histological slides were scored according to standardized National Toxicology Program (NTP) pathology codes. Morphometric analyses were performed on cross sections for each animal using image analysis software (Scion Image, Scion Corporation, Frederick, MD) and standard morphometric techniques. Briefly, the contour length of basal lamina underlying the luminal epithelium (LE) and corresponding area of LE was quantified for multiple, representative sectors of each section. Statistical analyses were performed using a two-way ANOVA with a Tukey's Honestly Significant Difference post hoc test, n=5, p<0.05 (SAS 9.1, SAS Institute Inc.).

Microarray Platform

Mouse and rat cDNA arrays were produced in-house using a LION Bioscience's Rat cDNA library (LION Bioscience, Heidelberg Germany) consisting of 8,567 clones representing 5,684 unique genes (Unigene Build #48) and mouse IMAGE Consortium clone library consisting of 13,362 clones representing 8,832 unique genes. Detailed protocols for microarray construction, labeling of the cDNA probe, sample hybridization and slide washing can be found at <u>http://dbzach.fst.msu.edu/interfaces/microarray.html</u>. Briefly, PCR amplified DNA was robotically arrayed onto epoxy coated glass slides (SCHOTT Louisville, KY), using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 32 (8 x 4) or 48 (12 x 4) Chipmaker 2 pins (Telechem, Atlanta, GA) for the rat and mouse arrays respectively, at the Genomics Technology Support Facility at Michigan State University (http://www.genomics.msu.edu).

Array Experimental Design

Temporal changes in gene expression were assessed using an independent reference design in which samples from o,p'-DDT treated animals were co-hybridized with Veh. Comparisons were performed on 3 biological replicates x 2 independent labelings of each sample (incorporating a dye swap) for each time point. Total RNA (15 µg) was reverse transcribed in the presence of Cy3- or Cy5-labeled dUTP (Amersham, Piscataway, NJ) to create fluor-labeled cDNA, which was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). In contrast, the mouse array experiments used a 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1 µg of total RNA for probe labeling, according to manufacturer's specifications. Cy3- and Cy5-labeled samples were mixed, vacuum concentrated (~1-2 µl) and resuspended in 32 µl of hybridization buffer (40% formamide, 4x SSC, 1% SDS) with 20 µg polydA and 20 µg of mouse COT-1 DNA (Invitrogen) as a competitor. This probe mixture was heated at 95°C for 2 min and was then hybridized to the array under a 22 x 40 mm LifterSlips (Erie Scientific Company, Portsmouth, NH) in a light protected and humidified hybridization chamber (Corning Inc., Corning, NY). Samples were hybridized for 18–24 h at 42°C in a water bath. Slides were then washed, dried by centrifugation and scanned at 635nm (Cy5) and 532 nm (Cy3) on a GenePix 4000B microarray scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 (Molecular Devices).

Array Data Normalization and Statistical Analysis

Data sets for rat and mouse EE have been previously published [12] and have been integrated into the current comparative analysis with o,p'-DDT data. As previously described with these data sets, o, p'-DDT microarray data were first examined using a quality assurance protocol prior to further analysis to ensure consistent, high quality data prior to normalization [13]. Data were normalized using a semi-parametric approach [13]. Model-based t-values were calculated from normalized data, comparing treated to Veh responses at each time-point. Empirical Bayes analysis was used to calculate posterior probabilities of activity (P1(t)-value) on a per gene and time-point basis using the modelbased t-value [14]. Genes were filtered for differential expression based on the P1(t)value which indicates increasing activity as the value approaches 1.0. A two-tiered set of criteria including a statistical P1(t) > 0.999 and |fold-change| > 1.5 was used as an initial selection filter of the expression data, and defined initial differentially expressed gene lists in both the rat and mouse. All data was deposited into dbZach (http://dbzach.fst.msu.edu), a MIAME compliant relational database that ensures proper data management and facilitates data analysis. Complete data sets with annotation and

P1(t) values are available as Supplementary Tables 1-4 at http://www.bch.msu.edu/~zacharet/publications/supplementary/index.html.

Expression Data Annotation and Coactivity Analysis

Features refer to individual cDNA clones spotted on the array and are assigned a GenBank accession number, gene name, symbol, and Entrez Gene ID where annotation is available. For the sake of brevity and consistency, genes are referenced by their official gene symbol as defined by NCBI. Rat and mouse orthologous gene pairs were derived from the publicly available Ensembl and HomoloGene databases. Comparing gene expression between species or ligand treatment involved examining the time, direction, and statistical significance of the change in expression on a gene by gene basis. Similarities and differences in gene expression patterns were designated as coactivesimilar direction (CAS), coactive-divergent direction (CAD), displaced active-similar direction (DAS), and displaced active-divergent direction (DAD). Comparative analysis was conducted using a multivariate correlation-based visualization application developed in-house. Additional analysis was performed using the 4-way Venn Diagram Generator (4VDG, http://www.pangloss.com/seidel/Protocols/venn4.cgi), which involved applying an initial relaxed filtering criteria (|fold change| > 1.3 and P1(t) > 0.99) to each data set, then entering respective Entrez Gene IDs (mouse IDs were used for rat where HomoloGene indicated orthology) into the 4VDG. The output categories were then filtered for only those genes which met more stringent criteria (|fold change| > 1.5 and P1(t) > 0.999) at any one time point. Expression data from $o_{,p}$ '-DDT studies above were compared to previously published data [12] from our group examining EE-elicited expression profiles utilizing the same models, experimental design and analyses.

QRT-PCR (Real Time) Analysis

For each sample, 1.0 µg of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0 μ l) was used as the template in a 30 μ l PCR reaction containing 0.1 μ M each of forward and reverse gene-specific primers designed using Primer3 (15), 3 mM MgCl₂, 1.0 mM dNTPs, 0.025 IU AmpliTag Gold and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Gene names, accession numbers, forward and reverse primer sequences and amplicon sizes are listed in Supplementary Table 5. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems) on an Applied Biosystems PRISM 7500 Sequence Detection System using the following conditions: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was performed to assess the specificity of the primers and the uniformity of the PCR generated products. Each plate contained duplicate standards of purified PCR products of known template concentration covering six orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number versus threshold cycle (Ct). No template controls (NTC) were also included on each plate. Samples with a Ct value within 2 standard deviations of the mean Ct values for the NTCs were considered below the limits of detection. The copy number of each unknown sample for each gene was standardized to the house-keeping gene, Rpl7 to control for differences in RNA loading, quality and cDNA synthesis. Statistical significance of induced or repressed genes was determined using two-way ANOVA followed by t-test for Veh treatment comparisons (SAS 9.1, SAS Institute Inc.). For graphing purposes, the



Figure 5.1

Dose Response Uterine Wet Weights (UWW). UWW was measured across several EE and o, p'-DDT doses in the mouse and rat. Fold change increase in wet weight is plotted. A dose response curve was fit to the data (GraphPad 4.0) to estimate EC₅₀ values. 300 mg/kg o, p'-DDT approximated the maximum response for the doses administered and was used in subsequent time course studies. O, p'-DDT exhibited much lower potency in inducing wet weight than EE, however efficacy was comparable in the mouse while subdued in the rat. Comparisons were made to control animals treated with sesame oil vehicle (n = 5).

relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

RESULTS

Uterine Wet Weight (UWW) and Water Content – UWWs were evaluated in uterotrophic assay dose-response studies in both species to determine the dose eliciting maximal physiological response (induction in uterine wet weight). Doses ranged from 1 to 300 mg/kg b.w. based upon previous studies [15]. In each species 300 mg/kg produced the highest induction (7.3-fold in mouse and 3.5-fold in rat) in UWW (Figure 5.1). Although this dose does not appear to saturate the response in the dose-response curve, higher doses were not deemed necessary for sufficient assessment of DDT-induced uterotrophy. DDT exhibits much lower potency with estimated EC₅₀ values of 58.6 and 137 mg/kg in the rat and mouse, respectively, in comparison to EE. However, the efficacy of o, p'-DDT response in the mouse was comparable to EE, achieving roughly 80% of maximal induction observed with EE, whereas the rat was more subdued resulting in only 42% of that observed in rat EE dose response studies. Based upon the dose response findings the 300 mg/kg dose was used in time-course studies resulting in significant increases in UWW as early as 4 h in the rat and 8 h in the mouse (Table 1). This early increase in weight in the rat can be attributed to increases in water weight as indicated by significant difference between wet and blotted weights at 4 and 8 h (Table 2). However, the rat exhibited very little increase in water content at 72 h compared to the mouse, which may explain the differences in UWW induction between the rat and mouse at the uterotrophic time point.

Table 5.1	Relative Uterine W	et Weights				
		Mouse			Rat	
Time (h)	VEH	EEa	DDT ^b	VEH	EEa	DDT ^b
2	0.386 ± 0.094	0.304 ± 0.072	0.394 ± 0.034	0.235 ± 0.039	0.219 ± 0.051	0.310 ± 0.026
4	0.399 ± 0.095	0.438 ± 0.091	0.458 ± 0.066	0.245 ± 0.061	0.323 ± 0.051*	$0.496 \pm 0.103^{*}$
8	0.363 ± 0.109	0.491 ± 0.052	$0.549 \pm 0.118^{*}$	0.275 ± 0.069	$0.356 \pm 0.063^*$	0.427 ± 0.098
12	0.306 ± 0.108	0.527 ± 0.104	0.465 ± 0.047	0.261 ± 0.068	0.370 ± 0.098*	0.545 ± 0.098*
18	0.451 ± 0.158	$0.876 \pm 0.241^*$	0.633 ± 0.172	0.233 ± 0.049	0.316 ± 0.065	0.475 ± 0.044*
24	0.329 ± 0.045	0.808 ± 0.182*	0.656 ± 0.103*	0.228 ± 0.065	0.412 ± 0.069*	$0.591 \pm 0.140^*$
72	0.382 ± 0.127	2.476 ± 0.599*	2.077 ± 0.929*	0.205 ± 0.048	$1.638 \pm 0.735^*$	$0.775 \pm 0.106^*$
Average re	slative weight (mg/g)) ± standard deviati	on. a n=8 compare	id to VEH of n=8.	b n=5 compared to	o VEH of n=5

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p < 0.05, 2-way ANOVA, Tukey's post hoc test

Table 5.2	Relative Uterine Wa	ater Content				
		Mouse			Rat	
Time (h)	Veh	EEa	DDT ^b	Veh	EEa	DDT ^b
2	0.073 ± 0.054	0.060 ± 0.043	0.066 ± 0.019	0.035 ± 0.007	0.053 ± 0.011	0.048 ± 0.013
4	0.076 ± 0.035	0.097 ± 0.036	0.091 ± 0.014	0.038 ± 0.014	0.108 ± 0.018*	0.130 ± 0.035*
8	0.073 ± 0.046	0.138 ± 0.041	0.102 ± 0.039	0.039 ± 0.009	$0.110 \pm 0.036^*$	$0.092 \pm 0.023^*$
12	0.053 ± 0.041	0.202 ± 0.073*	0.016 ± 0.096	0.035 ± 0.008	$0.099 \pm 0.037^*$	$0.120 \pm 0.033^{*}$
18	0.085 ± 0.052	0.269 ± 0.089*	0.119 ± 0.028	0.031 ± 0.009	0.064 ± 0.012	0.065 ± 0.008
24	0.069 ± 0.029	$0.186 \pm 0.057^*$	$0.140 \pm 0.031^*$	0.030 ± 0.008	0.075 ± 0.013	0.076 ± 0.026
72	0.105 ± 0.060	$0.804 \pm 0.428^{*}$	$0.541 \pm 0.346^*$	0.027 ± 0.007	$0.585 \pm 0.353^*$	0.077 ± 0.018
Average re	Mative weight (mg/g)	+ standard deviation	on a n=8 compare	ad to vehicle of n=	8 h n=5 compared	

Average relative weight (mg/g) ± standard deviation. a n=8 compared to vehicle of n=8. b n= vehicle of n=5. ^{*} p < 0.05, 2-way ANOVA, Tukey's post hoc test

Histopathological Assessment and Morphometry

Time course uterine samples from mouse and rat were also processed for H&E staining and histopathological assessment. Stromal edema was the most consistent observable treatment effect in both species throughout the time course, evident as early as 2 h in the rat and 4 h in the mouse (Tables 3 and 4) and sustained through 24 h in both species. As a consequence to stromal edema, fluid is transported through the epithelium, accumulates in the lumen and results in the water imbibition typical of estrogen induced uterotrophy at 72 h. Mild stromal hypertrophy was notably consistent in the rat between 12 and 72 h whereas histological examination of the mouse stroma revealed no significant increase until 72 h, matching the response to EE. Further, mild myometrial hypertrophy was observed at 24 and 72 h in the mouse but not in the rat. Surprisingly, no hyperplasia was recorded in the rat epithelium although previously observed at 18 and 24 h in EE time course studies [12]. However, hyperplasia was severe in the mouse at 72 h, consistent with a more pronounced $o_{,p}$ '-DDT-induced uterotrophic response. The presence of marked apoptosis in the luminal and glandular epithelium was present in rat but lacking in the mouse. Induction in luminal epithelial cell height, a classic marker of estrogen exposure in the uterus, was induced 1.8-fold in the rat and 1.5-fold in the mouse (Figure 5.2) compared to 2.8 and 2.4 after EE treatment in each species, respectively.

Comparative Analysis of Gene Expression

Temporal gene expression profiles in response to o,p'-DDT were generated in each species and compared to previously published EE time course data sets. Of the 8,734 mouse and 5,684 rat genes represented on the respective arrays (Figure 5.3A), o,p'-DDT elicited differential expression in a large proportion of uterine genes during the

Findings 2 4 8 12 18 24 72 EE 72 Edema (S) - Mild Mild Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild-Moderate Mild (5/5) Mild-Moderate Mild-Moderate (5/5) (6/5) Mild (5/5)	Histological					Time (hr)			
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Findings	2	4	∞	12	18	24	72	EE 72
Edema (S)	Mild	Marked	Moderate	Mild/Moderate	Moderate	Mild-Moderate		•
	(2/2)	(4/4)	(2/2)	(2/2)	(2/2)	(2/2)		
Hypertrophy (S)	ı	ı	ı	Mild (5/5)	Mild (3/5)	Mild-Moderate	Severe (5/5)	Severe (5/5)
						(2/2)		
Hypertrophy (E)	ı	ı	ı	·	ı	•	Marked (5/5)	Marked (5/5)
Hyperplasia (E)	1	ı	·	•	·	•	·	Mild (1/5)
Neutrophils (S)	ı	ı	ı	·	Mild (1/5)	ı	Mild (1/5)	•
Eosinophils (S,M)	ı	,	ı	·	•		Mild-Moderate	Moderate (2/5)
Apoptosis (E.G)	•	·	ı		,	Mild (1/5)	(5/5) Marked (5/5)	Mild-Marked
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Figure 5.2

Induction of Luminal Epithelial Height (LEH). LEH was quantified for each treatment group at 72 h and compared to vehicle controls. Mouse and rat induction was comparable between ligands but EE-induced LEH was significantly ($p \le 0.5$) greater than o_p '-DDT in rats. *= significantly different from Veh. *= significantly different from EE.

uterotrophic response. According to the two-step filtering criteria, 4,088 mouse and 2,499 rat genes were differentially expressed in at least one time point (Figure 5.3B). Approximately 75% of EE-regulated genes in the mouse and 90% in the rat were also regulated by o,p'-DDT. In order to better assess the similarity of two data sets, coactivity analysis was performed, whereby the times and directions of differential regulation were compared and genes were categorized accordingly. These categories are visualized in a coordinate correlation plot (Figure 5.3C) which depicts the highly correlated expression profiles of EE and o, p'-DDT in both fold change and P1(t) value across time. Of the 2,595 (Mm) and 2,258 (Rn) commonly regulated genes between EE and $o_{,p}$ '-DDT, a majority (80% in the mouse and 98% in the rat) exhibited coordinated temporal and directional regulation (CAS category), highlighting similar EE and o, p'-DDT-mediated gene regulation in the rodent uterus. A proportion of genes were also categorized as exhibiting divergent expression (CAD or DAD categories) between EE and o,p'-DDT, 429 in the mouse and 37 in the rat. Ortholog designations from HomoloGene were used to make cross-species comparisons, however, no overlapping genes were identified (Figure 5.3D).

A number of genes were selected for QRT-PCR analysis to confirm microarray results including the prototypical ER regulated gene Fos and Ca2 (Figure 5.4). Fos (FBJ osteosarcoma oncogene), an immediate early gene, was maximally induced at 2 h in both the mouse (13-fold) and rat (75-fold). Ca2 (carbonic anhydrase 2) has been previously shown [12] to exhibit divergent regulation between mouse and rat in response to ER ligands. However, Ca2 was induced in both species in response to o,p'-DDT despite exhibiting different temporal profiles. The mouse elicited a more robust response (11-fold

Figure 5.3

Comparative Analysis of Species-Conserved, Ligand-Specific Gene Expression. (A) cDNA microarrays were used containing 13,361 mouse clones representing 8,734 unique genes and 8,507 rat clones representing 5,684 unique genes. (B) Differentially expressed genes regulated by each ligand were identified using relaxed criteria (P1(t) > 0.99 and |fold change| > 1.3 at any time point) to minimize the likelihood of false-negatives that marginally failed to meet the selection criteria. Differentially expressed genes elicited by both EE and o,p'-DDT were analyzed for similarity in their temporal profiles by comparative analysis. Genes were designated as either CoActive-Similar direction (CAS), CoActive-Divergent direction (CAD), Displaced Active Similar direction (DAS), or Displaced Active Divergent direction (DAD) based on the relationship between the time and direction of differential regulation, and the significance (P1(t)) of the expression profile relative to the vehicle control. (C) The comparative results were plotted on a coordinate correlation graph. A majority of genes show positive correlation between ligands for both fold change and P1(t) value. (D) Cross species analysis of ligand-divergent (CAD) expression profiles indicate no conservation.

FIGURE 5.3





Figure 5.4

rat and human, hereafter referred to as Ca2 to represent all orthologs) is divergently regulated by EE in the mouse and rat, as previously reported. However, o_p '-DDT up regulated Ca2 in the rat, exhibiting a clear difference between EE and o_p '-DDT Quantitative Real Time PCR Confirmation of Fos and Ca2. The temporal gene expression of Fos, an estrogen-responsive immediate early gene, confirmed patterns observed in the microarray data. Carbonic anhydrase 2 (designated Car2 in the mouse and Ca2 in the treatment. N=5, Tukey's HSD post hoc test, p < 0.05 (*). at 72 h) while the rat showed relatively weak induction (1.8-fold, 8 h). In contrast, EE repressed rat Ca2 expression approximately 2-fold across most of the middle and late time points in the rat.

An integrated comparison of all four data sets was performed where differentially expressed orthologous genes (NCBI, HomoloGene) for each data set were entered into the 4-way Venn Diagram Generator (http://www.pangloss.com/seidel/Protocols/venn4.cgi). The number and identity of unique genes populating the overlaps of each data set was determined for common orthologs (Figure 5.5). The comparison of all four data sets resulted in 1,248 commonly regulated genes at any time point (Venn group I). Of the 1,248 common genes, 559 exhibited concerted directional and temporal expression patterns (CAS category). These genes were examined for functional category clusters and found to contain a broad spectrum of genes associated with cellular proliferation and differentiation, consistent with estrogen induced uterotrophy. Select examples of the 559 genes that exhibited similar profiles in terms of either fold-change (Ca3, C3, Fos, Igf1) or increase in copy number, regardless of fold change (Atf4, Dcn, Hspa4) are provided in Table 5.

Other subcategories of interest from the 4-way Venn analysis were groups N, D, B and K, which contain putatively unique responsive genes categorized by chemical or species, respectively. For example, group N contained 110 genes that were regulated in mouse and rat o,p'-DDT data sets; 51 of which were similarly regulated directionally and temporally (CAS), suggesting o,p'-DDT-specific responsive genes. These 51 genes were then examined for similar functional annotation using DAVID's web-based Gene Functional Classification tool (<u>http://david.abcc.ncifcrf.gov/home.jsp</u>). Despite no major

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Functional	Homolo	Gene	Gana Nama	Direction of	a i
Category	Gene ID	Symbol		Regulation	Time
Cell Cycle	37525	Ccnd2	cyclin D2	Down	Late
& Signaling	1355	Cdc25a	cell division cycle 25 homolog A (S. cerevisiae)	ď	Sustained
)	68203	Cdc2a	cell division cycle 2 homolog A (S. pombe)	5	Late
	1429	Csnk2a1	casein kinase 2, alpha 1 polypeptide	ð	Sustained
	3844	Fos	FBJ osteosarcoma oncogene	å	Early
	7326	G0s2	GO/G1 switch gene 2	d D	Sustained
	68226	Gspt1	G1 to S phase transition 1	5	Sustained
	515	lgf1	insulin-like growth factor 1	Ъ С	Sustained
	1679	Jun	Jun oncogene	Up	Early
Water/Ion	11183	Fxyd4	FXYD domain-containing ion transport regulator 4	Down	Sustained
Transport	68166	Aqp8	aquaporin 8	Чp	Sustained
Energetics	10059	Atad3a	ATPase family, AAA domain containing 3A	đ	Sustained
	1273	Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subu	đ	Sustained
	86109	Atp5I	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	5	Sustained
	37905	Cox5a	cytochrome c oxidase, subunit Va	đ	Sustained
	39658	Cox6b1	cytochrome c oxidase, subunit VIb polypeptide 1	ð	Sustained
	80	Cyba	cytochrome b-245, alpha polypeptide	ß	Sustained
	68675	Cycs	cytochrome c, somatic	đ	Sustained
	3337	Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	đ	Sustained
	3347	Ndufs5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	5	Sustained
	86345	Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2	d D	Sustained
Chaperone &	4696	Cct2	chaperonin subunit 2 (beta)	đ	Sustained
Heat Shock	4695	Cct4	chaperonin subunit 4 (delta)	å	Sustained
	6287	Cct5	chaperonin subunit 5 (epsilon)	đ	Sustained
	4694	Cct7	chaperonin subunit 7 (eta)	ð	Sustained
	21322	Hsp110	heat shock protein 110	å	Sustained
	68464	Hsp90aa1	heat shock protein 90kDa alpha (cytosolic), class A member 1	đ	Sustained
	1624	Hspa4	heat shock protein 4	å	Sustained
	68524	Hspa8	heat shock protein 8	ð	Sustained
	86105	Hspd1	heat shock protein 1 (chaperonin)	đ	Sustained
	5656	Tcp1	t-complex protein 1	đ	Sustained
a Early = 2, 4 or	8 hrs, Mid	= 8, 12, 18 c	or 24 hrs, Late = 72 hrs, Sustained = 3 or more consecutive timepoints.		

Table 5.5B. Sp	ecies and (Compound	Conserved Genes		
Functional	Homolo	Gene	Gano Nomo	Direction of	a
Category	Gene ID	Symbol		Regulation	Time
Transcription	1266	Atf4	activating transcription factor 4	å	Sustained
& RNA	6462	Crnkl1	Cm, crooked neck-like 1 (Drosophila)	5	Late
Processing	68487	Ddx39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	5	Sustained
I	74950	Hnrpab	heterogeneous nuclear ribonucleoprotein A/B	5	Sustained
	22991	Hnrpu	heterogeneous nuclear ribonucleoprotein U	5	Sustained
	37638	Pabpc1	poly A binding protein, cytoplasmic 1	5	Late
	4706	Prpf8	pre-mRNA processing factor 8	5	Sustained
	8101	Rere	arginine glutamic acid dipeptide (RE) repeats	Down	Sustained
	4289	Sap18	Sin3-associated polypeptide 18	S	Sustained
	6461	Sf1	splicing factor 1	5	Sustained
	6279	Sf3b3	splicing factor 3b, subunit 3	5	Sustained
	3714	Sfpq	splicing factor proline/glutamine rich	5	Mid
	292	Smn1	survival motor neuron 1	3	Mid
	4290	Smndc1	survival motor neuron domain containing 1	5	Mid
	38275	Tceb2	transcription elongation factor B (SIII), polypeptide 2	- D	Mid
	44649	Tomm20	runt related transcription factor 2	5	Sustained
Translation	1213	Aars	alanyl-tRNA synthetase	ß	Sustained
	20363	Eef1g	eukaryotic translation elongation factor 1 gamma	5	Sustained
	2904	Eit2s2	eukaryotic translation initiation factor 2, subunit 2 (beta)	5	Sustained
	2784	Eif3s4	eukaryotic translation initiation factor 3, subunit 4 (delta)	5	Sustained
	49610	Eif5	eukaryotic translation initiation factor 5	5	Sustained
	5870	Eprs	glutamyl-prolyl-tRNA synthetase	Up	Sustained
a Early = 2, 4 or	8 hrs, Mid =	= 8, 12, 18 с	xr 24 hrs, Late = 72 hrs, Sustained = 3 or more consecutive timepoints		

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Functional	Homolo Gene ID	Gene	Gene Name	Direction of Regulation	Time
Protein	2083	Psma4	proteasome (prosome, macropain) subunit, alpha type 4	,a	Sustained
Turnover	2087	Psmb1	proteasome (prosome, macropain) subunit, beta type 1	9	Mid
	55690	Psmb5	proteasome (prosome, macropain) subunit, beta type 5	5	Sustained
	2098	Psmc5	protease (prosome, macropain) 26S subunit, ATPase 5	5	Sustained
	2101	Psmd2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	-D	Sustained
	12212	Ube2f	ubiquitin-conjugating enzyme E2F (putative)	D.	Sustained
	43226	Ube213	ubiquitin-conjugating enzyme E2L 3	d D	Sustained
	2528	Usp1	ubiquitin specific peptdiase 1	3	Sustained
Cell	4675	Arpc1a	actin related protein 2/3 complex, subunit 1A	đ	Sustained
Architecture	37523	Capg	capping protein (actin filament), gelsolin-like	5	Late
	22430	Dcn	decorin	Down	Sustained
	56469	Des	desmin	9	Sustained
	21362	Dstn	destrin	S	Mid
	37844	Dynl11	dynein light chain LC8-type 1	g	Sustained
	40740	Krt13	keratin complex 1, acidic, gene 19	9	Sustained
	55643	Krt8	keratin 8	5	Sustained
	41321	Lmna	lamin A	3	Sustained
	2354	Spnb2	spectrin beta 2	Down	Sustained
	49734	Tubb2b	tubulin, beta 2b	DD	Mid
	66069	Tubb5	tubulin, beta 5	d	Sustained
Immune	68031	C3	complement component 3	dŊ	Late
Response	7662	Cd24a	CD24a antigen	ď	Late
	1292	Cfb	complement factor B	dD	Late
	2126	Ptprc	protein tyrosine phosphatase, receptor type, C	Ъ	Sustained
Apoptosis &	10683	Aven	apoptosis, caspase activation inhibitor	dŊ	Sustained
Cell Death	10658	Ciapin1	cytokine induced apoptosis inhibitor 1	ЧD	Sustained
	1951	Pdcd2	programmed cell death 2	dŊ	Sustained
	10506	Pdcd5	programmed cell death 5	DD	Mid

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over-represented functional categories, a few notable clusters were observed. Among them were three myosin related genes (Myo5a, Myo5b, Myh6), a cluster of proteolysis related genes (Mmp23, St14, Gzma, Cpxm1), apoptosis/cell death related genes (Apip, Gzma, Maged1, Sphk2) and general transcription-related cluster (Cdk9, Dnmt3a, Lyl1, Mafb, Pias2, Sertad2, Taf9b). All were up-regulated by o,p'-DDT but not EE, with the exception of Apip, which was down-regulated (Table 6).

Collectively, these results suggest that o,p'-DDT potentially mimics the effects of estrogen at high doses as evidenced by the classic induction in uterine wet weight, luminal epithelial cell height and water imbibition in both the mouse and the rat. Comparison of mouse and rat uterine gene expression profiles revealed high similarity in direction and time of regulation of o,p'-DDT and EE responsive genes. Approximately 560 genes exhibited highly conserved and temporally correlated expression across all four data sets, defining a robust gene set that is integral to ER-mediated uterotrophy in the rodent. The divergent regulation of Ca2 between EE and o,p'-DDT in the rat is not conserved in the mouse and was confirmed by QRT-PCR. Furthermore, a putative set of 51 species-conserved, o,p'-DDT-specific genes that exhibited similar profiles across species were identified. Putative differences may be attributed to cDNA print run differences or marginal cutoff criteria screening within respective genes, however the conservation of expression between two species given species-specific array platforms reduces that likelihood.

DISCUSSION

The rodent uterotrophic assay has been a classic bio-assay for assessing acute exposure to ER ligands. The enhanced uterotrophic assay employed in these studies



Figure 5.5

Four Way Venn Analysis of Active Genes. Each data set was converted into ortholog space and processed for activity using relaxed criteria prior to analysis using the 4-way Venn Diagram Generator (<u>http://www.pangloss.com/seidel/Protocols/venn4.cgi</u>). The Venn set was subsequently filtered for genes which met the initial, high-stringency criteria to ensure robust comparisons.

combined the physiological (UWW and water imbibition) and morphometric endpoints (LEH) observed after o,p'-DDT treatment with the early molecular events and modulation of gene expression. Although the 300 mg/kg dose used in the time course study greatly exceeds environmental exposure levels, it is important to understand the potential mechanisms through which contaminants such as DDT work, in order to extrapolate from acute to chronic exposures.

Increases in uterine weight approaching 2-fold induction were observed in dose response studies beginning at 10 and 30 mg/kg in rat and mouse respectively, consistent with previous reports [16]. However, the mouse showed higher efficacy of response at the highest dose of 300 mg/kg while the rat showed lower responsiveness. While gene expression profiling of the dose response samples at 72 h would be valuable, the temporal gene expression events preceding the large change in physiology provide more utility in comparative studies. For incorporation of the full range of genes affected by o,p'-DDT, the high dose of 300 mg/kg was used in the time course studies. The temporal inductions in UWW and water content were comparable between EE and o, p'-DDT for both species, with notable exception of the lack of water imbibition at 72 h in the rat. O,p'-DDTinduced changes in UWW and water content were observed at earlier time points in rat, but the mouse eventually mounted a larger response at 72 h including greater water imbibition (Table 1 and 2). Histopathological assessments of uterine response indicated little differences between EE and o, p'-DDT effects at the uterotrophic time point (72 h). However, subtle species-differences were noted, including indications of mild stromal edema, severe epithelial hyperplasia and marked epithelial apoptosis at 72 h, which were not observed in the rat. These results are consistent with the overall more efficacious

Functional	Homolo	Gene	Gene Name	Direction of	ъ і
Category	Gene ID	Symbol		Regulation	IIMe
Apoptosis &	6277	Apip	APAF1 interacting protein	Down	Early
Cell Death	21237	Gzma	granzyme A	g	Sustained
	32456	Sphk2	sphingosine kinase 2	ď	Sustained
	5077	Maged1	melanoma antigen, family D, 1	Чp	Sustained
Proteolysis	10485	Cpxm1	carboxypeptidase X 1 (M14 family)	å	Sustained
	3424	Mmp23	matrix metallopeptidase 23	ď	Mid
	7906	St14	suppression of tumorigenicity 14 (colon carcinoma)	d N	Sustained
Myosin	37626	Myh6	myosin, heavy polypeptide 6, cardiac muscle, alpha	đ	Sustained
Genes	20100	Myo5a	myosin Va	ď	Sustained
	49481	Myo5b	myosin Vb	Чp	Sustained
Transcription	55566	Cdk9	cyclin-dependent kinase 9 (CDC2-related kinase)	đ	Mid
	7294	Dnmt3a	DNA methyttransferase 3A	ď	Sustained
	4078	Ly1	lymphoblastomic leukemia	ð	Late
	31315	Mafb	isculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	d	Mid
	20979	Pias2	protein inhibitor of activated STAT 2	ď	Mid
	8843	Sertad2	SERTA domain containing 2	ď	Mid
	47969	Taf9b	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated fact	g	Sustained
		07 07 0			

a Early = 2, 4 or 8 hrs, Mid = 8, 12, 18 or 24 hrs, Late = 72 hrs, Sustained = 3 or more consecutive timepoints.

induction in UWW in the mouse compared to the rat. However, the lack of hyperplastic markers in the rat suggests that increases in UWW are more likely due to edema and hypertrophy rather than a proliferative response.

DDT has been reported to signal through ER-independent mechanisms via p38 MAPK pathways stimulating the AP-1 (activating protein-1) transcription factor [17]. DDT was shown to induce cell death via transcriptional control of TNF-alpha through this independent pathway. In the present study, o,p'-DDT gene expression profiles were analyzed for examples of genes that were conserved across both rodent species as being unique to or divergent from EE. TNF-alpha was induced ~1.4-fold by o,p'-DDT, but not EE in the mouse, and was not represented on the rat array. Furthermore, temporal gene expression profiles were notably similar between EE and o,p'-DDT in the high numbers of overlapping and correlated genes, as well as the low occurrence of species-conserved o,p'-DDT specific or divergent genes. Coactivity analysis revealed a large proportion (80% in the mouse, 98% in the rat) of commonly regulated genes between EE and o,p'-DDT, exhibiting highly correlated profiles within each species.

However, some divergently regulated genes (CAD category) were observed in the microarray data. For example, Zmynd11 (zinc finger, MYND domain containing 11) was designated as a CAD gene in the mouse for being repressed ~2-fold by EE at 18 and 24 h, while being induced 2-fold by o,p'-DDT between 8 and 24 h. This pattern was confirmed by QRT-PCR (data not shown). Yet, when lists of CAD orthologs were compared between species (Figure 5.3D), no overlaps were found, suggesting no species-conserved, o,p'-DDT divergent regulation.

Lastly, 4-way Venn analysis was performed to identify orthologs that exhibited conserved expression across all data sets, as well as genes exhibiting putative speciesconserved, compound-specific expression. Four-way Venn subset I consisted of 1,256 mouse-rat orthologs that were differentially expressed in all four data sets, 559 of which were well-correlated in their temporal expression profiles. A representative selection of these 559 genes exhibiting the most robust changes in fold change or total treatment difference (fluorescence intensity) is represented in Table 5. Functional annotation identified genes involved in cell cycle (Ccnd2, Cdc2a) and signaling molecules (Fos, Jun, Igfl); water and ion transport (Aqp8, Fxyd4); cellular energetics and electron transport (ATP synthase, cytochrome b/c subunits and NADH dehydrogenases); transcription factors and chromatin remodeling genes (Atf4, Rere, Sin3a); RNA processing and splicing factors (Smn1, Sf1, Tceb2, Hnrpab); translation initiation and elongation factors (Eif5, Eif2s2, Eef1g) and tRNA synthetases (Eprs, Aars); heat shock and chaperone proteins (Hsp90aa1, Hspa4, Tcp1, Cct2); cell structure related genes (tubulins, actins, keratins); immune response (C3, Ptprc, Cd24a); protein turnover (Psma4, Psmb1, Ube2f); and apoptosis and cell death control genes (Aven, Ciapin1, Pdcd2). Many of these responses have been previously observed in response to other ER ligands during rodent uterotrophy [9], [10], [18], [19], [11], but not characterized after exposure to o,p'-DDT. Massive proliferation and cell structure change, such as that involved during EE and o, p'-DDT-induced uterotrophy, requires increased expression and mobilization of cellular machinery indispensible for cell growth and division.

The 4-way Venn analysis also identified 110 genes which were differentially regulated in o,p'-DDT data sets alone and were further filtered for orthologs that

exhibited conserved temporal expression profiles between species, resulting in 51 putative species-conserved, o,p'-DDT-specific regulated genes. These genes were not over represented by any one functional annotation cluster, but fell into several smaller and more distantly related groups (Table 6). The genes related to apoptosis and proteolysis suggest an adaptive response to the large dose of o,p'-DDT in preventing cell over-growth or damage due to uncontrolled proliferative and xenobiotic stimulation. Myo5a and Myo5b are involved in intracellular secretory vesicle transport while Myh6 has been characterized almost exclusively in cardiac contractility, which makes interpretation difficult given that uterine contractile tissue exhibited myometrial hypertrophy only in the mouse.

Male exposure to DDT is a growing concern that has been linked to DDT's role in disrupting semen concentration via water resorption in the efferent ductules of the testis. Studies in mice have shown that ER is an important regulator of two of the major proteins involved in this process: Slc9a3 (or Nhe3, sodium/hydrogen exchanger member 3) and Ca2 (carbonic anhydrase 2) [20]. The export of protons by Slc9a3 is dependent upon proton generation via the Ca2-catalyzed hydrolysis of CO₂. While Slc9a3 was not represented on our custom microarrays, a family member Slc9a3r1 (sodium/hydrogen exchanger member 3, regulator 1) was substantially regulated by EE (1.5-fold induction at 2 h with sustained increase to 2.7-fold at 72 h) and o,p'-DDT (~3-fold induction between 2 and 24 h) in the rat, while the mouse exhibited differential expression only at 72 h (3-fold by EE and 1.8-fold by o,p'-DDT).

Ca2 is also important in the uterus for gland formation (adenogenesis) during uterine development [21]. However, the expression of uterine Ca2 seems to be dependent

upon ligand and species, as Ca2 was up-regulated by EE in the mouse and repressed in the rat, but induced by o,p'-DDT in both species. A functional AP-1 site, which is bound by a heterodimer of the Fos and Jun family members [22], has been characterized in the human Ca2 gene [23], which suggests a unique interaction of ER signaling at the Ca2 promoter. Additional species difference in ion transport was observed in the case of Fxyd4 (FXYD domain-containing ion transport regulator 4, a sodium/potassium pump subunit). Both species showed sustained down regulation between 12 and 24 h following the treatment with EE and DDT. However, at 72 h mouse expression was induced almost 3-fold in both EE and o,p'-DDT, while the rat exhibited maximal repression (3-fold in mouse and 5-fold in rat) at the same time point. Although it is unclear what specific role Slc9a3r1, Ca2 or Fxyd4 might play in modulating the water imbibition response, the moderate species difference in this physiological endpoint and the differential regulation of these genes between species and compounds suggests a causal role.

Global gene expression profiling in multiple species allows for important comparisons in identifying conserved and divergent mechanisms of action responsible for the observed physiological endpoints. Acute administration of o,p'-DDT closely mimics EE in the induction of the classical physiological and morphological endpoints during uterotrophy, as well as the accompanying molecular and gene expression events. Observations in the present study are contrasted with the results of o,p'-DDT-treated rodent livers [24] that elicited PXR/CAR-like mediated hepatic responses due to the higher presence of these receptors and lower expression of ER. This tissue specific behavior and compartmentalization of expression profiles highlights the importance of the cellular context for assessing systemic responsiveness to a given xenobiotic. The responsiveness of reproductive tissues to estrogens allows for high genome-coverage analysis of the gene expression profiles elicited in widely used rodent models. The extrapolation of biological information across species continues to be an area of uncertainty for most data collected in surrogate species. These studies provide experimental basis for understanding cross-species extrapolations of gene expression data between surrogate models and humans.

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CHAPTER SIX

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CHAPTER SIX

SPECIES COMPARISONS IN TRANSCRIPTOMICS: APPLICATIONS IN TOXICOLOGY

INTRODUCTION

Whole genome sequencing has advanced the biomedical sciences by elucidating the physical sequences of entire genomes for a number of model organisms. These advances were preceded by decades of research investigating the roles of genes, proteins and other metabolites in a variety of processes. The functional significance of each gene, protein and metabolite can now be investigated in the context of global interactions and relationships. The common genetic basis for all of biology (DNA \rightarrow mRNA \rightarrow protein) allows research tools and methodology to be shared, producing a wealth of information across a number of model organisms. This includes the exciting prospect of comparative analysis to identify conserved features important in development, homeostasis, disease, and toxicity. Cross-species comparisons also facilitate the elucidation of global interactions and relationships by identifying conserved and divergent responses important in the associated mechanisms. This chapter will focus on the comparative analysis of gene expression where there have been a significant number of recent advances.

Sequence isn't enough; the role of Transcriptomics

Comparative analysis assumes that important biological properties and responses are conserved across species and share common mechanisms [1]. This would include the structure and function of coding regions as well as associated regulatory elements (Figure 6.1). **Transcriptomics** (Table 1) characterizes the spatiotemporal changes in gene expression, and provides information on when and where genes are expressed. Global gene expression can be monitored using open platforms, such as differential display and serial analysis of gene expression (SAGE) which require little to no *a priori* knowledge about the organism. Alternatively, closed platform approaches can be used which take advantage of discrete sequence information available for several complete and incomplete genomes prior to experimentation.

What is functional orthology?

Functional annotation involves establishing relationships between gene sequences and their biological roles (Table 1). Focused biochemical assays, although tedious, are the "gold-standard" for determining gene function. However, a gene product may have multiple functions in different locations that could be dependent on interactions with other proteins. Consequently, a gene product could affect more than one biological process which would require several different assays to characterize all of its capabilities. Sequence information facilitates the extrapolation of experimentally-based functional annotations across species based on nucleotide or amino acid sequence similarity. Implicit in the extrapolation of functional annotation across species is the concept of **functional orthology**. Although the definitions of **homology, orthology** and **parology** have been debated [2], homology is commonly defined as the relationship between structurally related genes descendant from a common ancestor (Table 1). However, its not clear whether functional equivalence, structural similarity or common ancestry qualifies genes as orthologs. It has been argued that there is insufficient information on a



Figure 6.1

Functional Orthology. Orthology designations based upon coding region sequence homology in addition to other criteria are evaluated by expression analysis. Orthologous expression would suggest similar regulatory mechanisms whereas differential expression of orthologous genes suggests either incorrect orthology designations or divergent regulation.

Key Terminology				
Term	Definition			
transcriptomics	assessing global gene expression at the mRNA level (e.g. microarray analysis, SAGE, differential display, etc.)			
functional annotation	attributing molecular function, biological process or tissue location to a specific gene			
functional orthology	property of orthologs that exhibit similar molecular function, biological process and tissue location			
homolog	structurally related gene descendant from a common ancestor			
paralog	homolog within the same species			
ortholog	homolog between species			
orthologous	property of two orthologs exhibiting similar gene expression			
co-expression	patterns across experiment parameters			
experimental	independent variable which is tested (e.g. treatment, time, dose,			
parameter	Joisease state, developmental stage, tissue location, etc.)			

TABLE 6.1 Key Terminology for Comparative Toxicogenomics

gene by gene basis to accurately determine the timing of speciation and gene duplication events that gave rise to the contemporary slate of genomes. In particular, the analysis of structure-function relationships among highly divergent proteins usually proceeds in the absence of this information. Consequently, it can not be determined with certainty whether two contemporary proteins are orthologs or paralogs [Gerlt and Babbit in [2]]. In many cases this uncertainty can be mitigated by comparing the structural similarity of the define orthologous relationships (Homologene; genes to http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene, Ensembl: http://www.ensembl.org/index.html). These measures of similarity can then be complemented by spatiotemporal expression data to investigate orthologous expression defined as putative orthologs exhibiting comparable patterns of regulation and expression. Differentially expressed genes are those that exhibit significant change in response to different experimental parameters, such as treatment (e.g., vehicle, chemical, drug, other manipulations), dose (level of the experimental manipulation), time, developmental stage or disease state. If a pair of orthologous genes are differentially expressed and respond in a comparable manner under the same conditions, their regulation can be referred to as orthologous expression.

This chapter will examine comparative gene expression analysis and its utility in elucidating mechanisms of action. Diverse experimental and comparative methods as well as available annotation and interpretative tools and resources will also be presented. Furthermore, an assessment of current limitations and needs will be discussed to frame the challenges associated with cross species comparisons.



Figure 6.2

Importance and Applications of Species Comparisons. The benefits of cross-species comparisons holds the potential extend knowledge to Human Medicine, Agriculture, Pesticides, Ecology, Toxicology and Risk Assessment.

OBJECTIVES

A variety of model organisms have been used in basic research. In many cases, it is assumed that the biological information collected in one species is transferable across species including humans. This also has far reaching implications for agriculture, pesticide control and ecology (Figure 6.2). A goal of comparative genomics is to refine and inform the use of surrogate species and their relevance in advancing our understanding of the mechanisms involved in development, homeostasis, disease and toxicity in order to improve human health. This involves transferring functional gene annotation from one species to another with confidence.

Regulatory policies regarding drugs, chemicals and food are largely based on safety and efficacy studies using model organisms. When extrapolating data from model organisms, uncertainty factors (ranging from 1 to 10) are applied to account for incomplete information regarding the similarity of response between species. These data gaps can be attributed to species-specific differences in protein function (*i.e.* binding affinities, enzyme kinetics) or control (*i.e.* DNA regulatory elements, protein-protein interactions, methylation) (Figure 6.1). For example, toxicity studies show that hamsters are exquisitely sensitive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), whereas the guinea pig is nearly resistant. The toxicity of TCDD is mediated by the aryl hydrocarbon receptor (AhR), and sequence comparisons between hamster and guinea pig AhRs identified an expanded glutamine-rich domain in the C-terminus that correlates with reduced toxicity [3]. Differences in avian toxicity to TCDD can also be partially attributed to differences in TCDD-AhR-binding affinity [4] but does not completely explain the broad rang of species sensitivities.

Orthology Criteria							
	Description of Methodia	Information Source					
Sequence	reginrocal RLAST best hit	nucleotide sequence					
Similarity		amino acid sequence					
Synteny	conserved order of genes	whole genome					
Cynteny	in the genome	sequence					
Phylogenetic Tree Matching	organism-level relatedness based upon non-molecular data	taxonomy					
Functional Complementarity	conservation of molecular function	biochemical evidence					

TABLE 6.2 Criteria for Determining Orthology across Genomes

Pharmacokinetic (PK) and pharmacodynamic (PD) studies also facilitate the interpretation of toxicity findings and support refinements in mechanistically-based human risk-assessments. PK data minimizes uncertainties inherent in route-to-route, high-to-low dose, and species-to-species extrapolations [5, 6]. Consequently, cross-species comparison studies are of great importance in determining degrees of functional orthology. Greater confidence in orthologous relationships will not only identify important conserved responses in efficacy and toxicity but will also reduce uncertainties associated with extrapolating model data to humans.

CONSIDERATIONS

In order to conduct informative gene expression comparisons, specific and reliable orthologous gene relationships must be established. Orthologous relationships can be predicted based on sequence similarity, synteny, phylogenetic tree matching, and functional complementation (Table 2). Several online resources are available (Table 3) which utilize these criteria with differing algorithms and stringency levels to provide ortholog predictions.

A significant impediment to comparative genomics is accounting for one-to-many or many-to-many relationships between orthologs and paralogs, which is further complicated when complete genome sequences are not available. Although it is always possible to identify a reciprocal best hit "ortholog", without a complete genome sequence, the true ortholog may not yet be sequenced. To optimize comparisons a tiered approach can be implemented whereby orthology criteria are set loosely to initially include all possible relationships. False-positive orthologs can be subsequently screened

Orthology Resources								
R. MILL	Sequence	Vinteriv	Chylogent	Eunctional Complementation	Algorithm	Number of Species		
HomoloGene	RBH ^a	yes	sequence	-	yes	≥3		
Ensembl	RBH	yes	species	-	yes	≥3		
EGO (Eukaryotic Gene Orthology)	RBH	no	no	-	yes	≥3		
InParanoid	RBH	yes	no	-	yes	pairwise		
PhIGs (Phylogenetically Inferred Groups)	-	no	species	-	yes	≥3		
OrthoMCL	RBH	no	no	-	Markov Clustering	≥3		
HCOP (HGNC ^b Comparison of Orthology Predictions)	RBH	yes	species	-	yes	≥3		
KOBAS (KO ^C -Based Annotation System)	-	no	no	GO ^d Terms	yes	≥3		

TABLE 6.3 Resources for Determining Orthology

a Reciprocal Best BLAST Hit. b HUGO Gene Nomenclature Committee.

c Kegg Orthology. d Gene Ontology

out by further filtering and the identification of divergent responses by implementing more stringent orthology criteria, under the assumption that orthologs will exhibit comparable expression patterns. In addition, discretion is needed in balancing the tradeoff between the number of genomes to be compared and the size and veracity of identified orthologs. In general, the more species included in the search the fewer orthologs will be identified. However, provided a consistent strategy is used to map orthologs, bias error is less likely. Moreover, with more focused gene-specific, hypothesis-driven investigations, increasing stringency in orthology determination may be implemented to validate cross-species extrapolations. Furthermore, ortholog assignments will improve as genomes are completed and refined, gene annotation improves and additional genome sequences from other models become available.

In addition to sequence similarity, the degree to which putative orthologs exhibit similar behavior across different experimental conditions provides further evidence of orthology based on conserved regulation. However, consensus on defining orthologous expression has not been established but may include comparisons of tissue- or cell typespecific gene expression profiles, in which 1) direction, 2) magnitude, 3) time and duration, and 4) shape of response curve may be used as criteria. In many cases, correlation analyses can be used to measure similarities in direction, magnitude and time, depending upon the distance metric utilized. Determining which quantitative measures can be used to minimize subjective assessments of orthologous expression and how many of these criteria need to be met has not been established. Nevertheless, if conserved regulation of gene expression defines orthologous expression, then gene expression regulation under different conditions and in response to different stimuli (Figure 6.3) will



Figure 6.3

Stimulus Targeted Workflow. Microarray data derived from responses to stimuli as opposed to correlation across tissues will result in more physiologically-based determinations of orthologous expression. Important and integral steps involve merging phenotypic and histomorphological endpoints with specific gene expressions in order to phenotypically link the profiles. allow for more robust determinations of orthologous expression. This requires some knowledge regarding what types of stimuli effect changes in specific gene families or molecular processes. A distinction must be made between monitoring a gene's basal level of expression across several uninitiated tissue types verses a single tissue type which is responding to some stimulus or environmental change. However, there may be significant differences in the constitutive level of expression of a gene across models which may alter its response to a change in environmental condition or stimulus. Both the basal levels and stimulated responses are necessary for proper assessment.

Examples of microarray-based methods of examining gene expression across species have followed three different approaches, namely:

1) Same-Species Hybridization, Cross-Platform comparison: (one to one) comparing data between two or more species-specific array experiments

2) Cross-Species Hybridization, Same-Platform comparisons: (many to one) hybridizing biological samples from multiple species to array targets of a single species

3) Mixed-Species Hybridization, Same-Platform comparison: (one to many) hybridizing biological samples from one species to array targets of multiple species.

Most comparisons are made between data sets derived from same-species hybridizations. For example, mouse samples hybridized to mouse-based arrays compared to a human dataset obtained using human arrays. An important consideration is to determine whether normalization occurs separately or together and when to merge data sets. For example, a novel strategy was used to compare the expression of human breast tumor and

chemically-induced rat mammary tumor samples in order to validate the rat mammary tumor model [7]. Using this approach, 2,305 rat orthologs were used to classify human tumors derived from array data suggesting that rat primary tumors share comparable signatures with low to intermediate grade estrogen receptor positive human breast cancer. Moreover, the study validated chemically-induced rat mammary tumors as a model of human disease.

Many factors including differing experimental timing, resources or logistics, confound the merging and normalization of raw microarray data. Independent normalization was used to identify conserved and divergent orthologous uterine gene expression during uterotrophy in rats and mice treated with ethynylestradiol using the same study design [8]. Parallel but species-specific statistical analyses identified 153 orthologous pairs which exhibited conserved temporal responses. Furthermore, there was sufficient orthologous expression evidence to support the transfer of functional annotation from 44 characterized mouse genes to previously unannotated rat ESTs based upon sequence homology and co-expression patterns was possible, demonstrating a novel utility of cross-species analysis.

Several groups have sought to circumvent the problem of limited microarray resources for non-traditional models by performing direct cross-hybridization experiments using labeled cDNAs from one species (ape, pig, cow, mouse, salmon) [9-16] and hybridizing to array probes from a related organism with more developed annotation. This cross-hybridization approach assumes that cDNA probes are of sufficient length and homology to overcome inter-species differences in gene sequence. For example, rabbit RNA samples have been cross-hybridized to mouse [17]. Other

studies [9, 12, 18] have conducted cross-hybridizations using multiple biological and technical replicates, and independent quantitative real time PCR verification with moderate success.

As oligonucleotide arrays (Affymetrix, Agilent, CodeLink) have become more prevalent and utilized, questions have arisen regarding the species-specificity of smaller probes. Cross-hybridizations between mouse and human samples on a human oligonucleotide arrays were conducted to examine a dual-species chimeric tissue model of transplanted human hepatocytes in mouse liver. This study addressed the degree to which incidental and undesired mouse tissue would contribute to the human sample hybridizations to human arrays [19]. Specific cross-reactive probes were identified and a method to monitor the relative proportions to which each species' tissue content contributed to the expression data was developed.

Cross-species hybridization can also involve printing orthologous cDNAs from multiple species onto a single array. Samples from represented species are then hybridized to identify same-species and cross-species interactions on the same array. Analysis of oocyte expression in the cow, mouse and frog found that cross-species hybridizations are highly reproducible and that the expression of a number of orthologs is conserved [20]. These results were subsequently verified by gene- and species-specific quantitative real time PCR and further species-specific array platform experiments. Although cross-hybridization experiments make interspecies comparisons much easier, there still remains a lack of consensus regarding its reliability and long term utility.

Conservation of gene sequence and regulation for a number of pathways and responses is expected for comparable responses. However, given the increasing number

of gene expression studies and screening algorithms which select for conserved responses, there will inevitably be examples of divergent orthologous expression (i.e., one ortholog is induced while the other is not responsive or is repressed) that require closer scrutiny to exclude orthology misclassifications, artifacts and false negatives. Overall, it is easier to identify conserved orthologous expression as opposed divergent regulation. Several factors may be responsible for divergent orthologous gene Species-differences in trans-acting factors or RNases may modulate expression. transcription or mRNA stability. Likewise, the degeneracy of *cis*-acting regulatory elements (cREs) such as transcription factor binding sites may also account for regulatory differences. Methylation status, chromatin structure or other epigenetic modifications could also play a role. It is therefore important to further investigate divergent expression patterns to elucidate the regulatory mechanisms involved. Characterization of divergent orthologous gene expression will also facilitate future data interpretation as well as the designation of functional orthology.

Due to the static relationship between a gene's expression and its putative regulatory motifs, in addition to the availability of genomic sequence data, the role of *c*REs can also be examined. Several *in silico* genomic sequence search algorithms and experimental approaches have been developed to identify and associate *c*REs with gene regulation. Supervised methods involve the identification of known response elements by computationally scanning proximal, regulatory genomic sequences for consensus response elements based on a position weight matrix (PWM) approach [21]. Transcription factor binding site databases and web resources (i.e. TRANSFAC, http://www.gene-regulation.com/pub/databases.html) provide consensus motifs for PWM

development. An alternative, unsupervised approach involves generating unique 5-15 nucleotide "words" from proximal/regulatory genomic sequences (i.e. total genome or upstream promoter regions) that can then be used to determine the frequency of over represented of words/motifs within the regulatory sequence of genes exhibiting comparable expression patterns when compared to random sequences [22].

Protein-DNA interactions can be examined experimentally using chromatin immuno-precipitation (ChIP) to identify genomic regions bound by transcription factors of interest. Genome-wide ChIP analysis, commonly referred to as ChIP-on-chip or ChIPchip, uses a microarray strategy to identify all protein-DNA interactions at a specific time point [23]. Similar to SAGE, precipitated chromatin can also be concatermized and sequenced providing an unsupervised strategy to identify protein-DNA interactions [24-26]. However, there is poor correlation between ChIP evidence of a protein-DNA interaction and transcriptional activity. Complementary, global gene expression profiling, computational regulatory motif searches and ChIP analyses facilitate a more comprehensive interpretation of the data.

The manner in which one pursues further evidence to explain divergent expression of orthologs will depend largely upon the resources available. Bioinformatic approaches require genomic sequence data, computing power and programming capabilities while ChIP-chip approaches require antibodies with high affinity and specificity as well as specialized array platforms or access to high throughput sequencing facilities. These complementary methods are crucial for identifying comparable patterns of gene expression involving conserved mechanisms of regulation that will support conclusions regarding the orthologous expression.

LIMITATIONS

Several factors limit cross-species analyses, such as: 1) incomplete genome sequence data, 2) unstable gene annotation with complementary functional annotation, 3) the complexities of orthology mapping, 4) concerns regarding reporting standards, 5) limited relevant human gene expression data, and 6) inadequate tools and resources for data integration. For instance, incomplete sequence information for a particular species significantly compromises the ability to identify orthologous genes with certainty, thus limiting comprehensive comparisons. For many species (e.g., cow, pig, sheep, chicken, dog, and horse), annotation is limited to a few hundred genes, consisting mainly of ESTs and computationally predicted mRNA [27-29]. Thus, orthology mapping against genomes with mature annotation (e.g., human, mouse, or rat) is frequently performed to interpret expression data.

Several factors compromise orthology mapping resulting in a lack of consensus on the most appropriate way to determine orthology. The presence of large paralogous gene families resulting in "one-to-many" or "many-to-many" relationships between species also complicates orthology assignments. Ambiguities in orthology mapping resulting from poor resolution of homologous gene families and isotypes further limit the ability to assess cross species responses.

Comparative gene expression studies also require appropriate study designs that include sufficient replication to support statistically rigorous comparisons. Although the cost of microarrays continues to decreases, this is often mitigated by the increasing cost of newer technologies, higher genome coverage, and required QA/QC robustness.

Moreover, a related concern is the reporting standards needed to facilitate sharing of expression data. More specifically, standards that communicate the types and amounts of detail required to reproduce the experimental design. However, ambiguity in the definition and description of proposed standards (i.e., MIAME) has resulted in different interpretations and a lack of consensus regarding their implementation resulting in the existence of MIAME-compliant public repositories with different reporting requirements [30, 31].

While direct comparisons of specific tissue or organ responses between species are desirable, genetic heterogeneity and the availability of appropriate human samples significant limit cross-species comparisons that include humans. Most often human tissues are only available following surgical removal of diseased or compromised tissue, depending on patient compliance and the surgeon's discretion. Furthermore, studies with model species can be more precisely controlled with greater latitude regarding treatment regimens and dose ranges in order to obtain a more comprehensive assessment. Although human cell culture systems are available, the ability of in vitro systems to accurately model *in vivo* responses has not been adequately demonstrated.

There is increasing access to several software packages as well as free web-based tools for data mining, analysis, annotation, and visualization. However, few of these solutions explicitly address cross-species comparisons, facilitate orthology designations or address orthologous expression. The lack of robust cross-species studies may contribute to the paucity cross-species analysis tools. More recently some tools with inter- or cross-species functionalities (Comparative Toxicogenomics Database, (http://ctd.mdibl.org/); Integrative Array Analyzer, (http://zhoulab.usc.edu/iArray

<u>Analyzer.htm</u>); Resourcerer, (<u>http://www.tigr.org/tigr-scripts/magic/r1.pl</u>); yMGV, (<u>http://transcriptome.ens.fr/ymgv/</u>) have been made available. However, many of these limitations seem are only functions of time as efforts to address genomic sequencing and annotation needs as well as integrated approaches to orthology mapping appear to be in development.

CONCLUSIONS

Cross-species analyses can provide compelling information that significantly advances our understanding of the mechanisms of action of disease, drug efficacy and toxicity. Furthermore, complete knowledge of species specific and conserved mechanisms will increase the efficiency of drug development and estimates of risk to human health. While comprehensive comparative studies are in their infancy, the required infrastructure and resources needed to support these studies continues to develop. In addition, increases in genomic data, array platform variety, coverage and reliability; maturation of annotation and increased understanding and compliance to reporting standards in addition to improved bioinformatic tools are expected. These key advances will afford future investigations with refined perspectives on cross-species extrapolations that will significantly improve efforts to improve human health.

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE RESEARCH

CONSERVATION OF ER SIGNALING

The preceding studies document the gene expression profiles that accompany estrogen induced uterotrophy in the rodent. These studies utilize a comprehensive time course study design that assesses uterine response in an acute time frame. This temporal resolution allows for the monitoring of changes in early gene expression cascades that might be induced, peak and subside within a matter of hours. Thus coactivity analysis was developed to identify the time sensitive gene expression changes and evaluate between two species or ER ligands the types of relationships defined by the CAS, CAD, DAS and DAD categories.

A prominent feature of all of the gene expression profiles evaluated in these studies is the high degree of overlap in genes regulated by EE, TAM and o,p'-DDT. Furthermore, the high proportion of the mouse or rat genomes being differentially expressed by a single treatment (time points 2 to 24 h) points to the large scale conserved nature of estrogen signaling during uterotrophy. Functional categories represented in each of the six data sets repeatedly point to up regulation of genes associated with proliferation and differentiation including 1) cellular materials: the basic building blocks of cells, consisting of membranes, organelles, nucleotide and protein synthesis; 2) cellular energy: the power house genes that generate energy currency such as ATP and NADH in pathways like the TCA cycle and electron transport; 3) cellular instructions: executing the uterotrophic program requires concerted and massive processivity of the central dogma:

DNA to mRNA to protein, without compromising cellular integrity, allowing DNA replication mistakes, cellular oxidative stress imbalance or other cell cycle check point mistakes. Thus, the central hypothesis of these studies has been proven true given the substantial subset of EE, TAM and o,p'-DDT regulated genes which are found to be in common and highly correlated.

Estrogen induced uterotrophy is likely one of the largest tissue-wide physiological responses (after perinatal development) in higher mammals. Thus, the characterization of the initial gene expression events is a starting point to understanding a broad range of physiological programs involving wide spread proliferation and change in cell type, including cancer and embryonic development.

DIVERGENT RESPONSES

Although the majority of gene expression results were highly similar across species and chemical, comparative analysis allowed for the use of species and chemical as a screen for identifying unique or divergently regulated genes. For example, Chapter 3, Table 3 lists putative divergently regulated genes between species in response to EE treatment. These results were later tested by QRT-PCR and all but one (carbonic anhydrase 2) failed to confirm a divergent temporal profile. This is to be expected given the thousands of genes being tested and data analysis filters and screens which are designed to select for those genes with high probability of being a false positive. Several reasons could explain errant behavior, most likely owing to cross-contamination of wells in a cDNA clone set microtiter plate during handling. However, it is usually the case that discounting all data suspected to be artifactual will inhibit the discovery of truly novel

findings. Such was the case with carbonic anhydrase 2. Microarray data indicated a CoActive-Displaced (CAD) relationship between species for both EE and TAM data sets (induced in the mouse and repressed in the rat) while o,p'-DDT induced Ca2 in both the mouse and rat. This peculiar expression behavior in response to different ER ligands in mouse and rat may be explained by cross talk of o,p'-DDT's ER dependent and independent signaling pathways.

Ca2 is well known for its homeostatic role in osteocytes in regulating bone resorption. The human CAII promoter contains an AP-1 site which was shown to be directly activated by Fos/AP-1 and is strongly regulated during osteoclast differentiation/bone resorption [1]. In the rat, removal of estrogen by ovariectomy is associated with increased expression of Ca2 in osteoclasts. This is consistent with our observations of EE and TAM down regulating Ca2 in the uterus. However, in the mouse, Ca2 plays an important role in fluid resorption in the efferent ductules of the testis. ERa knockout mice exhibit decreased Ca2 expression relative to wildtype. Furthermore, Ca2 has been shown to be essential for uterine adenogenesis (gland formation) in the developing mouse [114]. Collectively, Ca2's species- and ligand-divergent regulation during uterotrophy and its important pleiotropic roles in bone homeostasis, male fertility and uterine gland development highlight an important relationship between estrogen signaling and Ca2's potentially key role in mediating adverse health effects by estrogenic endocrine disruptors.

GENOME COVERAGE

A limitation of the preceding studies is the lack of full genome coverage on the custom in-house cDNA microarrays. At the time of construction (approximately 2002) the cDNA

clones for the mouse and rat microarrays were selected for maximal genome representation of well annotated genes and overlap of orthologs between human, mouse and rat clones. For the majority of the time since then, our in-house microarray clone sets have afforded us the experimental liberty to generate unhindered quantities of expression data within our budget constraints; in contrast to other researchers performing microarray experiments that were forced to be selective in the number of treatment groups or replicates to be performed. However, with the maturation of human and mouse genome annotations and increasing annotations of the rat in recent years, the current genome coverage of the cDNA microarrays does not facilitate more comprehensive conclusions that could be drawn from true "genome-wide" coverage. Yet, the current data sets remain an adequate representation of the most mature genome annotations and provide insights into the genomic trends observed for each species.

SURROGATES TO HUMANS

Possibly a more interesting hypothesis that arises from the preceding studies than the first is the question of which rodent species better predicts human uterine response to TAM and o,p'-DDT? Given the limitations of performing controlled experiments with human subjects, a number of promising alternatives are available. The first is the use of human endometrial cells in culture. Ishikawa cells, a human endometrial adenocarcinoma cell line [2], express ER and have been used extensively in estrogen studies [3]. ECC1 [4] and HEC-1 [5] also express ER and are viable options. Comparisons of whole uterus homogenates to an epithelial cell line would have its limitations; however these comparisons would begin to bridge the gap between rodent surrogates and human response. Human primary cell cultures of uterine or endometrial cells would be an ideal comparator. Human uterine primary smooth muscle cell cultures [6] and endothelium/trophoblast co-cultures are being utilized to study implantation related endpoints. Their use in characterizing human uterine response to ER ligands would provide broad insights into cross-species extrapolations.

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