Tox 21
Developmental Toxicology

Susan Makris

US EPA, Office of Research and Development
National Center for Environmental Assessment
Washington, DC

Origins of Tox21

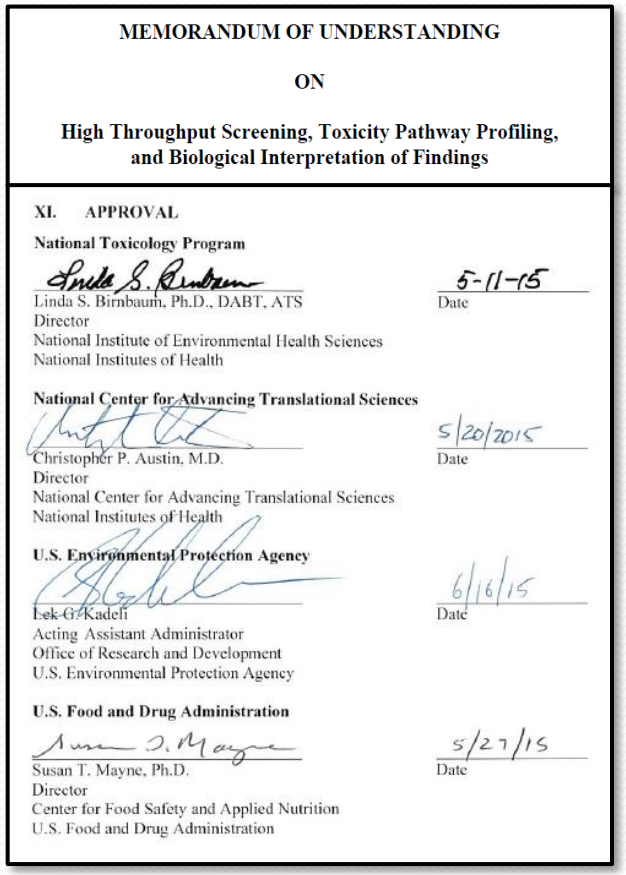
"Toxicity Testing in the 21st Century"

- Limitations in the current testing paradigm
  - Historical increase in:
    - Number of tests
    - Cost of testing
    - Use of laboratory animals
    - Time to develop and review data
  - Difficult to apply to risk assessment due to inability to fully address complex issues such as:
    - Life stage sensitivity
    - Mixtures and cumulative exposures
    - Varying exposure scenarios
    - Understanding of mechanism of toxicity and implications in assessing dose-response
    - Characterization of uncertainty

NRC, 2007

NRC recommended a transformation in toxicity testing and risk assessment that focuses on toxicity pathways

Formation of U.S. Tox21 Federal Partnership - 2008



MEMORANDUM OF UNDERSTANDING

ON

High Throughput Screening, Toxicity Pathway Profiling, and Biological Interpretation of Findings

XI. APPROVAL

National Toxicology Program

5-11-15
Date

Linda S. Birnbaum, Ph.D., DABT, ATS
Director
National Institute of Environmental Health Sciences
National Institutes of Health

National Center for Advancing Translational Sciences

5/20/2015
Date

Christopher P. Austin, M.D.
Director
National Center for Advancing Translational Sciences
National Institutes of Health

U.S. Environmental Protection Agency

6/16/15
Date

Lek G. Kadeli
Acting Assistant Administrator
Office of Research and Development
U.S. Environmental Protection Agency

U.S. Food and Drug Administration

5/27/15
Date

Susan T. Mayne, Ph.D.
Director
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

MOU Signed February, 2008; Revised July, 2010

- National Institute of Environmental Health Sciences (NIEHS)
- National Center for Advancing Translational Sciences (NCATS)
- U.S. Environmental Protection Agency (EPA)
- U.S. Food and Drug Administration (FDA)

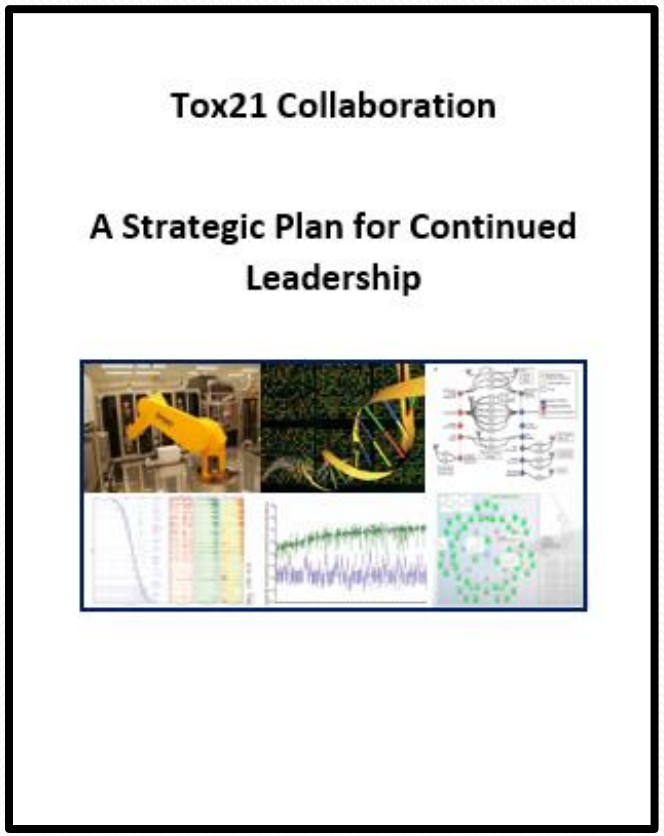
<u>A Successful Interagency Collaboration</u>:

- Thousands of chemicals tested in 70 assays and over 50 relevant pathways (primarily HTP testing)
- Public release of millions of data points
- 200 peer reviewed articles in 56 journals
- Data now being used for regulatory decisions

https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21

Thomas et al. 2018 ALTEX

Tox21 Strategic and Operational Plan - 2018



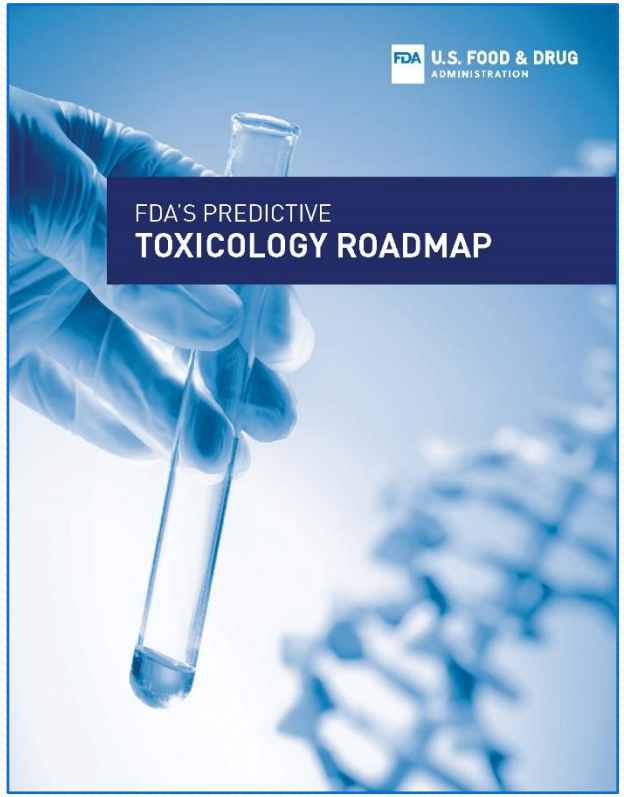
Areas of Focus

- Developing and deploying alternative test systems that are predictive of human toxicity and dose response
- Addressing key technical limitations of current high throughput screening systems
- Consolidating chemical library management and developing more focused libraries
- Curating and characterizing legacy animal toxicity studies for continued comparison to high-throughput screening results
- Validating high-throughput assays, integrated assay batteries, computational models, 3-D organ-like model systems, and other emerging Tox21 approaches
- Refining and deploying high-throughput methods for characterizing pharmacokinetics to better predict the relationship between target tissue concentrations and external doses of chemicals.

https://www.epa.gov/sites/production/files/2017-08/documents/tox21_fact_sheet_v7.pdf

Agency-Specific Roadmaps Published

FDA Predictive Toxicology Roadmap (Dec 2017)

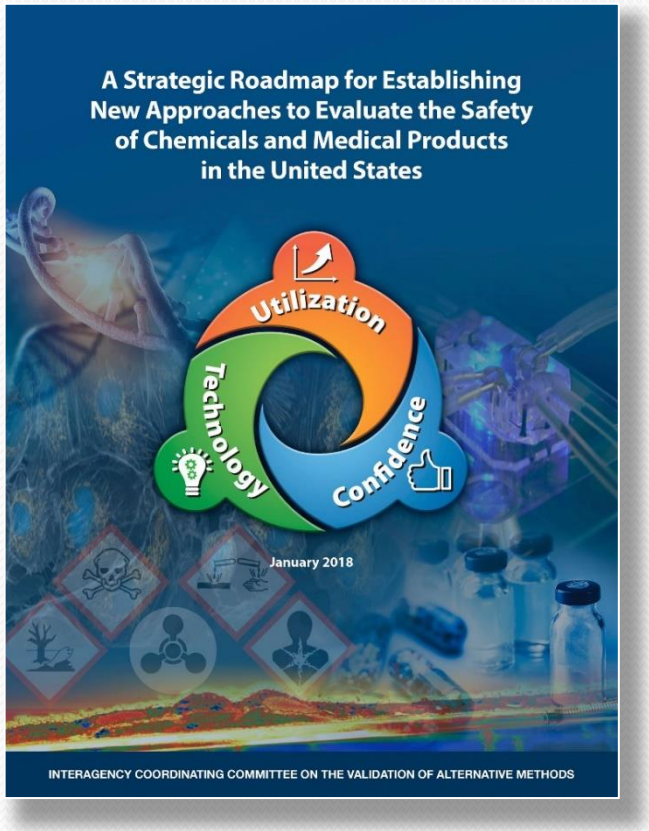


https://blogs.fda.gov/fdavoice/index.php/2017/12/fda-launches-predictive-toxicology-roadmap-to-enable-advances-in-toxicity-testing/

Focus

- Reliable interpretation and application for product development and/or regulatory decisions
- Clear context of use
- Importance of multi-sector partnerships and collaborations to identify, develop, validate, and integrate assays into risk assessment

ICCVAM Strategic Roadmap (Jan 2018)



https://ntp.niehs.nih.gov/go/natl-strategy

Tox 21 Collaborative Projects

- Cell Line Selection for High-throughput Transcriptomics (Sipes, Harrill, Setzer)
- Acetylcholinesterase (AChE) Inhibitors Screening (Xia, Santillo)
- In Vitro Disposition of Tox21 Chemicals (DeVito, Friedman)
- High-Throughput (Ferguson, Harrill, Xia)
- Predictive Modeling of Developmental Toxicity with Human Pluripotent Stem Cells (Knudsen, Kleinstreuer, Lumen)
- Incorporating Genetic Susceptibility into Developmental Neurotoxicity Screening via Population Diversity (Harrill, Behl)
- Performance Based Validation of Tox21 Assays (Houck, Judson, Kleinstreuer)
- Retrofitting Existing Tox21 HTS Assays with Metabolic Capability (Xia, Witt, Simmons)

https://www.epa.gov/chemical-research/tox21-cross-program-projects

An EPA Statutory Mandate for Chemical Testing

The 1976 Toxic Substances Control Act (TSCA) was amended by the **Frank R. Lautenberg Chemical Safety for the 21st Century Act** in 2016:

- new requirements and deadlines for actions related to the regulation of new and existing chemical substances.
- new subsection under Section 4 (*Testing of Chemical Substances and Mixtures*); particularly, Section 4 (h) entitled *Reduction of Testing on Vertebrates*

4(h)(2) - ***Implementation of Alternative Testing Methods***—To promote the development and timely incorporation of new scientifically valid test methods and strategies that are not based on vertebrate animals, the Administrator ***shall –***

4(h)(2)(A) - "not later than 2 years after the date of enactment….develop a strategic plan to ***promote the development and implementation of alternative test methods and strategies*** to reduce, refine, or replace vertebrate animal testing and provide information of equivalent or better scientific quality and relevance for assessing risks of injury to health or the environment of chemical substances or mixtures through, …"

https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/strategic-plan-reduce-use-vertebrate-animals-chemical

Federal Register Notice, June 2015

"Endocrine Disruptor Screening Program: Use of High Throughput Assays and Computational Tools"

http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2015-0305-0001

EDSP Tier 1 Battery of Assays	Model Alternative Development
Estrogen Receptor (ER) Binding	ER Model
Estrogen Receptor Transactivation (ERTA)	ER Model
Uterotrophic	ER Model
Androgen Receptor (AR) Binding	AR Model
Hershberger	AR Model
Aromatase	STR Model
Steroidogenesis (STR)	STR Model
Female Rat Pubertal	ER, STR & THY Models
Male Rat Pubertal	AR, STR & THY Models
Fish Short Term Reproduction	ER, AR & STR Models
Amphibian Metamorphosis	THY Model

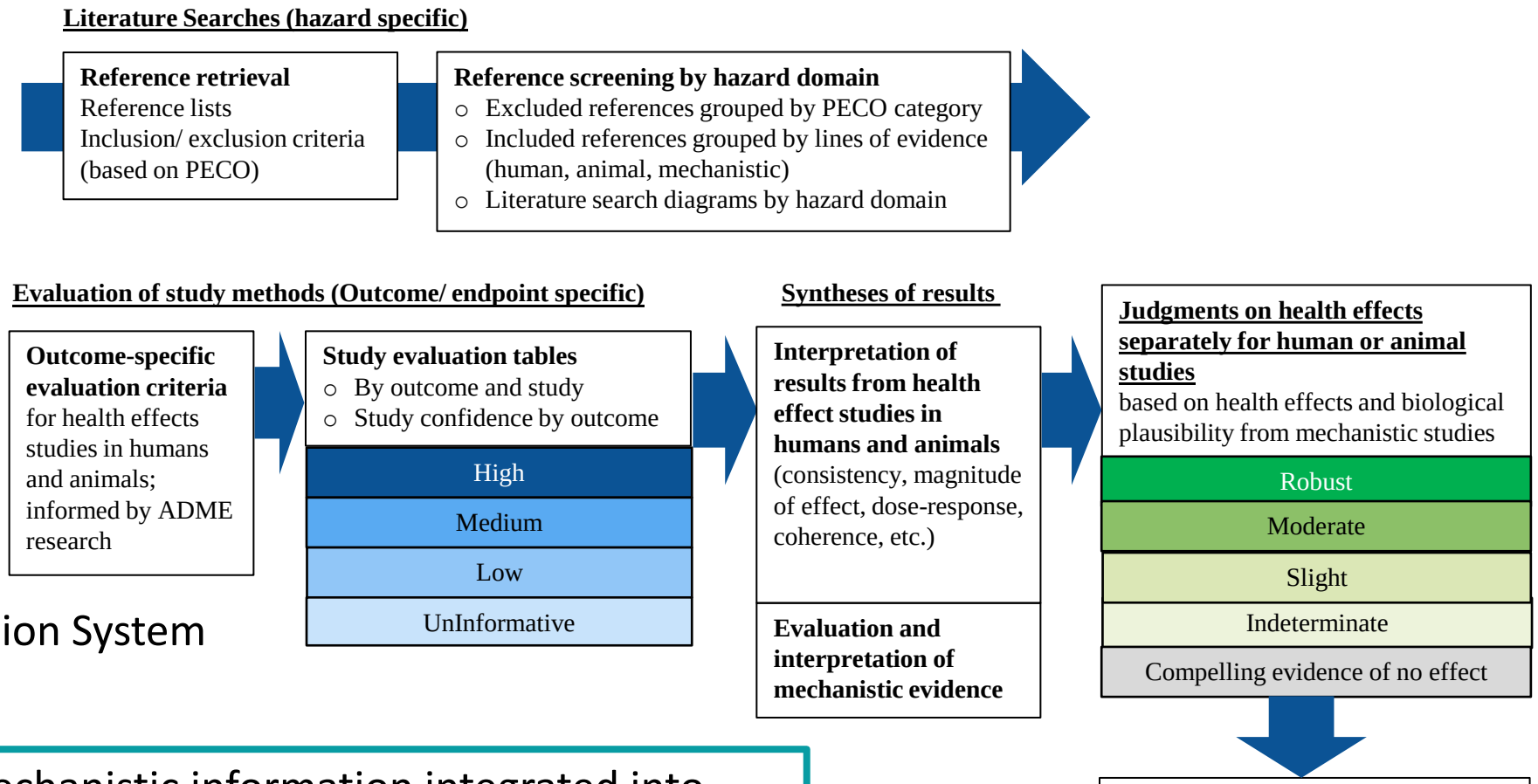
https://www.epa.gov/endocrine-disruption/use-high-throughput-assays-and-computational-tools-endocrine-disruptor

https://www.regulations.gov/docket?D=EPA-HQ-OPP-2017-0214

Issues Typically Considered in the Evaluation and Interpretation of Regulatory Developmental Toxicity Data

- Bioavailability in the maternal animal (systemic exposure)
- Route(s) of exposure of the agent in vivo and any route-related differences in metabolism
- Ability of the agent or active metabolite to cross the placenta: fetal exposure
- Potential for the agent to cause death, altered growth structural abnormalities or functional effects in the offspring; effects of maternal toxicity or stress as a mitigating factor
- Life stage sensitivity: quantitative or qualitative effects
- Differences in toxicokinetic parameters (absorption, distribution, metabolism, storage, or excretion) in the fetus compared to adults
- Differences in toxicodynamic parameters in the fetus compared to adults: different targets or level of response
- Windows of susceptibility in the developing organism
- Dose-response (NOAELs/LOAELs/BMDs)

EPA IRIS Systematic Review Process



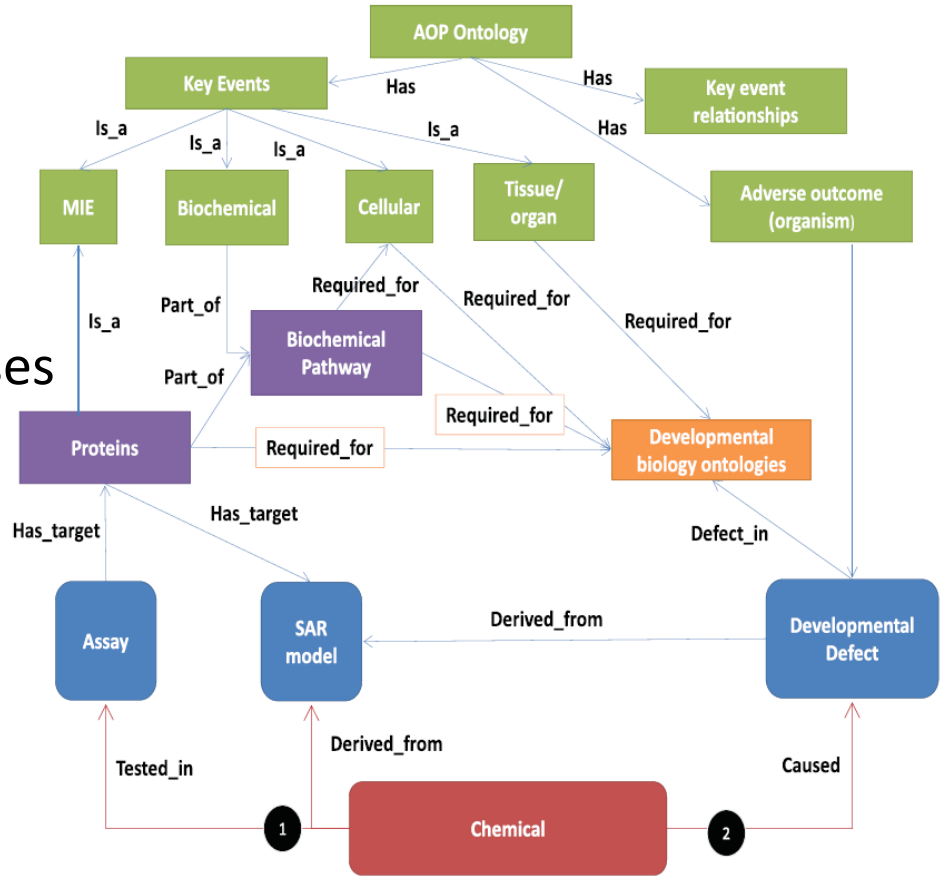
IRIS = Integrated Risk Information System

Examples of chemicals with mechanistic information integrated into devtox characterization:

- TCE: putative AOP for disruption of valvulo-septal morphogenesis was integrated into the WOE evaluation supporting TCE-related cardiac malformations (Makris et al., 2016)
- Phthalates: testosterone reduction during critical period in late gestation resulted in malformations of the male reproductive system

Developmental Toxicity Ontology

- Ontology = a way to classify terms, how they relate to broader concepts and their interrelationships
- A developmental toxicity ontology can span multiple levels of organization and is based on:
  - Knowledge of developmental biology
  - Mode of action/ adverse outcome pathways
- Challenges
  - Role of potency (separating adaptive vs. adverse responses)
  - Maternal toxicity as a driver or confounder of in vivo responses
  - Importance of developmental stage susceptibility



DOI: 10.1002/bdr2.1189

ORIGINAL RESEARCH ARTICLE

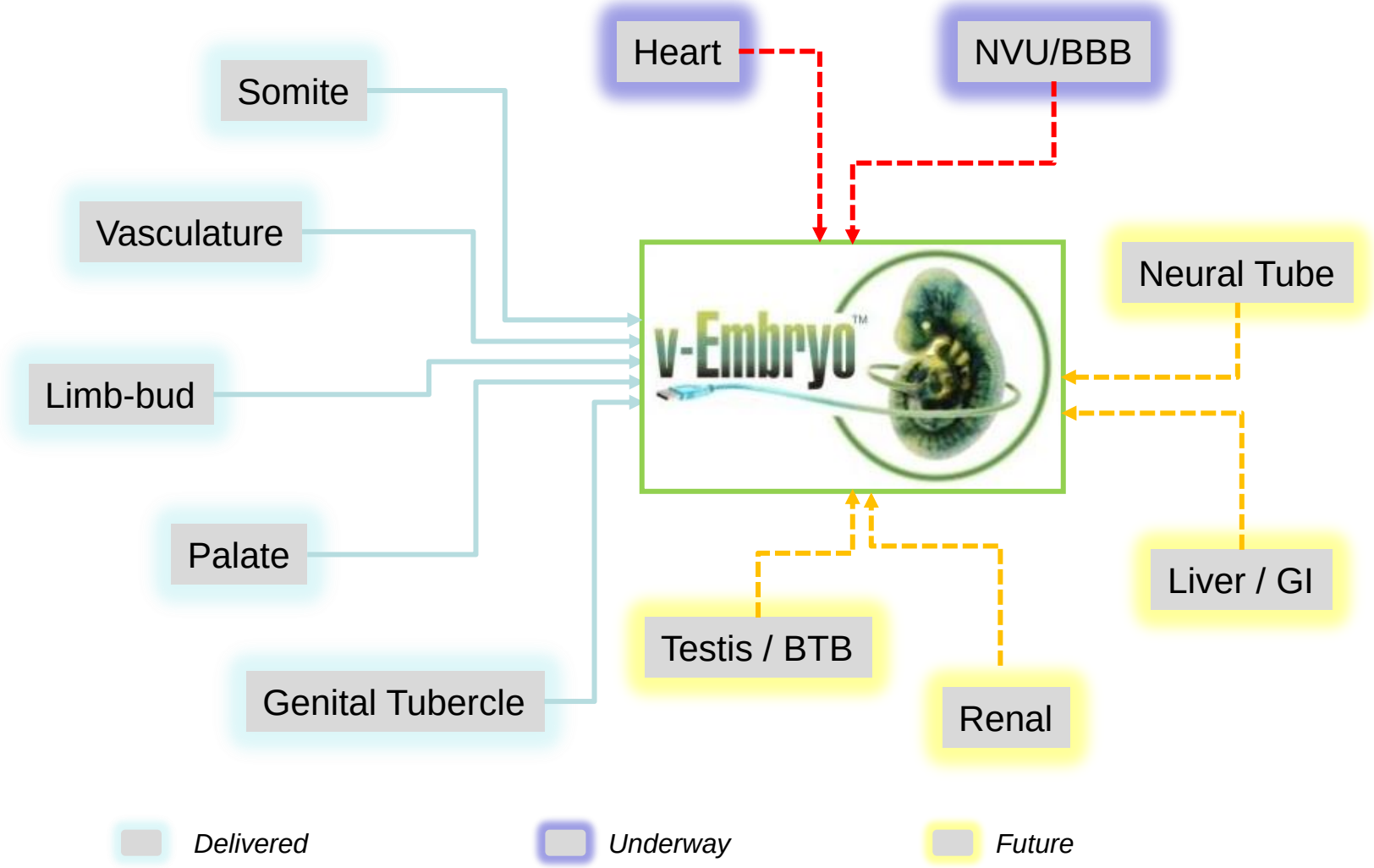
WILEY Birth Defects Research THE TERATOLOGY SOCIETY EST. 1960

Building a developmental toxicity ontology

Nancy Baker[1] | Alan Boobis[2] | Lyle Burgoon[3] | Edward Carney[4]* |
Richard Currie[5] | Ellen Fritsche[6] | Thomas Knudsen[7] | Madeleine Laffont[8] |
Aldert H. Piersma[9] | Alan Poole[8] | Steffen Schneider[10] | George Daston[11]

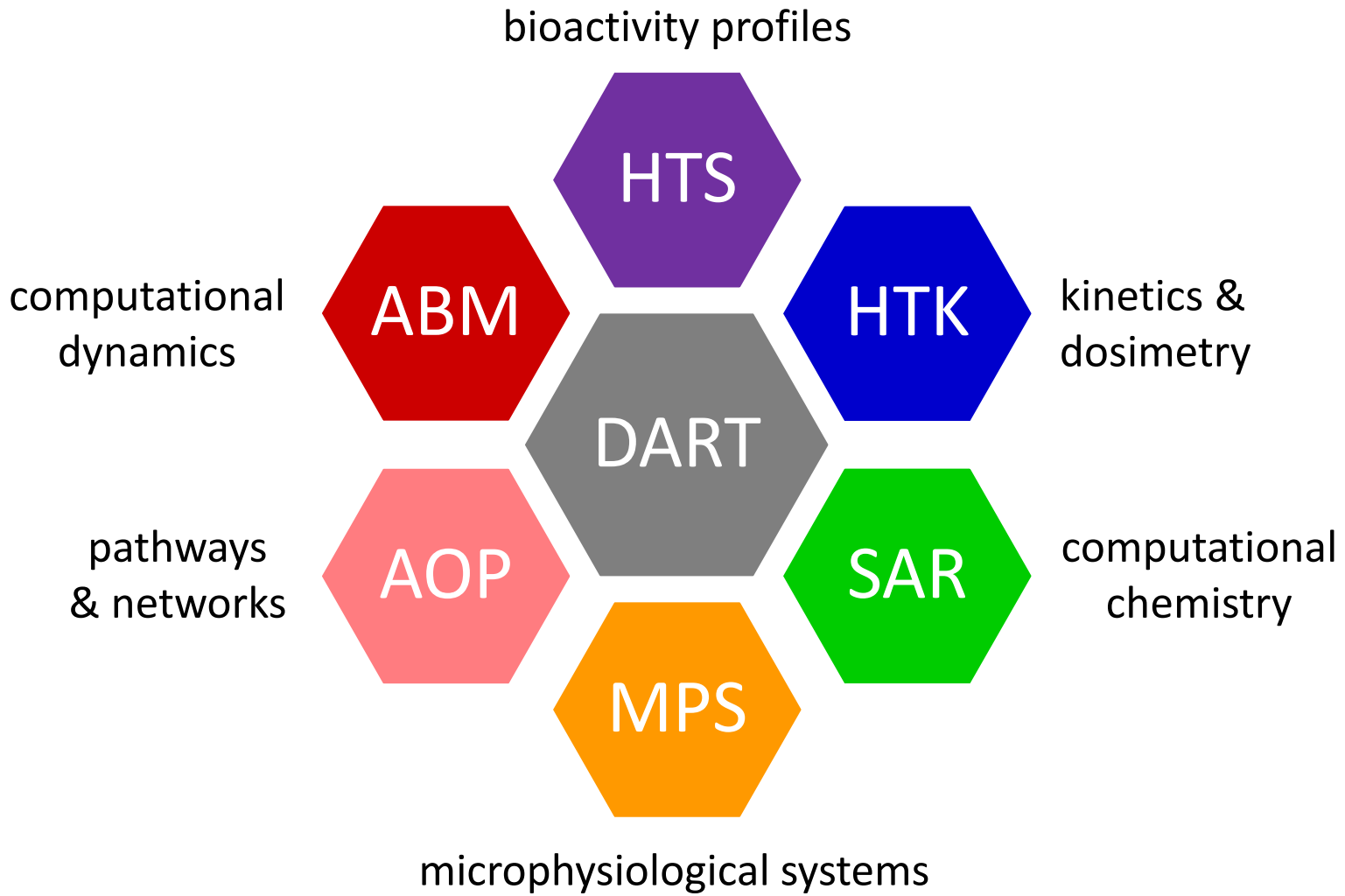
Baker et al. 2018

Moving Toward a 'Virtual Embryo'



- *Hester et al. (2011) PLoS Comp Bio; Dias et al (2014) Science*
- *Kleinstreuer et al. (2013) PLoS Comp Bio.*
- *Ahir et al. (MS in preparation)*
- *Hutson et al. (2017) Chem Res Toxicol.*
- *Leung et al. (2016) Reprod Toxicol.*
- *Zurlinden/Saili et al. (on-going)*
- *Hunter et al. (on-going)*
- *(Future research)*

IATA: Computational Synthesis and Integration



HTS – high throughput screen
HTK – high throughput kinetics
SAR – structure activity relationship
MPS – microphysiological systems
AOP – adverse outcome pathway
ABM – agent based model

Performance Check: In Vitro to In Vivo

- ToxCast_STM anchored to 42 DevTox benchmark compounds aimed at assessing alternative models[1] and having information on pregnancy risk.
- Overall accuracy of 78.6% (0.65 sensitivity, 1.00 specificity, MCC = 0.647).
- Consistent with Palmer et al. (2013) pharma-trained model 77% accuracy (0.57 sensitivity, 1.00 specificity).

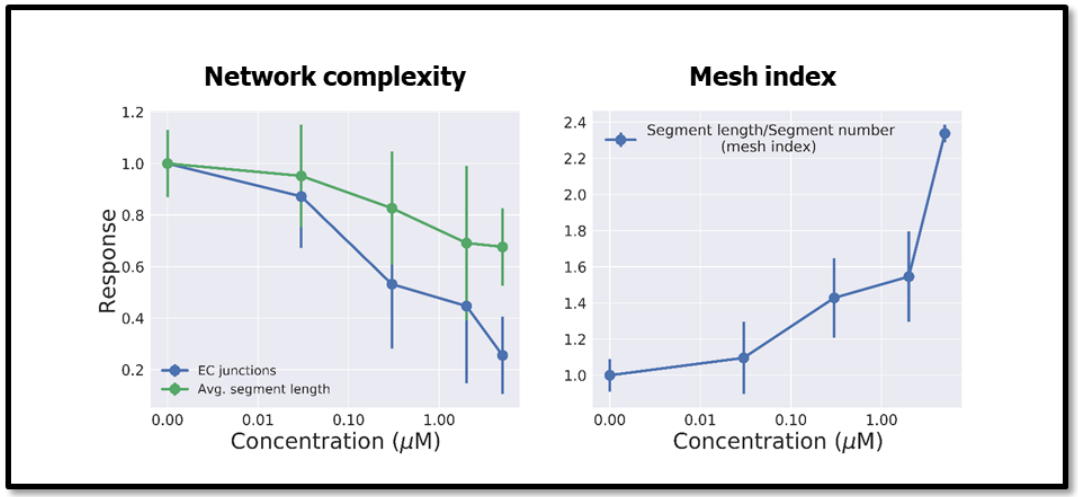
[1] Genschow et al. 2002; West et al. 2010; Daston et al. 2014; Augustine-Rauch et al. 2016; Wise et al. 2016

NCCT, manuscript in preparation

CASRN	Chemical	HTC[1] (µM)	CV[2] (µM)	TI[3] (µM)	Preg.class[4]	STM class[5]
302-79-4	all-trans-Retinoic acid	10	NA	0.003	X	TP
69-74-9	Cytarabine hydrochloride	1	0.083	0.054	D	TP
59-05-2	Methotrexate	1	0.062	0.059	X	TP
147-24-0	Diphenhydramine hydrochloride	100	3.76	0.588	B	TP
50-35-1	Thalidomide	100	NA	1.27	X	TP
51-21-8	5-Fluorouracil	1	1.45	2.02	D	TP
298-46-4	Carbamazepine	100	NA	2.29	C	TP
55-98-1	Busulfan	100	4.91	2.31	D	TP
13292-46-1	Rifampicin	10	NA	2.46	C	TP
19774-82-4	Amiodarone hydrochloride	10	NA	5.1	D	TP
75330-75-5	Lovastatin	20	NA	5.1	X	TP
3056-17-5	Stavudine	100	NA	32.5	C	TP
2392-39-4	Dexamethasone sodium phosphate	100	21.8	37.7	C	TP
53-86-1	Indomethacin	100	44.1	72.7	D	TP
127-07-1	Hydroxyurea	1000	237	74.9	D	TP
127-01-1	Valproic acid	1000	271	155	D	TP
4376-20-9	MEHP	500	NA	167	D	TP
57-41-0	5,5-Diphenylhydantoin	100	NA	NA	D	FN
51-52-5	6-Propyl-2-thiouracil	100	NA	NA	D	FN
10043-35-3	Boric acid	40.7	NA	NA	NTP	FN
4449-51-8	Cyclopamine	10	NA	NA	D	FN
6055-19-2	Cyclophosphamide monohydrate	20	NA*	NA	D	FN
56-53-1	Diethylstilbestrol	10	NA	NA	X	FN
107-21-1	Ethylene glycol	100000	NA	NA	NTP	FN
57-30-7	Phenobarbitol sodium	100	NA*	NA	D	FN
81-81-2	Warfarin	100	NA	NA	X	FN
69-72-7	Salicylic acid	1000	1795	513	C	TN
103-90-2	Acetaminophen	100	NA*	NA	B	TN
79-06-1	Acrylamide	36	NA	NA	NTP	TN
50-78-2	Aspirin	100	NA*	NA	C	TN
80-05-7	Bisphenol A	100	39.4	NA	NTP	TN
94-26-8	Butylparaben	100	NA	NA	GRAS	TN
58-08-2	Caffeine	500	NA	NA	B	TN
464-49-3	D-Camphor	20	NA	NA	C	TN
131-11-3	Dimethyl phthalate	100	NA	NA	NTP	TN
59-30-3	Folic acid	100	NA	NA	A	TN
54-85-3	Isoniazid	8.8	NA*	NA	C	TN
57-55-6	1,2-Propylene glycol	1000000	246664	327552	NTP	TN
68-26-8	Retinol	10	NA	NA	A	TN
81-07-2	Saccharin	100	NA	NA	A	TN
134-03-2	Sodium L-ascorbate	20	NA*	NA	A	TN
599-79-1	Sulfasalazine	100	NA*	NA	B	TN
	True Positive Rate (sensitivity)		*0.29*	*0.65*		
	True Negative Rate (specificity)		*0.94*	*1*		
	Overall Accuracy		***55.00%***	***78.60%***	***(MCC = 0.647)***	

Performance Check: In Silico to In Vitro

Computational prediction (cNVU)

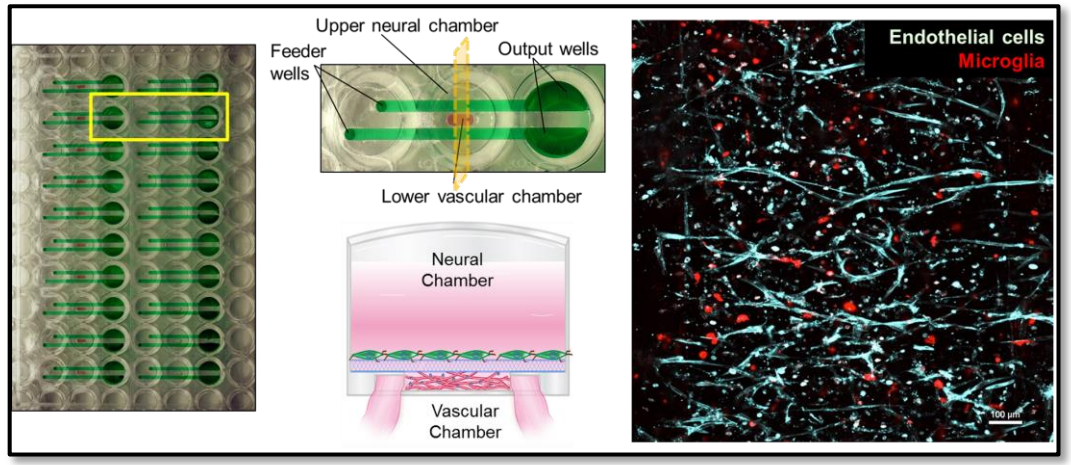


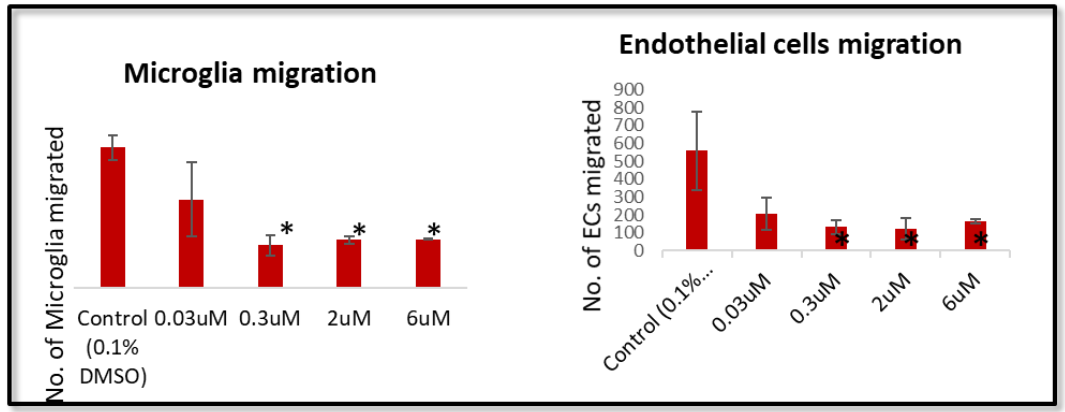
Critical concentration:

- predicted *in silico* ~0.5 µM
- observed *in vitro* ~0.3 µM

Todd Zurlinden, Kate Saili - NCCT

Biomimetic reconstruction (hNVU)





Murphy, W Daly, G Kaushick – U Wisconsin (HMAPS)

Performance Check: Cross Assays

Profiling the ToxCast library with pluripotent embryonic stem cell assays

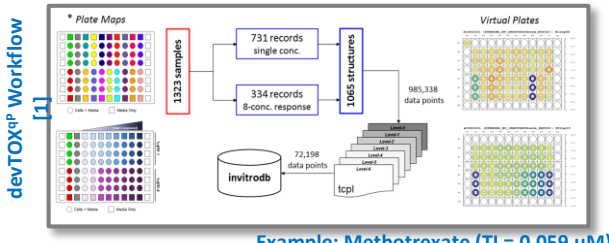
Thomas B. Knudsen[1] Todd J. Zurlinden[1], and E. Sidney Hunter[2]
U.S. EPA, Office of Research and Development [1]NCCT and [2]NHEERL

Introduction

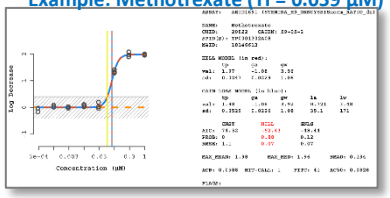
ToxCast chemicals were profiled for developmental toxicity potential in two embryonic stem cell assays and processed in the ToxCast data analysis pipeline (tcpl):

[1] human pluripotent H9 stem cell-based (hESC) assay monitoring a metabolic biomarker [Palmer et al. 2013, BDRB];
[2] mouse differentiating embryonic stem cell (mESC) adherent assay [Barrier et al. 2011, Reprod Tox].

hESC (pluripotent) assay



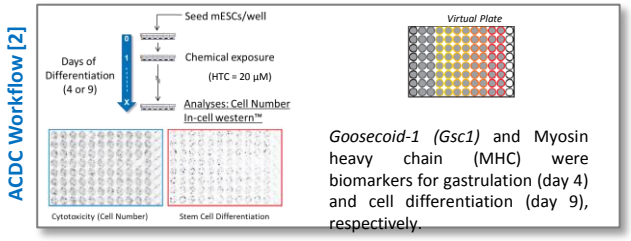
Example: Methotrexate (TI = 0.059 µM)



- ↓ ornithine/cysteine in the day 3 secretome predicts µM threshold for teratogenicity (TI) [1];
- point of departure for cell viability equates to 11% reduction in cell number.

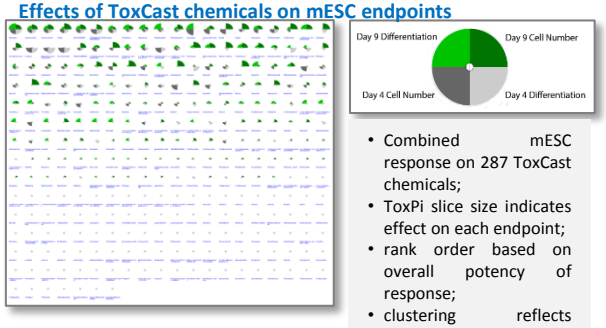
- TI was recorded for 181 chemicals (17% of 1065 tested); model performance used 42 benchmark compounds and ToxRefDB prenatal studies in rats and/or rabbits (dLEL ≤ 200 mg/kg/day) [manuscript in preparation].

mESC (differentiation) assay



Goosecoid-1 (Gsc1) and Myosin heavy chain (MHC) were biomarkers for gastrulation (day 4) and cell differentiation (day 9), respectively.

Effects of ToxCast chemicals on mESC endpoints

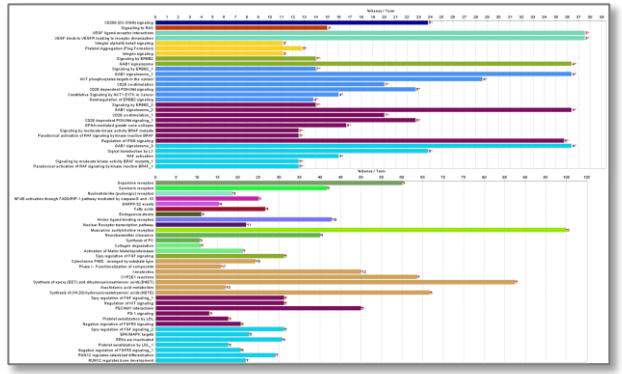


- Combined mESC response on 287 ToxCast chemicals;
- ToxPi slice size indicates effect on each endpoint;
- rank order based on overall potency of response;
- clustering reflects similarity in the response pattern.

Mining the ToxCast dataset to define assay sensitivity

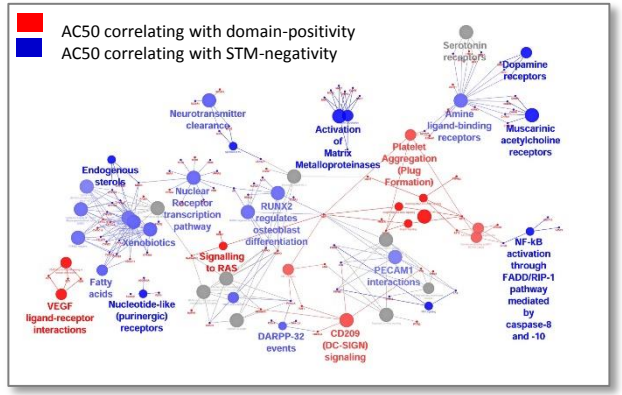
To gain insight into the biological pathways and targets associated with the stem cell responses, machine-learning was used to mine correlations to 337 enzymatic and receptor signaling assays in the ToxCast NovaScreen dataset (NVS). Each NVS assay was enriched for an AC50 correlation against a hESC-positive or hESC-negative outcome, weighted by an assay-specific logistic regression model, processed through the Reactome HSA Pathway Browser (v3.5, database release 63), and independently enriched for significant pathway associations with the ClueGO plug-in to Cytoscape v3.4 (Bonferroni-corrected p ≤ 0.05, minimum 3 genes for a pathway identifier).

Sensitive Domain (86 NVS targets)
Insensitive Domain (131 NVS targets)



Domain Network (hESC):

- enriched pathway interactions mapped with the Cluepedia plug-in to Cytoscape.
- positive-response examples: inhibition of BRAF signaling, adrenocorticoids (GR, MR);
- negative-response examples: female hormone receptors (ESR1, PR), muscarinic receptors (M1-5), MMPs.
- mESC examples: p53 signaling sensitive, IL6 signaling not.



hESC model performance

stringency filter applied to the in vivo anchor

	benchmark	none	low	medium	high
TP	17	85	60	35	19
FP	0	14	37	23	9
FN	9	217	127	51	11
TN	16	116	208	176	88
n	**42**	**432**	**432**	**285**	**127**
Sensitivity	0.654	0.281	0.321	0.407	0.633
Specificity	1.000	0.892	0.849	0.884	0.907
Accuracy	78.6%	46.5%	62.0%	74.0%	84.3%
MCC	0.647	0.190	0.202	0.332	0.554

mESC model performance

low	medium	high
95	23	1
40	40	32
126	35	5
54	52	44
315	150	82
0.430	0.397	0.167
0.300	0.565	0.579
47.3%	50.0%	54.9%
0.004	-0.038	-0.135

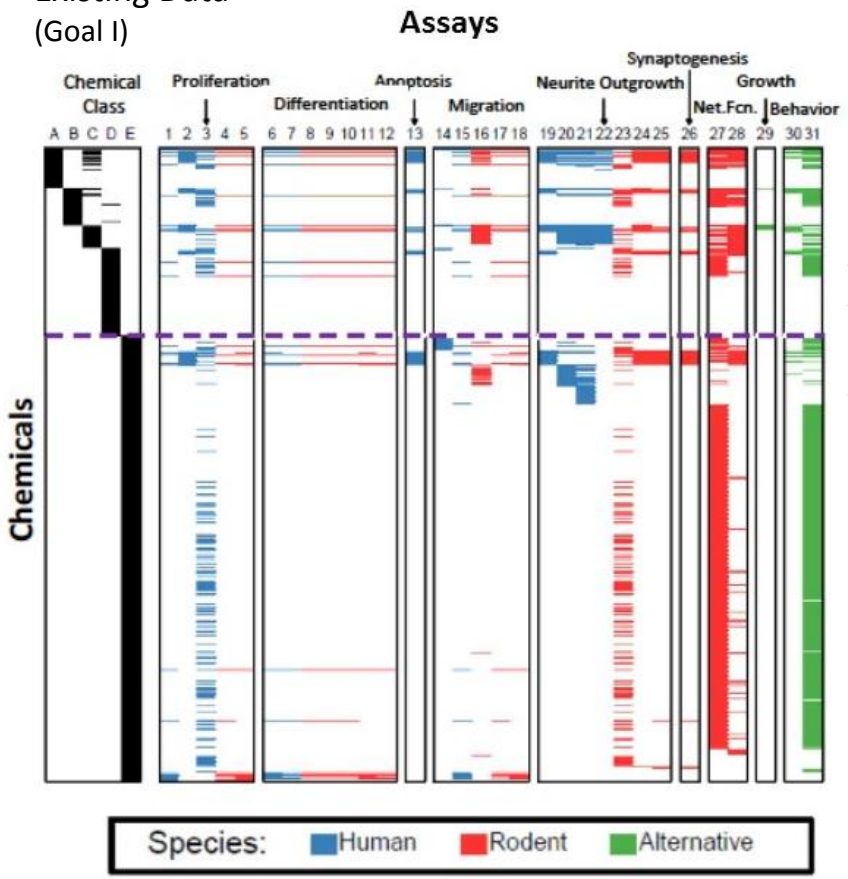
- positive mESC response recorded for 95 chemicals (30.1% of 315 with ToxRefDB prenatal rat or rabbit studies);
- 221 dLEL-positives: *Gsc1* picked up 28% and MHC picked up 25% (overlap = 11% for *Gsc1* on day 4 + MHC on day 9).

Summary

- ToxCast chemicals were classified for potential developmental toxicity using the hESC devTOX^qP platform from Stemina Biomarker Discovery [1] or an adherent mESC assay [2].
- Performance against prenatal animal studies (ToxRefDB) improved from 62% to >84% accuracy as the level of confidence in the *in vivo* anchoring result (dLEL) increased.
- Characterizing the applicability domain at a pathway level sets the stage for new approach methodologies predicting developmental toxicity without vertebrate animal testing.

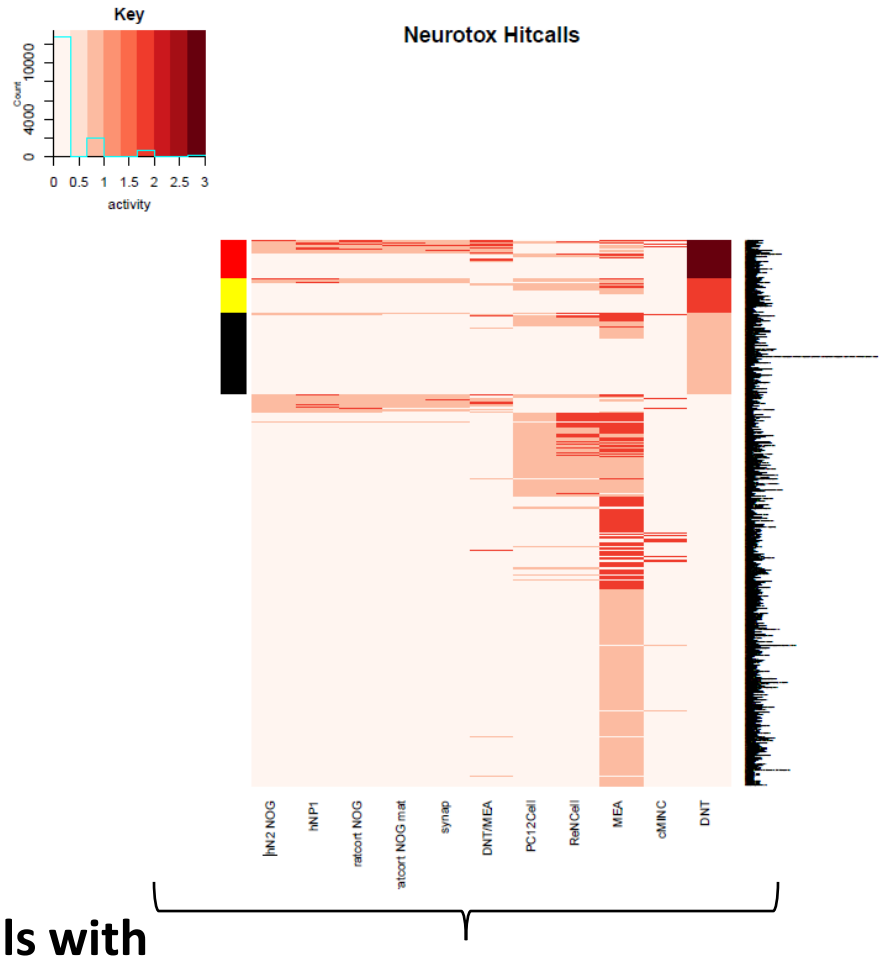
EPA Developmental Neurotoxicity Research Plan

Compile
Existing Data
(Goal I)



GAPS

Expand
Chemical
Universe
(Goal II)



Data Translation
(Goal III)

Goal I: Compile existing data and identify gaps (focus on chemicals with in vivo data: Mundy et al. 2015)
Goal II: Expand the universe of compounds tested
Goal III: Data translation and accessibility
Goal IV: Provide a biological context for enhanced interpretation of DNT


Neurotoxicology and Teratology 52 (2015) 25–35
Contents lists available at ScienceDirect
Neurotoxicology and Teratology
journal homepage: www.elsevier.com/locate/neutera
Review article
Expanding the test set: Chemicals with potential to disrupt mammalian brain development
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ToxCast/Tox21 data are located in the **CompTox Chemicals Dashboard**:
https://actor.epa.gov/dashboard/