Annual Meeting of the European Teratology Society (ETS)
Elsevier 2020 Award Lecture
September 14, 2021

Molecular Characterization of a Toxicological Tipping Point during Human Stem Cell Differentiation

Reproductive Toxicology 91 (2020) 1–13

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Molecular characterization of a toxicological tipping point during human stem cell differentiation

Katerine S. Saili[a], Todor Antonijevic[a,b,c], Todd J. Zurlinden[a], Imran Shah[a], Chad Deisenroth[a], Thomas B. Knudsen[a,*]

[a] National Center for Computational Toxicology (NCCT), U.S. Environmental Protection Agency, Office of Research & Development, 109 T.W. Alexander Drive, Research Triangle Park, NC 27711, United States
[b] Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN 37830, United States
[c] TruStempin, Katy, TX 77494, United States

ARTICLE INFO

Keywords:
All-trans retinoic acid
Endoderm
FOXA2
Tipping point
Differentiation
iPSC
SOX17
HOMER

ABSTRACT

Endoderm gives rise to the gut tube in the early embryo. We differentiated human induced pluripotent stem cells (hiPSCs) to embryonic endoderm to identify a "tipping point" at which the developing system did not recover from perturbations caused by exposure to all-trans retinoic acid (ATRA). Differentiating hiPSC-derived endoderm exposed to five concentrations of ATRA between 0.001 and 10 μM at 6 h, 96 h, or 192 h was assessed for forkhead box A2 (FOXA2) protein expression and global gene transcript expression. A tipping point of 17 ± 11 nM was identified where patterns of differentially expressed genes supported a developmental trajectory shift indicating a potential teratogenic outcome. This concentration is between women's endogenous ATRA blood plasma levels and teratogenic levels of circulating isotretinoin, an ATRA isomer used to treat acne. Taken together, these data suggest that this approach is a sensitive method to identify a point of departure for chemicals that impact early embryonic development.

1. Introduction

The ToxCast™ high throughput screening (HTS) program [1–4] is a powerful approach to identify molecular targets of environmental contaminants. By applying systems biology modeling approaches to the HTS data, effects on cells, tissues, and organ systems can be deduced. Although modeling approaches allow extrapolation from adult cell lines to developmental processes, pathways involved in embryonic development are not directly represented in ToxCast™. To address this, we investigated the suitability of a differentiation assay with human induced pluripotent stem cells (hiPSCs) for modeling tissue differentiation during human embryonic development (Fig. 1). Endogenesis, the formation of endoderm, was selected due to the role of endoderm in giving rise to visceral organs derived from the gut tube including thyroid, thymus, lung, pancreas, liver, gastrointestinal system, prostate, and the visceral yolk sac. The critical role of endogenesis in proper embryonic development establishes this system as a model to screen and identify chemicals that may disrupt embryonic patterning leading to birth defects.

Endogenesis occurs during the primitive streak stage of gastrulation when epiblast-derived ectodermal cells migrate ventrally to invade the hypoblast-derived extraembryonic (yolk sac) endoderm while transitioning to an embryonic endodermal state via the process of epithelial to mesenchymal transition (EMT) [5,6]. These events mark the establishment of the three germ layers at the earliest stage of embryogenesis in week three of human development (gestational age week 5) [7]. The anterior primitive streak gives rise to embryonic endoderm [8], in turn leading to formation of the gut tube, comprising foregut, midgut, and hindgut. The foregut is the source of anterior organs: thyroid, thymus, lungs, stomach, liver, and pancreas; the midgut becomes the small intestine; the hindgut becomes the large intestine. In addition to the gut tube-derived organs, the anterior primitive streak also gives rise to extra-embryonic endoderm, also known as yolk sac or visceral endoderm, to be incorporated into the gut epithelium [8]. The posterior primitive streak gives rise to mesoderm also through an EMT [9].

The establishment of the three germ layers from the primitive streak begins with ectoderm, which is the first layer to form and often referred to as the default cell fate from which mesoderm or endoderm (mesendoderm) is induced [10,11]. There are several well-established molecular signaling events that facilitate germ layer specification. Hepatocyte growth factor (HGF) expression favors ectoderm differentiation while inhibiting endoderm formation [12]; while Wnt, bone

* Corresponding author at: 109T.W. Alexander Drive (D-143-02), Research Triangle Park, NC 27711, United States.
E-mail address: knudsen.thomas@epa.gov (T.B. Knudsen).

https://doi.org/10.1016/j.reprotox.2019.10.001
Received 4 June 2019; Received in revised form 23 September 2019; Accepted 1 October 2019
Available online 07 October 2019
0890-6238/ © 2019 Published by Elsevier Inc.

USEPA – Office of Research and Development
Center for Computational Toxicology and Exposure
Chemical Safety for Sustainability (CSS) Research Program
Research Triangle Park, NC 27711
knudsen.thomas@epa.gov

DISCLAIMER: The views expressed are those of the presenters and do not reflect Agency policy.

Project Team

Kate Saili, PhD
Postdoctoral Fellow
(now *OAR-OAQPS*)
Lead author

Imran Shah, PhD
CCTE
Computational Biologist
Tipping points

Todor Antonijevic, PhD
Postdoctoral Fellow
(now *ToxStrategies*)
Boolean analysis

Chad Deisenroth, PhD
CCTE
Cell Biologist
High content imaging

Todd Zurlinden, PhD
Postdoctoral Fellow
(now *ORD-CPHEA*)
Bioinformatics

Tom Knudsen, PhD
CCTE
Developmental Biologist
Corresponding author

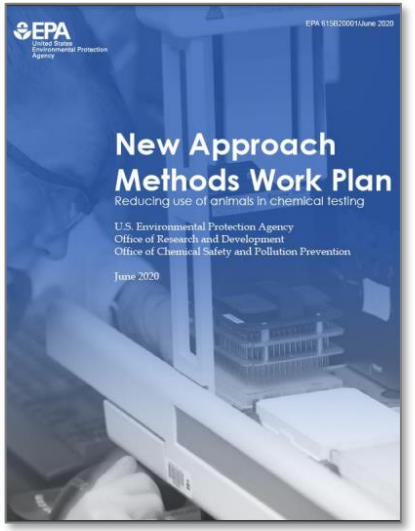
Vala Sciences, Inc. performed the chemical exposures and sample collection under U.S. Environmental Protection Agency contract #EPD13054.

Subcontractor: human iPSC line from Allele Biotechnology and Pharmaceuticals, Inc.

Subcontractor: Cofactor Genomics performed the library construction and RNA-sequencing.

Thanks to David Belair, Logan Everett, John Cowden, and RTX reviewers for helpful comments on the manuscript.

New Approach Methods (NAMs)



- NAMs refer to *in vitro* data and *in silico* models for toxicological assessment with less reliance on animal testing, thus accelerating data acquisition with fewer resources.

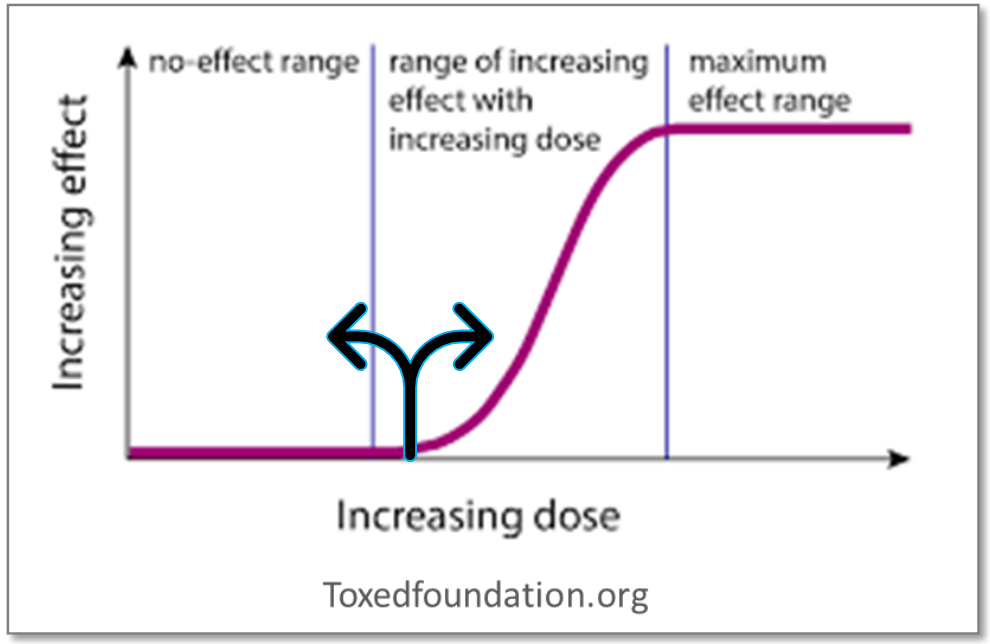
- An explosion of *in vitro* data from high-throughput screening (HTS) and high-content imaging (HCI) enable profiling large chemical libraries for molecular and cellular determinants of bioactivity.

- However, de-scaling the embryo into complex data-sets from *in vitro* profiling brings the challenge of re-composing the full complexity of anatomical development for NAM-based hazard evaluation.

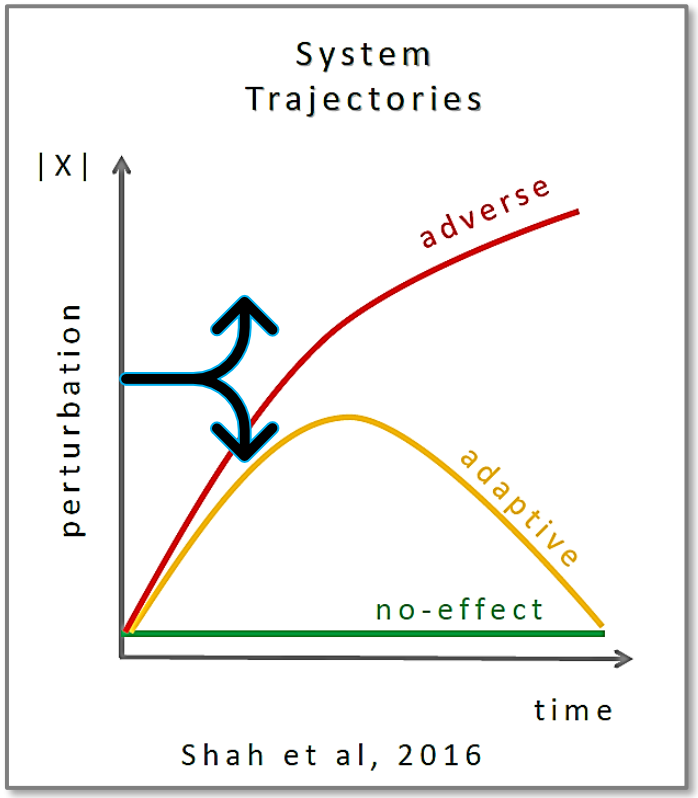
- Dosimetry being a key regulatory driver motivates the need for computational models that can be used to establish a health-protective 'critical response' from complex *in vitro* bioactivity data.

Critical response dosimetry

Point of departure (POD) is the dose at which a biological response is first observed (e.g, LOAEL, BMD_5) and is a basis for reference dose extrapolations in risk assessment.



Toxicological tipping point (TTP) represents the chemical concentration where a biological system (e.g., tissue, organ, or cultured cell) is perturbed beyond its return to homeostasis.



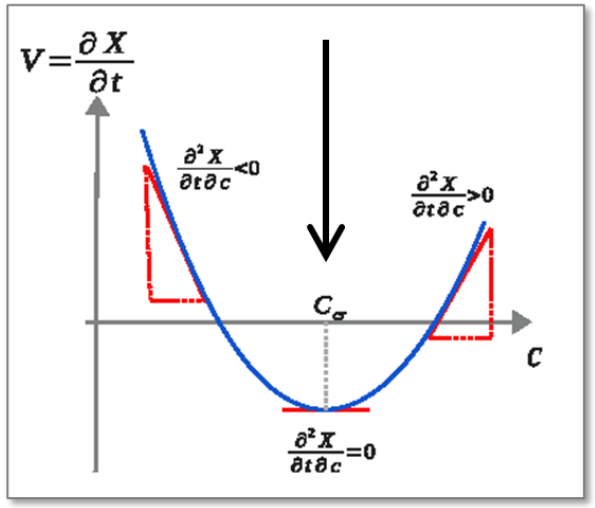
Tipping point: *critical point between **adaptive** and **adverse** response trajectories*

- HepG2 is an immortal **endodermal** cell line established from a 15-yr old hepatoma patient.
- HepG2 cells screened with 967 ToxCast chemicals (0.4 to 200 µM) across 6-, 24- and 72-hr.
- Concentration-dependent changes measured by automated HCI on 10 fluorescent labels for:
  - p53 nuclear accumulation
  - stress kinase activation (phospho-JUN)
  - oxidative stress (phospho-H2A.x)
  - cytoskeletal changes (TUBA)
  - mitochondrial polarization (CMXRos)
  - mitochondrial mass (net content)
  - cell cycle arrest
  - mitotic arrest
  - nuclear size
  - cell count

Shah et al. (2016) Env Hlth Persp

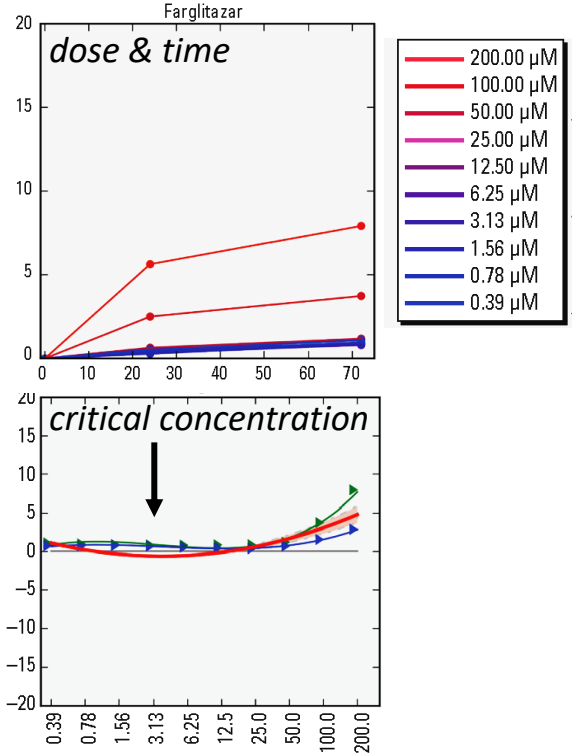
What does a toxicological tipping point look like mathematically?

Critical Concentration



Mathematical model from 336 TTP trajectories in HepG2 cells.

Example



Goals of this study

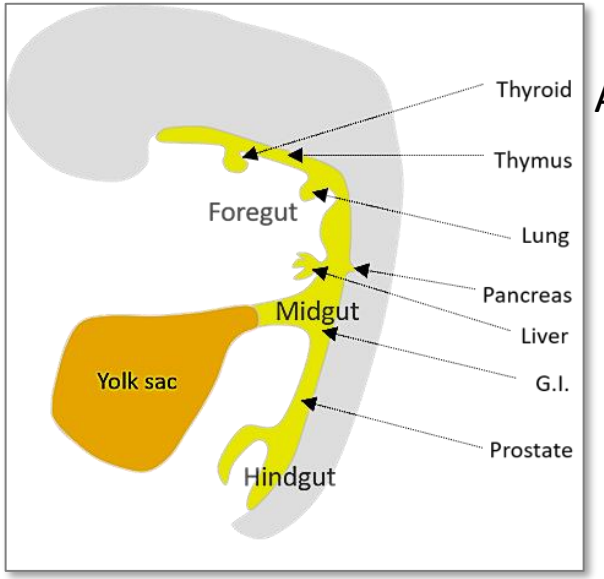
Would the TTP model compute a critical response in a differentiating cell lineage?

1. Establish a human iPSC assay for **endoderm** specification and differentiation (endogenesis):

   - *Antero-Posterior patterning of definitive endoderm into regional domains (foregut, midgut, hindgut) is locally controlled by mesodermal signals;*
   - *all-trans-retinoic acid (ATRA) and various WNT, FGF, and BMP family members recapitulate this progression in hPSC systems.*

2. Explore the 'tipping point' concept in iPSCs directed to endodermal differentiation (with activin) utilizing ATRA as a reference perturbagen.

   - *ATRA signaling contributes to endodermal patterning of the gut tube (posteriorizes) and alters SOX17 expression in hPSCs.*
   - *SOX17 is a key transcription factor for endodermal patterning, as SOX17-nullizygous mouse embryos lack midgut and hindgut structures.*

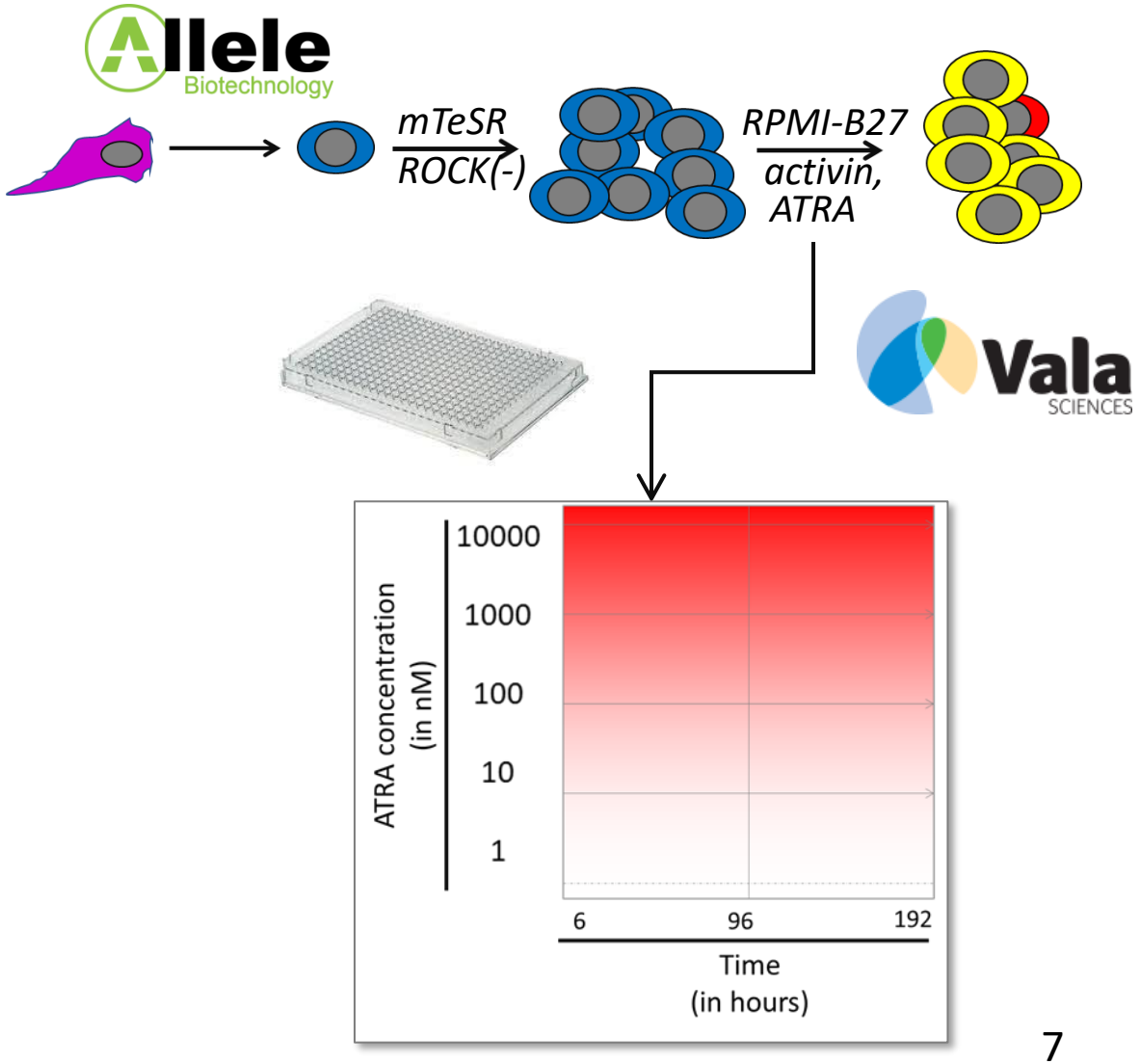


ATRA concentration thresholds:

- non-morphogenetic: < 1 nM
- morphogenetic: 3-7 nM
- dysmorphogenic: >30 nM
- broadly teratogenic: >1000 nM

Endogenesis assay

- iPSCs (ABP-SC-HDFAIPS) reprogrammed from adult female fibroblasts.
- Maintained feeder-free in mTeSR medium with ROCK inhibitor.
- Seeded on 384 plates coated with Matrigel (1.5K cells/well); ROCK inhibitor removed at 24h.
- At 48h switched to defined differentiation medium (RPMI-B27 with 100 ng/ml activin).
- ATRA exposure at 0.001, 0.01, 0.1, 1, 10 µM in 0.1% DMSO, medium renewed daily.
- Cultures processed at 6h, 96h (4-days),or 192h (8-days) for HCI (n=3) and RNAseq analysis (n=2).

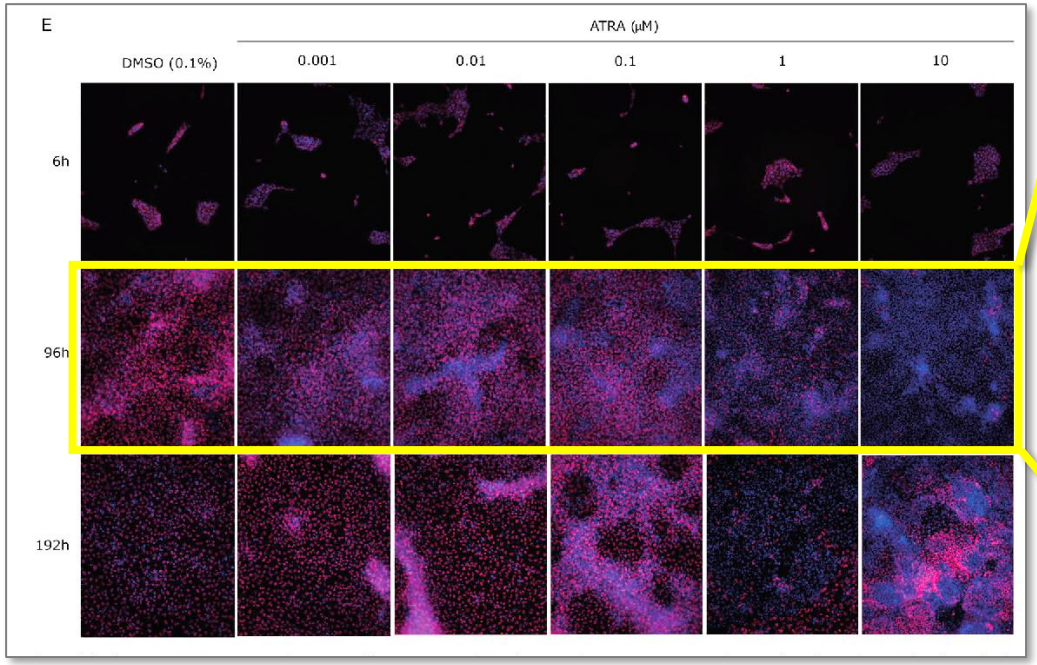


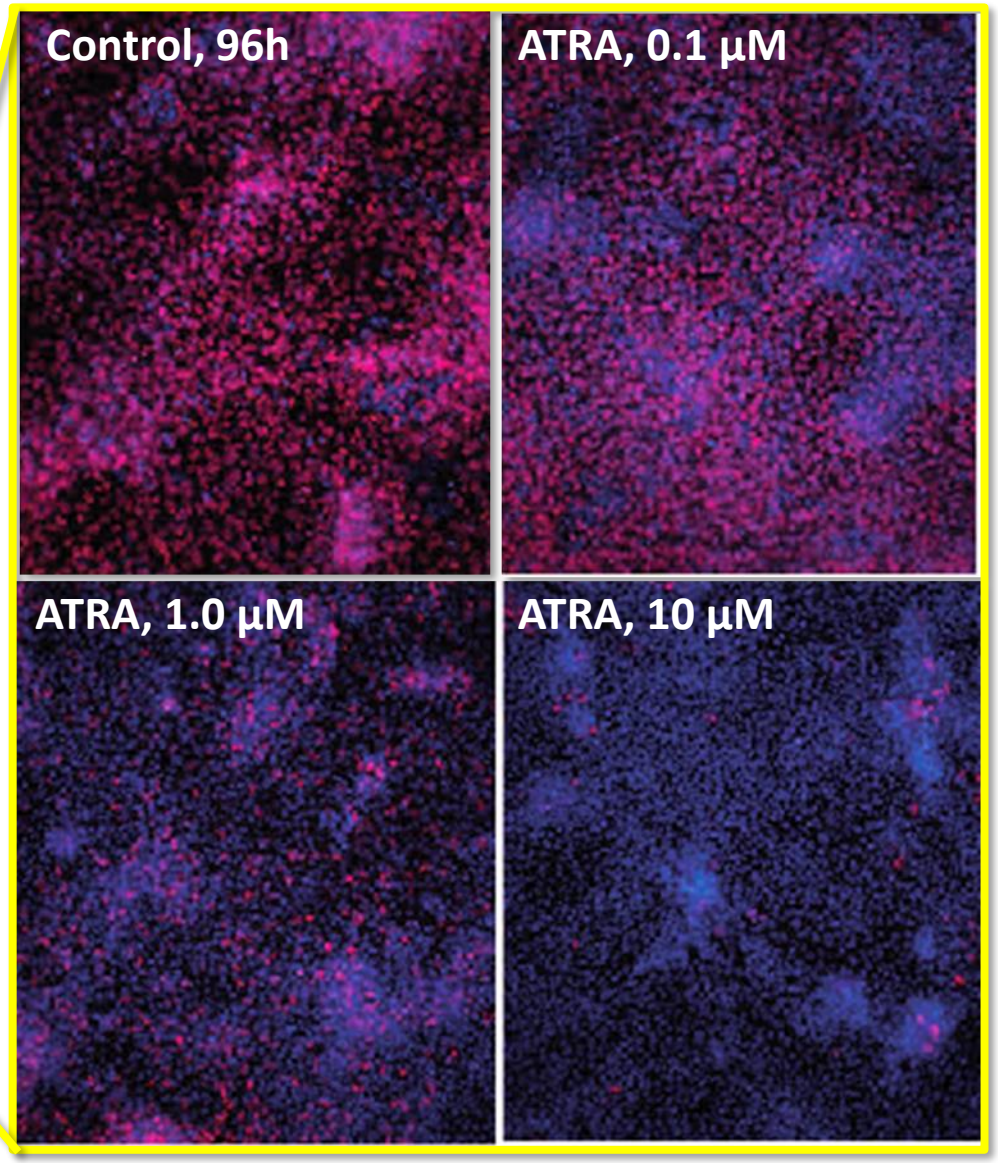
HCI analysis: *ATRA-dependent effects on hiPSCs directed to endodermal differentiation*

Concentration-dependent changes over 192h measured in 4 channels (CellProfiler 2.2.0):

FOXA2
Hoechst

- definitive endoderm (FOXA2)
- mitochondria (Mitotracker)
- cell death (ImageIT-Dead)
- cell number (Hoechst)

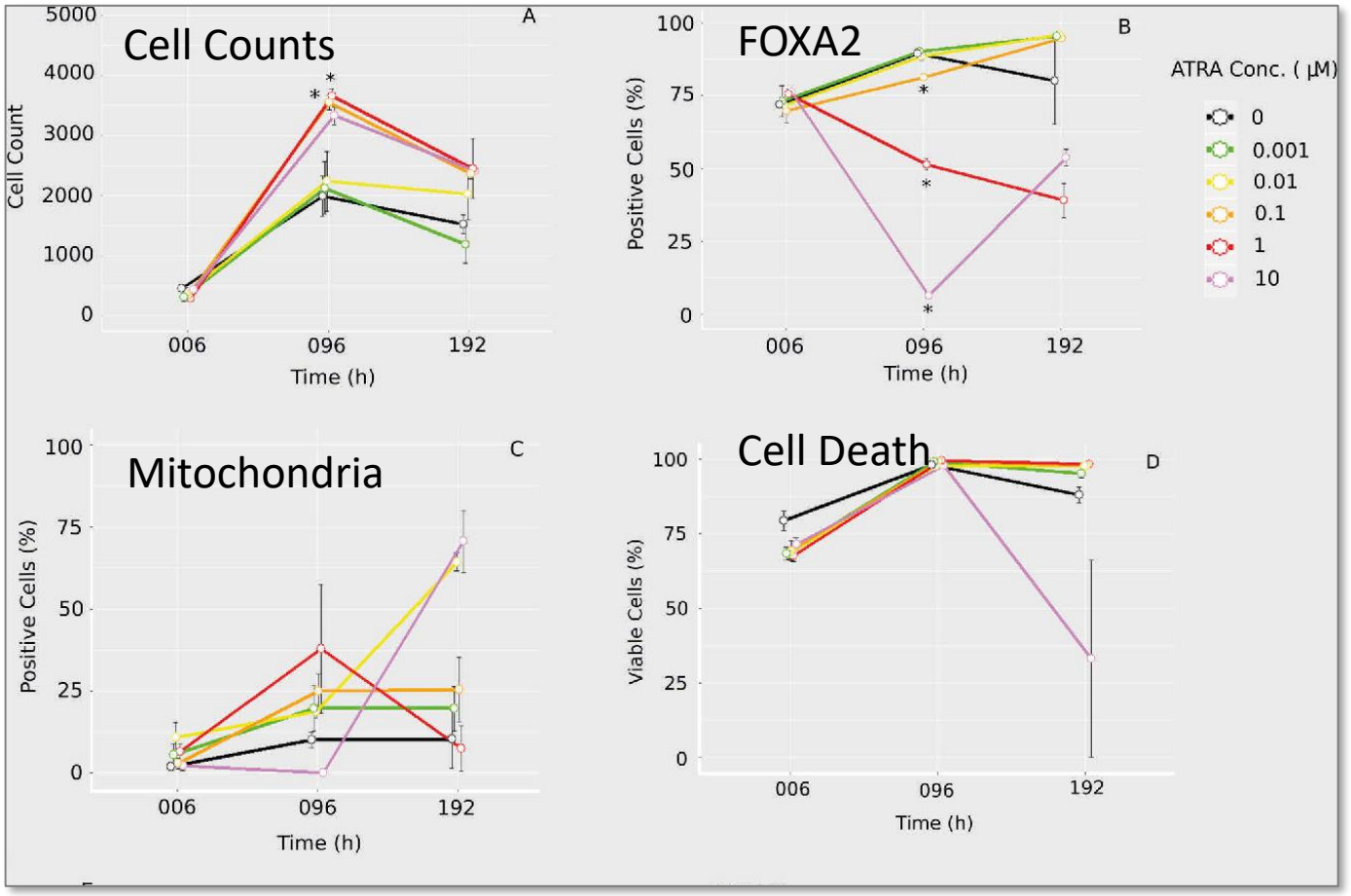




ATRA concentration response trajectories

- Confluence reached at 96h, but higher cell counts observed with ATRA ≥ 100 nM.
- ATRA suppression of FOXA2-positive cells was significant at 100 nM and higher.
- Non-significant trend on mitochondrial fluorochroming below 10 µM.
- No effect on cell death below 10 µM or before 192h.

ATRA perturbed endogenesis
in a time and concentration dependent manner.



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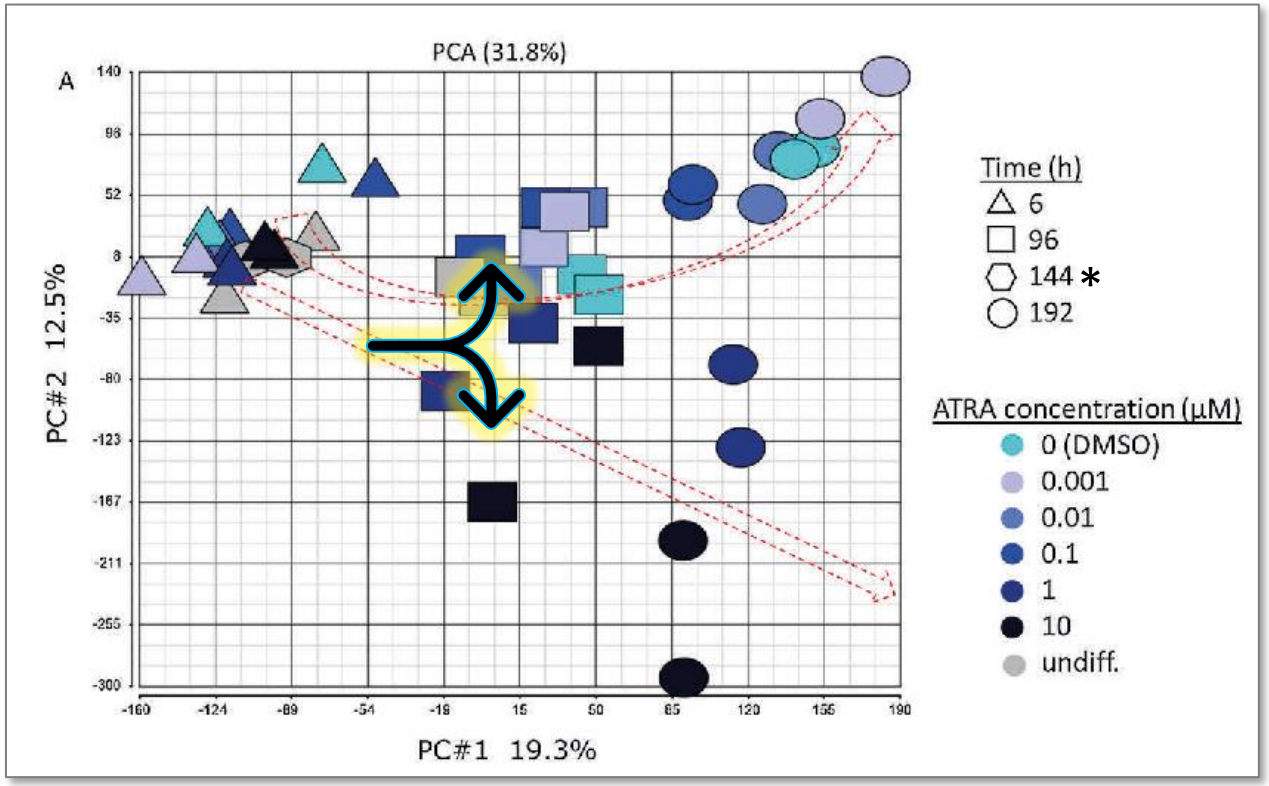
Transcriptomic response to ATRA exposure

What would the molecular profile of a developmental tipping point look like?

- Total RNA sampled from two biological replicates per treatment.
- cDNA libraries constructed after depleting rRNA and mtRNA contamination.
- RNA-sequencing 75bp single-end reads >30M read depth on an Illumina NextSeq500.
- Raw and processed data deposited in the Gene Expression Omnibus (GSE131921).
- Reads aligned to the human genome (GRCh38) for Principal Component Analysis (PCA).

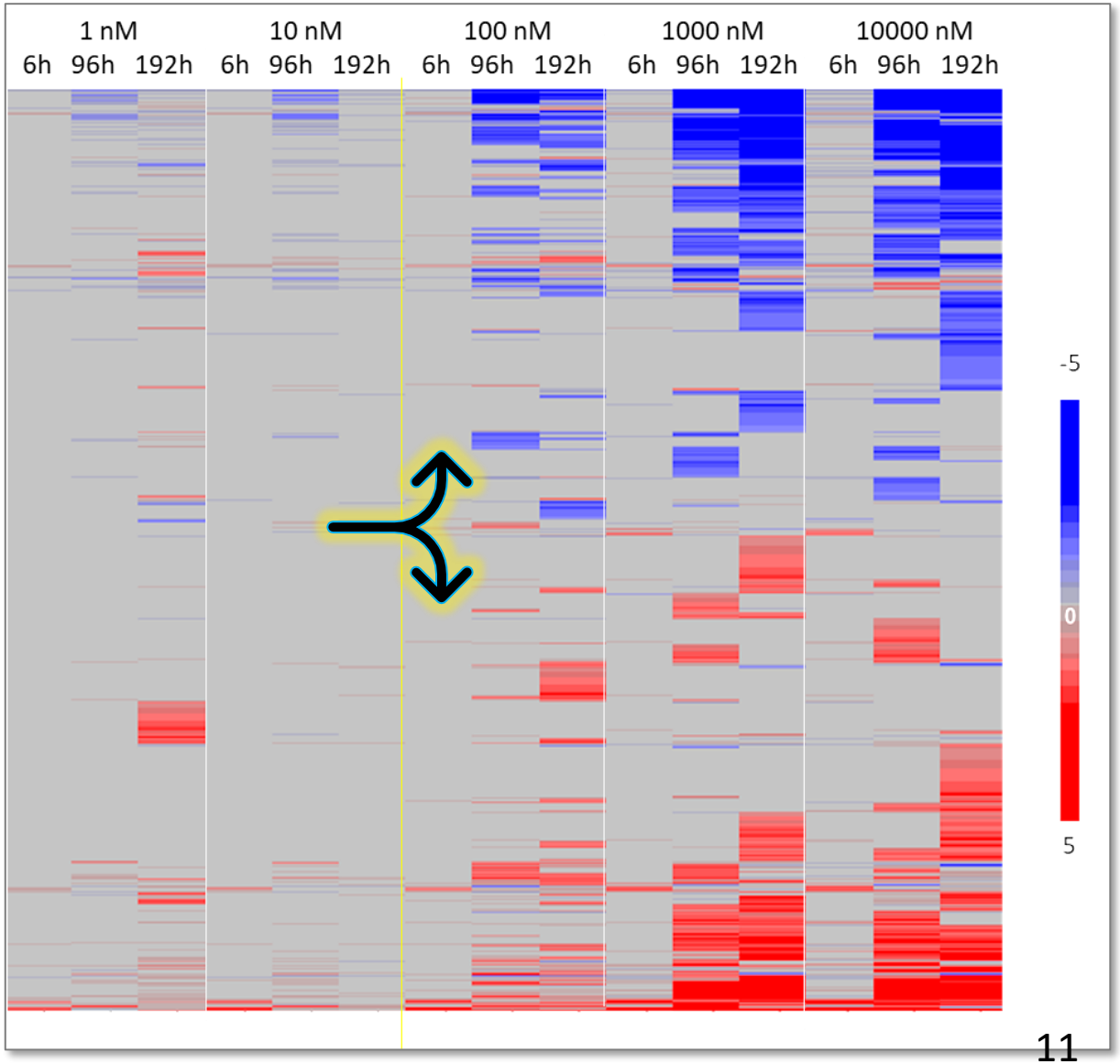
PCA of unfiltered count data for 58,051 aligned fragments



** 144h controls because undifferentiated cultures did not survive past 6-days*

Differentially Expressed Genes (DEGs)

- 9997 DEGs (median counts > 5, abs(log2FC) > 1, and Benjamini-Hochberg adjustment <0.05).
- Clustered by gene expression changes in ATRA relative to control samples.
- DEGs resolve to increased (red) and decreased (blue) expression at 96h with ATRA >10 nM.



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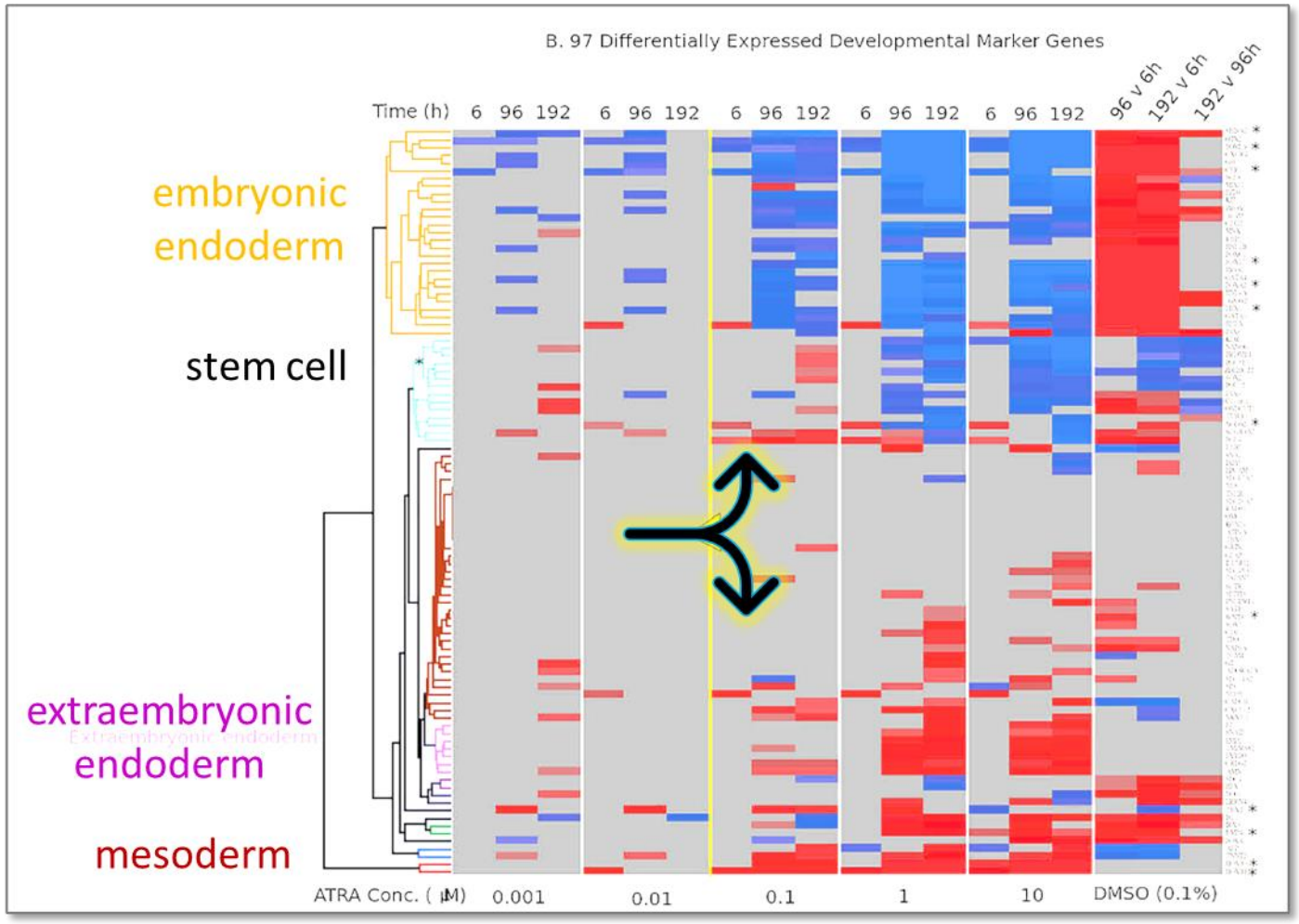
Developmental marker genes

- Heat-map of 97 developmental marker genes selected from the literature.
- Covers all three primary germ layers from gastrulation to organogenesis.
- ATRA >10 nM suppressed definitive endoderm (*FOXA2*, *SOX17*, *GATA4*).
- Concomitant increase in primitive streak and mesoderm (*HOXB1, HOXB3, TBXT*).
- ATRA > 10 nM progressively shifted differentiation toward mesendoderm.



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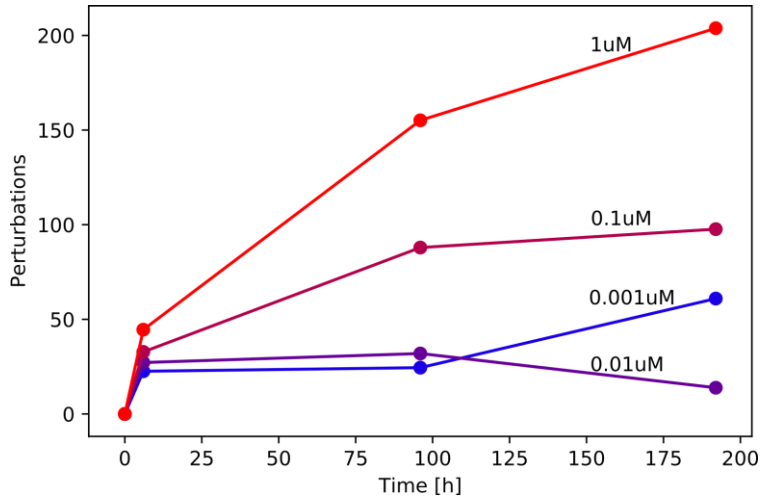
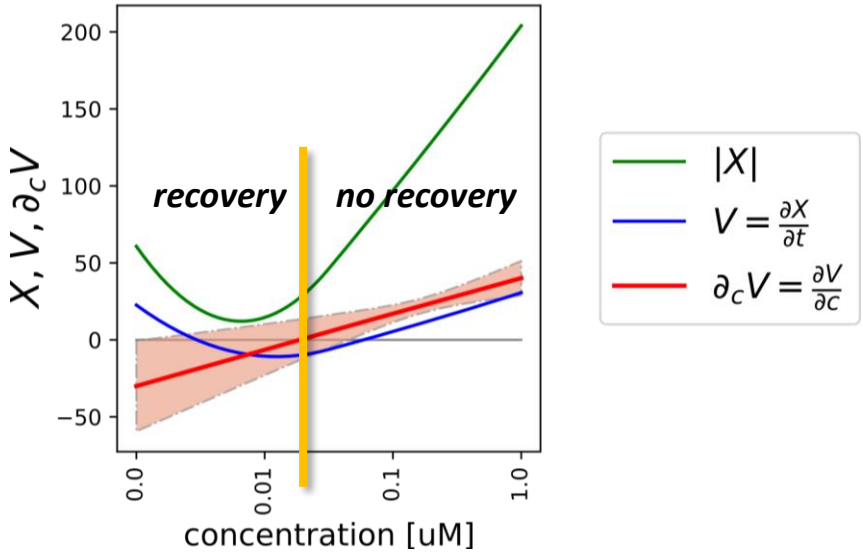
Tipping point computation

- HCI analysis suggested a tipping point between 10 and 100 nM.
- Definitive tipping point was computed on 8340 DEGs at or below 1000 nM ATRA.
- ***|X|*** perturbations, ***V*** temporality, and ***∂cV*** concentration derivative.
- TTP = 17 (± 11 SD) nM, inferring a critical concentration affecting endogenesis.





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ATRA concentration thresholds

Regional and nominal ATRA concentration thresholds reported in different studies on morphogenesis, differentiation, and pregnancy.

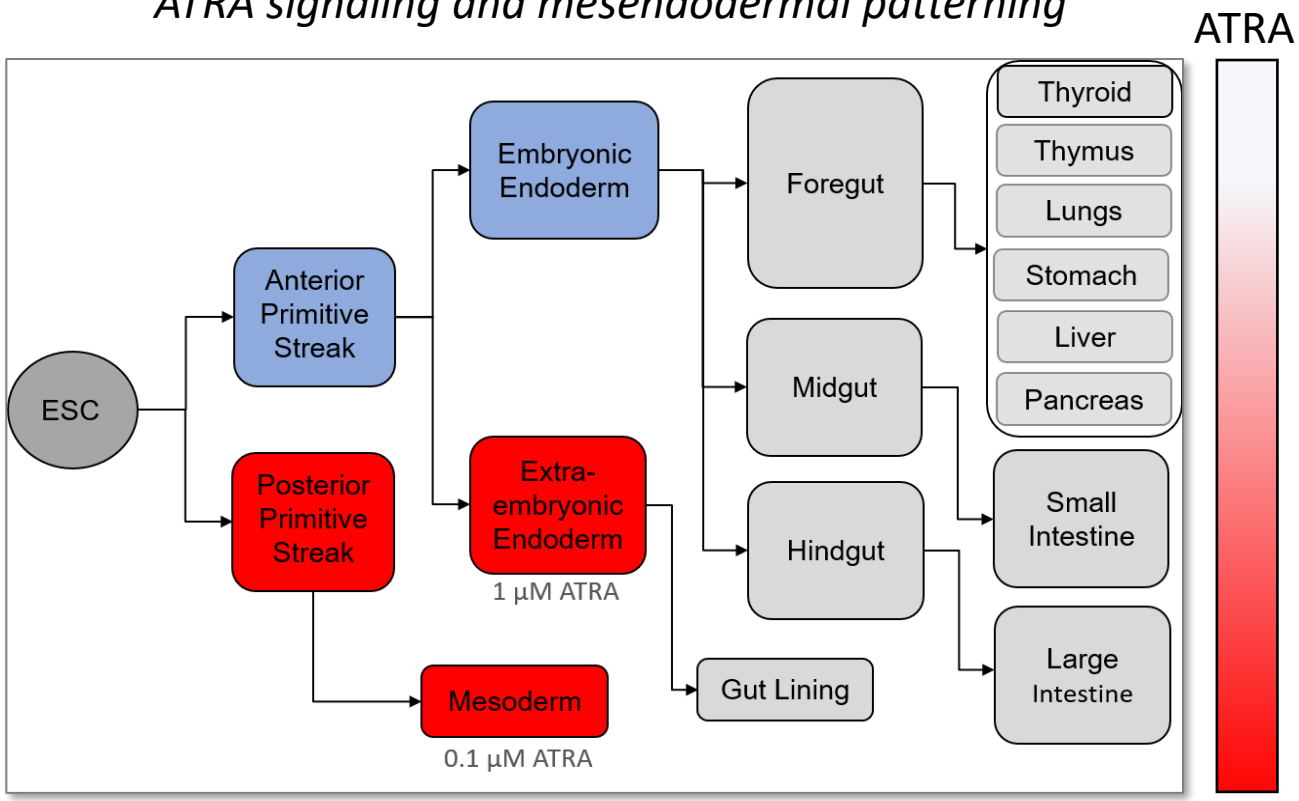
Dosimetric	Conc.	Indication	Reference
baseline ATRA (5 somite zebrafish embryo)	< 1 nM	non-morphogenetic	(Shimozono, Iimura et al. 2013)
maternal serum (animal study)	1.7 nM	non-teratogenic	(Daston, Beyer et al. 2014)
devTOX^{qp} assay (pluripotent hESC)	3.0 nM	teratogenic threshold	(Zurlinden, Saili et al. 2020)
normal plasma concentration	5.0 nM	physiological (adult)	(Napoli, Posch et al. 1991)
axial gradient (5 somite zebrafish embryo)	6.0 nM	morphogenetic signal	(Shimozono, Iimura et al. 2013)
endodermal differentiation (h-iPSC)	17 nM	toxicological tipping point	(Saili, Antonijevic et al. 2019)
devTOX^{qp} assay (pluripotent h-iPSC)	19 nM	DevTox potential	(Palmer, Smith et al. 2017)
genetic perturbation (mouse)	30 nM	altered homeostasis	(Helms, Thaller et al. 1994)
maternal serum (animal study)	30 nM	teratogenic potential	(Daston, Beyer et al. 2014)
limb-bud (GD 10.5 mouse embryo)	30 nM	physiological (embryo)	(Horton and Maden 1995)
pharmacological kinetics	1,000 nM	efficacious (therapeutic)	(Helms, Thaller et al. 1994)
limb-bud (GD 11 mouse embryo)	1,500 nM	weakly teratogenic dose	(Satre and Kochhar 1989)
limb-bud (GD 10.5 mouse embryo)	12,500 nM	fully teratogenic dose	(Horton and Maden 1995)

SOURCE: Knudsen et al. (2021), Reprod Toxicol

Endodermal trajectories

- ATRA above 17 nM shifts differentiation away from embryonic endoderm in favor of mesoderm (and extraembryonic endoderm).
- The suite of 97 germ layer marker genes (e.g., Fox, Sox, & Hox) captures the shift.
- *In vivo*, ATRA signaling contributes to midgut and hindgut patterning [Bayha et al. (2009) PLoS] …
- … *Sox17*-null mice display lethal deficiencies in these endodermal segments [Kanai-Azuma et al. (2002), Development].

ATRA signaling and mesendodermal patterning



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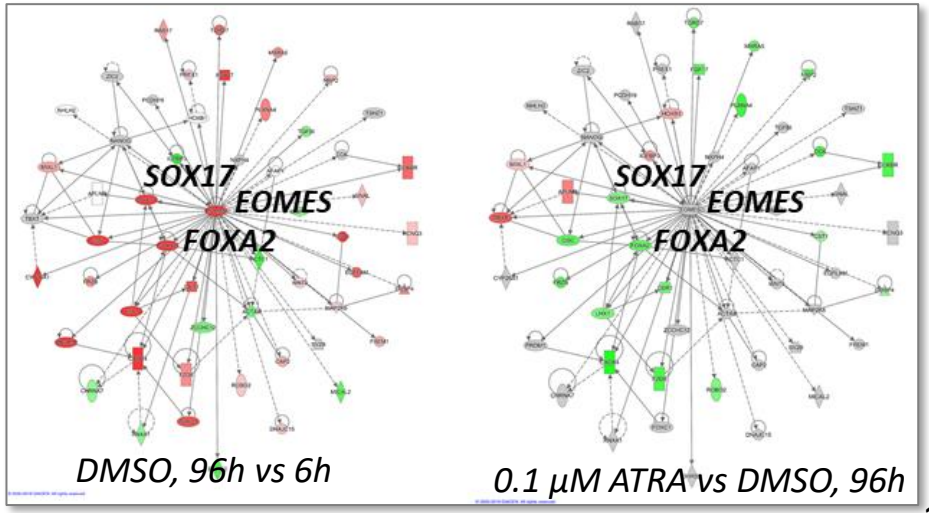
Pathway analysis

What pathways might be dysregulated at the tipping point?

- We used Ingenuity Pathway Analysis (IPA) to infer upstream regulators of DEG patterns at 96h.
- *Eomesodermin* (*EOMES*) emerged as a top candidate for dysregulation at the tipping point.
- *EOMES* is a T-box gene critical for mesendoderm specification at the primitive streak stage.
- IPA predicted a strong inhibition state for EOMES-dependent regulation @ 100 nM ATRA versus a strong activation state in time-matched controls.

Concentration	Upstream Regulator	Expr Log Ratio	Predicted Activation State	Activation z-score	p-value of overlap
DMSO (96h v 6h)	progesterone		Activated	2.148	1.33E-15
DMSO (96h v 6h)	EOMES	6.906	Activated	3.685	7.46E-15
DMSO (96h v 6h)	SOX2	0	Inhibited	-2.731	7.95E-14
DMSO (96h v 6h)	topotecan		Inhibited	-3.923	1.85E-12
DMSO (96h v 6h)	dexamethasone		Activated	3.013	4.58E-12
DMSO (96h v 6h)	tretinoin		Activated	2.51	1.01E-11
0.1 µM (96h)	EOMES	-1.017	Inhibited	-2.549	1.22E-15
0.1 µM (96h)	dexamethasone		Inhibited	-2.512	1.62E-13
0.1 µM (96h)	LHX1	-2.049	Inhibited	-2.774	2.81E-11



DMSO, 96h vs 6h *0.1 µM ATRA vs DMSO, 96h*

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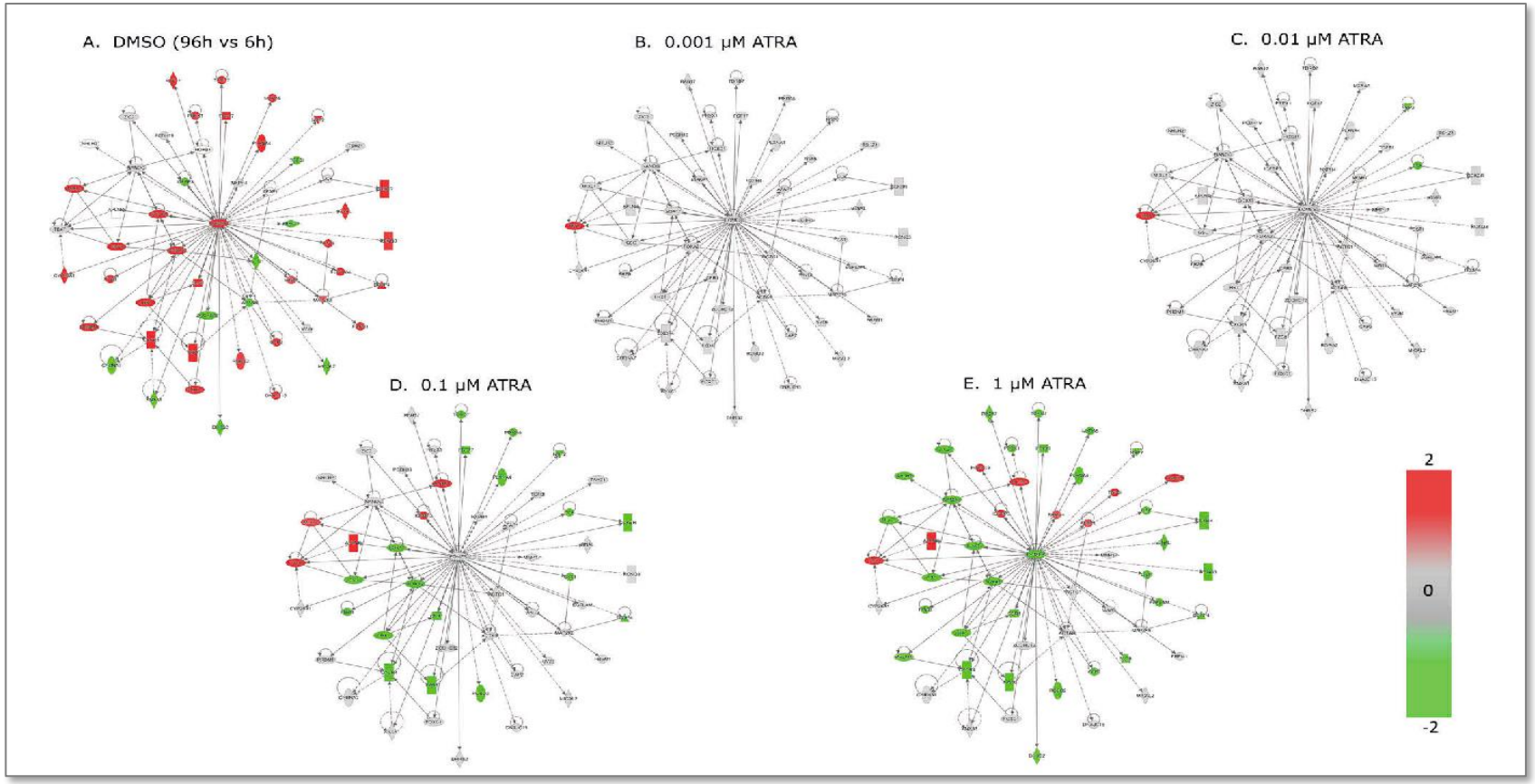
Hypothesis

EOMES is central to a tipping point network that accounts for a shift in developmental trajectory of multipotent mesendoderm from endoderm towards mesoderm, which may be linked to ATRA-dependent effects on the Nodal pathway.



Value and Limitations

- This study serves as proof-of-concept that plausible targets in a ‘toxicological tipping point’ can be defined at the molecular level through monitoring regulation of the embryonic transcriptome.
- Although we focused on the influence of ATRA on endogenesis, the approach is scalable to other chemical compounds, cell lineages, and biological processes relevant to hazard evaluation for predictive toxicology.
- Challenges remain for operationalizing this concept in a complex system capable of self-organization (morphogenesis) and canalization (developmental buffering).
- ‘Adaptation’ in this case implies how well a pluripotent system driven toward endodermal differentiation holds to its default trajectory during ATRA exposure, where the tipping point was computed 17 nM.
- ‘Adversity’ implies loss of fault tolerance as the nominal concentration of ATRA led to missing or incorrect cell phenotypes, potentially due to its known effects on cell signaling through the Nodal pathway.
- The findings raise fundamental questions of network dynamics, such as the nature of the primary ‘attractor states’ linked to morphoregulatory pathways.

Thank You!

Kate Saili, PhD
Postdoctoral Fellow
(now *OAR-OAQPS*)
Lead author

Imran Shah, PhD
CCTE
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Developmental Biologist
Corresponding author

Reproductive Toxicology 91 (2020) 1–13

Contents lists available at ScienceDirect

Reproductive Toxicology

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[a] National Center for Computational Toxicology (NCCT), U.S. Environmental Protection Agency, Office of Research & Development, 109 T.W. Alexander Drive, Research Triangle Park, NC 27711, United States
[b] Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN 37830, United States
[c] ToxStrategies, Katy, TX 77494, United States

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1. Introduction

The ToxCast™ high throughput screening (HTS) program [1–4] is a powerful approach to identify molecular targets of environmental contaminants. By applying systems biology modeling approaches to the HTS data, effects on cells, tissues, and organ systems can be deduced. Although modeling approaches allow extrapolation from adult cell lines to developmental processes, pathways involved in embryonic development are not directly represented in ToxCast™. To address this, we investigated the suitability of a differentiation assay with human induced pluripotent stem cells (hiPSCs) for modeling tissue differentiation during human embryonic development (Fig. 1). Endogenesis, the formation of endoderm, was selected due to the role of endoderm in giving rise to visceral organs derived from the gut tube including thyroid, thymus, lung, pancreas, liver, gastrointestinal system, prostate, and the visceral yolk sac. The critical role of endogenesis in proper embryonic development establishes this system as a model to screen and identify chemicals that may disrupt embryonic patterning leading to birth defects.

Endogenesis occurs during the primitive streak stage of gastrulation when epiblast-derived ectodermal cells migrate ventrally to invade the hypoblast-derived extraembryonic (yolk sac) endoderm while transitioning to an embryonic endodermal state via the process of epithelial to mesenchymal transition (EMT) [5,6]. These events mark the establishment of the three germ layers at the earliest stage of embryogenesis in week three of human development (gestational age week 5) [7]. The anterior primitive streak gives rise to embryonic endoderm [3], in turn leading to formation of the gut tube, comprising foregut, midgut, and hindgut. The foregut is the source of anterior organs: thyroid, thymus, lungs, stomach, liver, and pancreas; the midgut becomes the small intestine; the hindgut becomes the large intestine. In addition to the gut tube-derived organs, the anterior primitive streak also gives rise to extra-embryonic endoderm, also known as yolk sac or visceral endoderm, to be incorporated into the gut epithelium [8]. The posterior primitive streak gives rise to mesoderm also through an EMT [9].

The establishment of the three germ layers from the primitive streak begins with ectoderm, which is the first layer to form and often referred to as the default cell fate from which mesoderm or endoderm (mesendoderm) is induced [10,11]. There are several well-established molecular signaling events that facilitate germ layer specification. Fibroblast growth factor (FGF) expression favors ectoderm differentiation while inhibiting endoderm formation [12]; while Wnt, bone

* Corresponding author at: 109T.W. Alexander Drive (D-143-02), Research Triangle Park, NC 27711, United States.
E-mail address: knudsen.thomas@epa.gov (T.B. Knudsen).

https://doi.org/10.1016/j.reprotox.2019.10.001
Received 4 June 2019; Received in revised form 23 September 2019; Accepted 1 October 2019
Available online 07 October 2019
0890-6238/ © 2019 Published by Elsevier Inc.

DOI: 10.1016/j.reprotox.2019.10.001