March 3 discussion with Unilever
(presentation for NAMs symposium, SOT 2021)

Predictive DART:

Synthetic Microsystems, Computational Intelligence, and Artificial Life

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

Disclosures

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https://stemcells.nih.gov/research/registry.htm

Funding: our research with human pluripotent stem cell lines (hPSCs) was performed under EPA's *Chemical Safety for Sustainability Research Program, Research Area 5 'Virtual Tissue Models' (VTMs).*

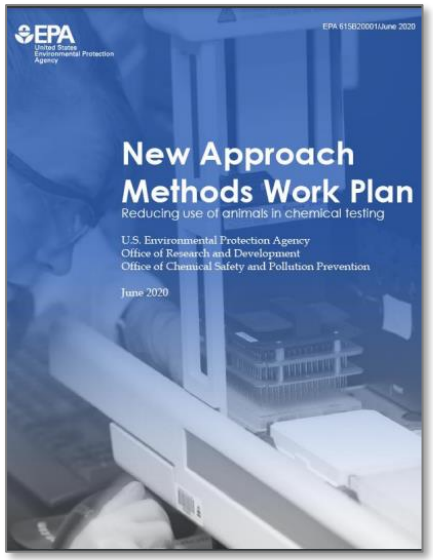
Compliance*:* work involving established hPSC lines is compliant with Executive Order 13505 (issued 2009) to ensure that is ethically responsible, scientifically worthy, and conducted in accordance with applicable law.

The H9 cell line is registered in the NIH Human Embryonic Stem Cell Registry: WA09 (H9): NIH Approval Number: NIHhESC-10-0062 (EPA contract EP-D-13-055 with Stemina Biomarker Discovery).

Other pluripotent stem cell lines: endodermal hPSC line from Allele Biotech #ABPSC-HDFAIPS (EPA contract EP-D-13-054 with Vala Sciences, Inc.).

Shifting toxicity testing to animal-free alternatives

- **June 2016:** *Frank R. Lautenberg Chemical Safety for the 21st Century Act* enacted to advance chemical safety evaluations with novel methods that reduce testing on vertebrate animals and are translatable to vulnerable populations / lifestages.
- **September 2019:** directive issued by USEPA Administrator Wheeler set a vision to reduce mammalian study requests 30% by the year 2025 and eliminate them by 2035.
- **June 2020:** USEPA work plan to accelerate scientifically valid *New Approach Methods* (NAMs) for assessing toxicity of large numbers of chemicals with less reliance on animal testing.



- **Science challenge:** build confidence in the predictive power of computational models and computer simulation for human-relevant pathways underlying developmental toxicity.

Developmental toxicity

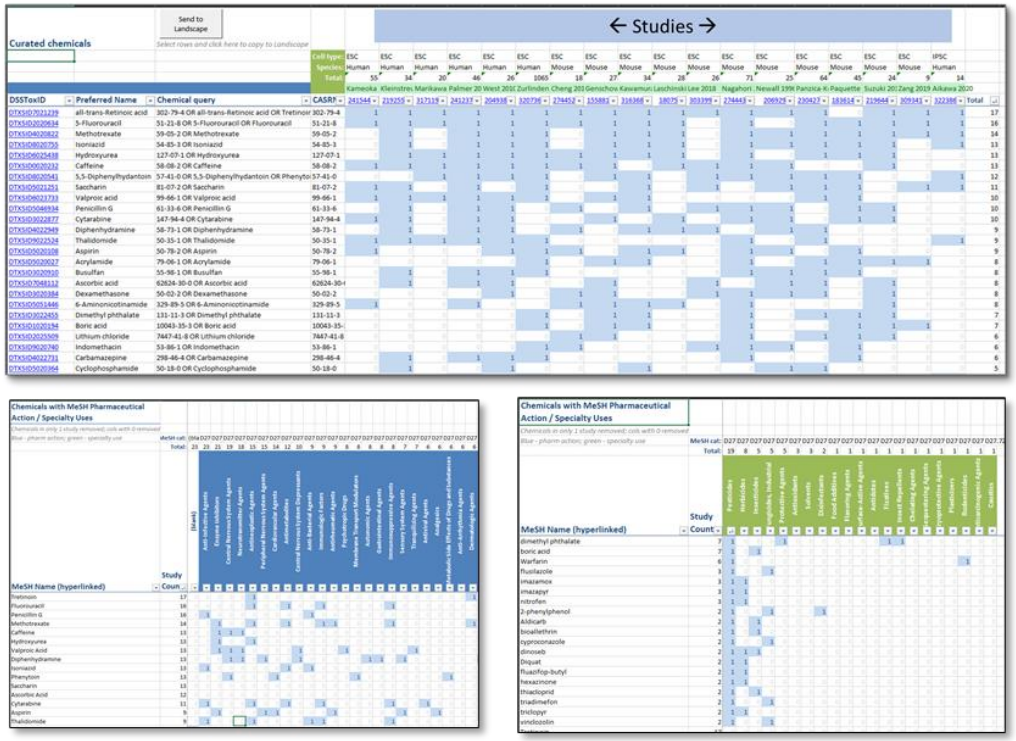
- Observation of fetal outcome in pregnant animal studies, most often in testing rats and/or rabbits, is the accepted means of developmental hazard identification.
- A guideline prenatal developmental toxicity study (e.g., OECD 414) is mechanistically complex, requires many animals, and potentially confounded by maternal effects.
- *In vitro* assays and *in silico* models that reflect key aspects of embryo-fetal development will be indispensable for NAM-based detection of developmental hazard potential.
- *In vitro* profiling of human pluripotent stem cell (hPSC) lines is an active area of investigation and one of the most promising alternatives to pregnant animal testing.

Novel features of PSC lines

- **Self-renewal:** cells replicate themselves indefinitely when cultured under appropriate growth factor conditions.
- **Pluripotency:** cells have the potential to form most of the different cell types comprising the embryo/fetus.
- **Autopoiesis:** capacity to self-organize into rudimentary tissues and more complex organoid structures.

PSC lines established from the embryoblast (mouse, human) can recapitulate **some** of the biology driving embryogenesis during the period covered by guideline prenatal studies (e.g., OECD TG 414).

Conceptual and practical considerations



Abstract Sifter, SWIFT, MeSH terms, Chemicals Dashboard, …

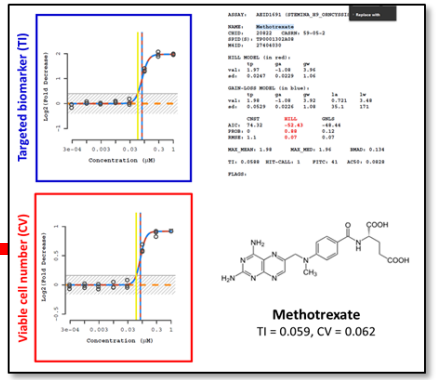
- **Detailed literature review:** survey of extant ESC assays used to classify developmental toxicants:
  - chemical domain
  - biological domain
  - standardized protocols
  - reproducibility
  - biomarker readouts
  - predictive power.

1,533 records in PubMed reduced to 333 (AI for relevance) and 192 (manual curation).

- **1,250 annotated chemicals (through 2020):**
  - 18 publications tested ≥ 10 compounds (primary)
  - 174 publications tested 1-9 (evidentiary support)
  - most frequently represented: ATRA, 5-FU, MTX.

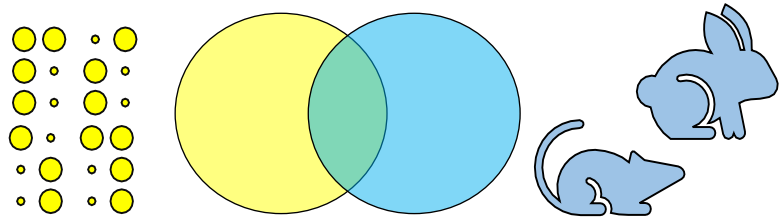
Piersma et al., manuscript in preparation

ToxCast HTS Profiling with the devTOX^{qP} Assay



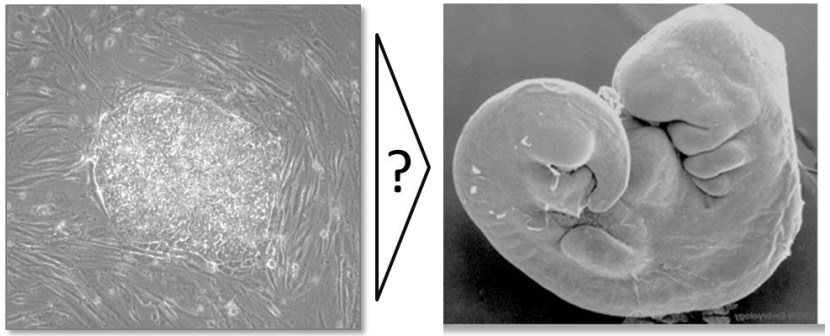
- Pluripotent human (H9) stem cell-based biomarker assay for developmental toxicity screening developed by Stemina Biomarker Discovery [*Palmer et al., 2013, BDRB*] ...
- … defined developmental toxicity potential by the concentration of a test chemical reducing the ratio of ornithine (secreted) to cystine (utilized) to a critical level (77% accuracy).
- We used DevTOX^{qP} to test 1065 ToxCast chemicals for teratogenicity index (TI) and pipelined the dataset into EPA's CompTox Chemicals Dashboard [*Zurlinden et al., 2020, Toxicol Sci*] …
- … and observed a 19.2% positivity rate across the 1065 chemicals tested, with performance reaching 79%–82% balanced accuracy to well-curated teratogens and non-teratogens.

Bridging animal-human studies



- Query of prenatal developmental studies in EPA's ToxRefDB database found adverse fetal outcome for 53 of 283 (18.7%) chemicals tested in pregnant rats and rabbits.
- Profiling 1065 ToxCast chemicals with a pluripotent stem cell (hPSC) assay showed a 19.2% positivity rate for teratogenic potential [Zurlinden et al. 2020].
- Closely matches the 18.7% positivity rate from concordant animal studies, but only a subset of the positives are detected by both *in vitro* and *in vivo* platforms.
- Discordance: (i) biology missed by the hPSC platform; (ii) concurrence of fetal outcomes with maternal toxicity; (iii) mesoscopic properties of complex systems.

Can an hPSC assay live up to the NAM challenge?

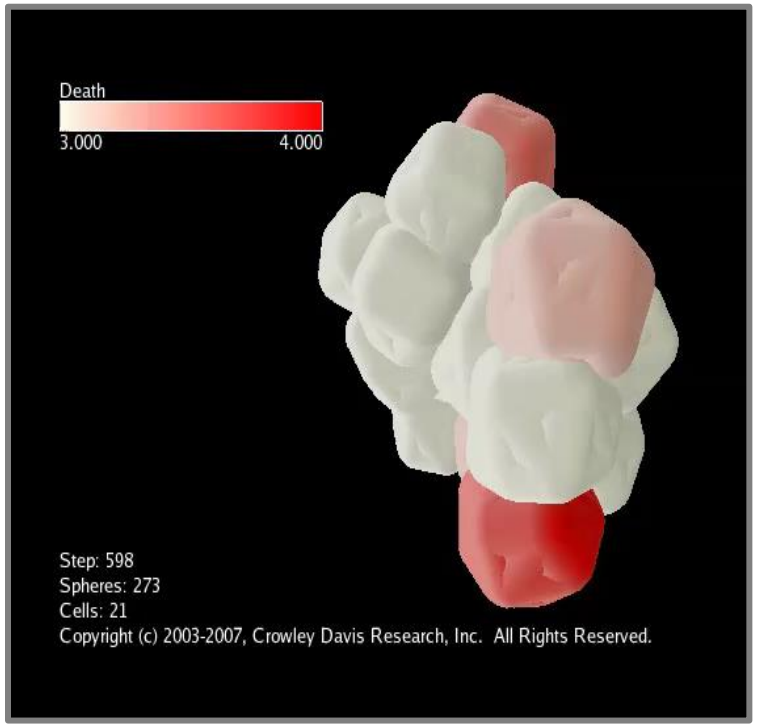


Motivation for a building a more synoptic view to improve mechanistic understanding of developmental processes and toxicities around hPSCs.

- does not encompass the full complexity of anatomical development;
- blind to the precise spatial-temporal control of cell-cell interactions *in vivo* ;
- misses developmental effects secondary to maternal or placental toxicity;
- uncertainty of post-organogenesis vulnerability and post-natal manifestations;
- cross-species extrapolation (mESC to human, hESC to animals);
- limited xenobiotic metabolism and other ADME considerations (toxicokinetics);
- uncertainties in translatability to the intact embryo (toxicodynamics).

A more synoptic view …

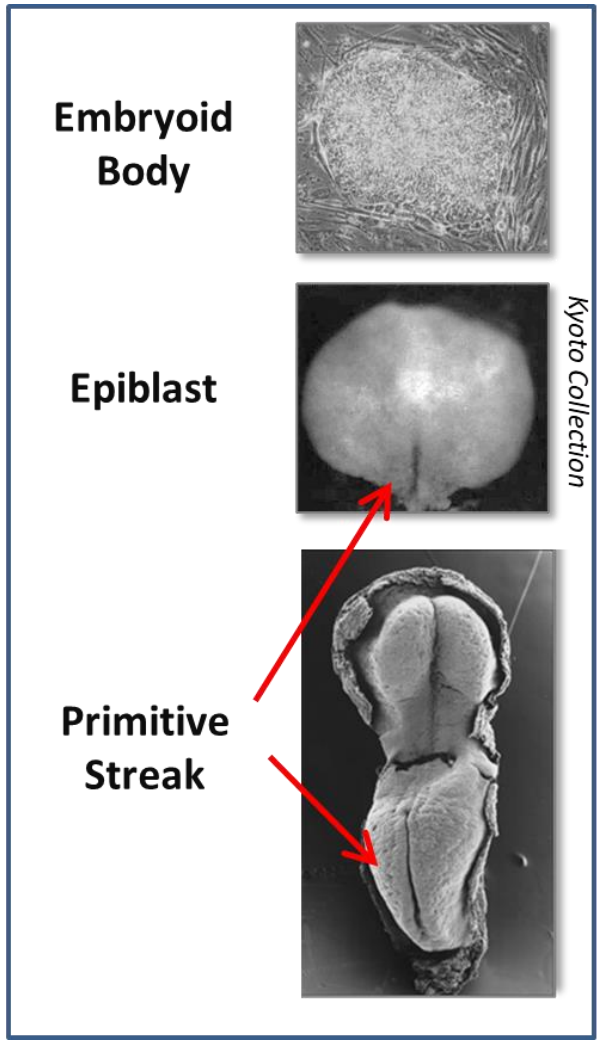
Anatomical homeostasis in a self-regulating 'Virtual Embryo'



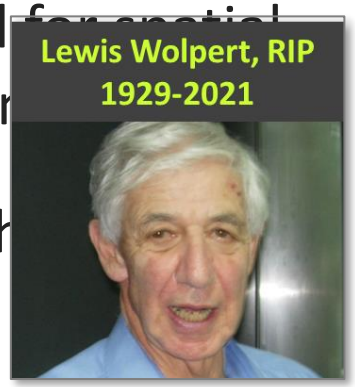
SOURCE: Andersen, Newman and Otter (2006) Am. Assoc. Artif. Intel.

- **synthetic microsystems:** recapitulate the microphysiology and cellular behaviors of a physical system.
- **computational intelligence:** biological-inspired algorithms use fuzzy logic to fill in missing or incomplete information.
- **artificial life:** computer simulation of biological processes evolved through automation, control networks.

Gastrulating embryo: *remarkable example of a self-organizing system*



- The molecular biology and behavior of hESCs in culture most closely resembles the **epiblast** of an early embryo during 'gastrulation'.
- The hallmark of gastrulation in *Vertebrata* is **primitive streak** formation through which the genomic body plan is set up.
- Cell migration through the primitive streak is essential for spatial organization, regional specification, and lineage determ
- Although cultured hESCs can form most cell types in th lack **positional information** of an intact epiblast.

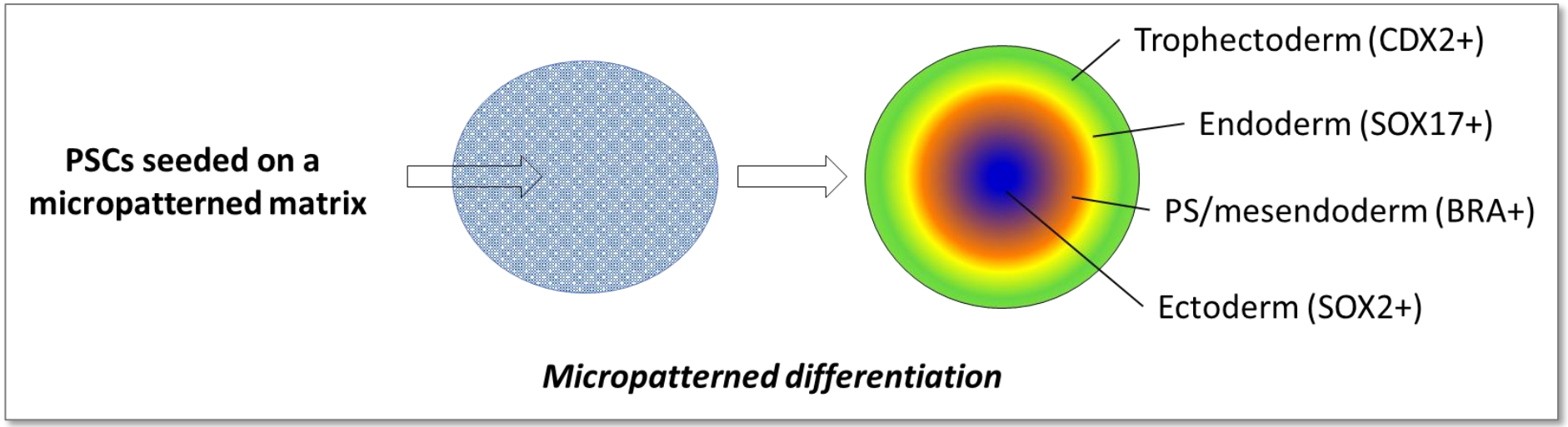


"It is not birth, marriage, or death, but **gastrulation** which is truly the most important time in your life." *- Lewis Wolpert*

Geometric confinement

- Randomly seeded PSCs readily generate primary germ layers in culture; however, patterns of differentiation are heterogeneous and spatially disordered.

- PSCs differentiated on a micropatterned surface express lineage-specific markers and self-organize in symmetrical domains.



Martinez Arias et al. (2014) Development; Warmflash et al. (2014) Nat Meth

Cellular dynamics and signaling in the epiblast

Key regulatory signals

STATE 1 - Naïve Pluripotency (self-renewal)

LIF/STAT3	ESC pluripotency signal
OCT4, SOX2, NANOG	pluripotency core triad
PI3K/AKT/MEK/ERK	signal transduction

STATE 2 - Primed Pluripotency (patterning)

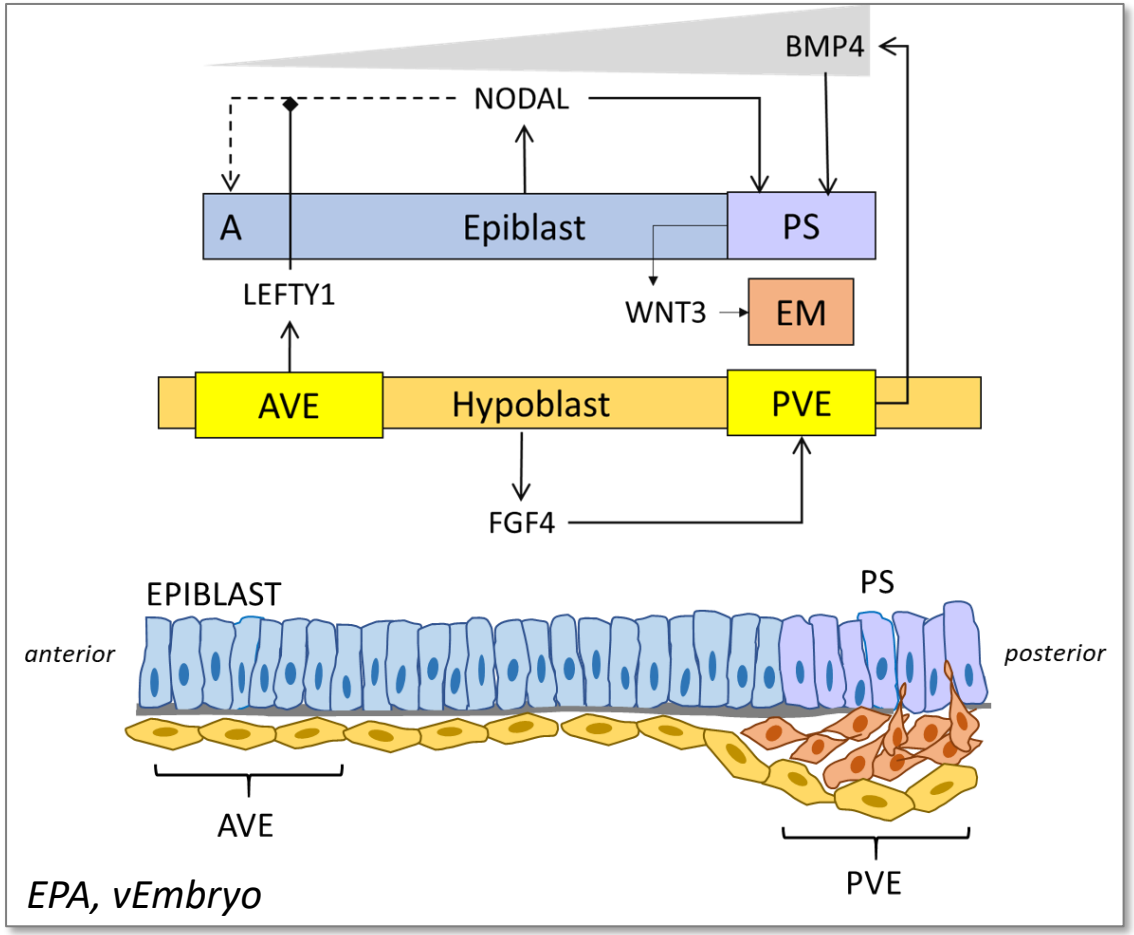
FGF4	maintains *Bmp4* in ExE (GD 5.5)
BMP4	primes posterior cell fate
WNT3 *	pinpoints PS & propels pAVE
NODAL	induces primitive streak (GD 6.25)
LEFTY1/CER1	NODAL antagonists in AVE

STATE 3 – Determination (gastrulation)

ACTIVIN A	mesoendoderm formation
FGF8	Hox clock
ATRA	mutual antagonism with Fgf8
…	…

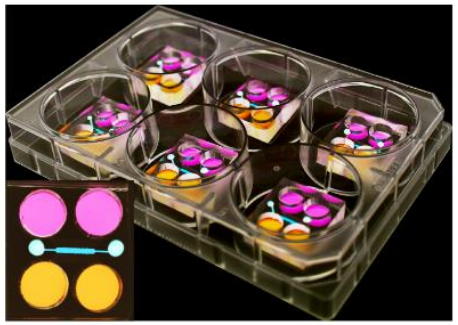
SOURCE: Tam et al. (2006) Curr Opin Gen Dev

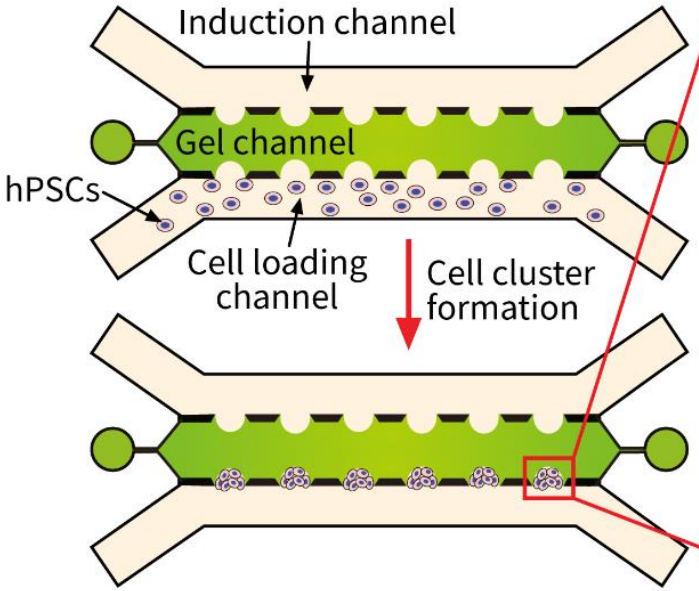
Morphological programming logic

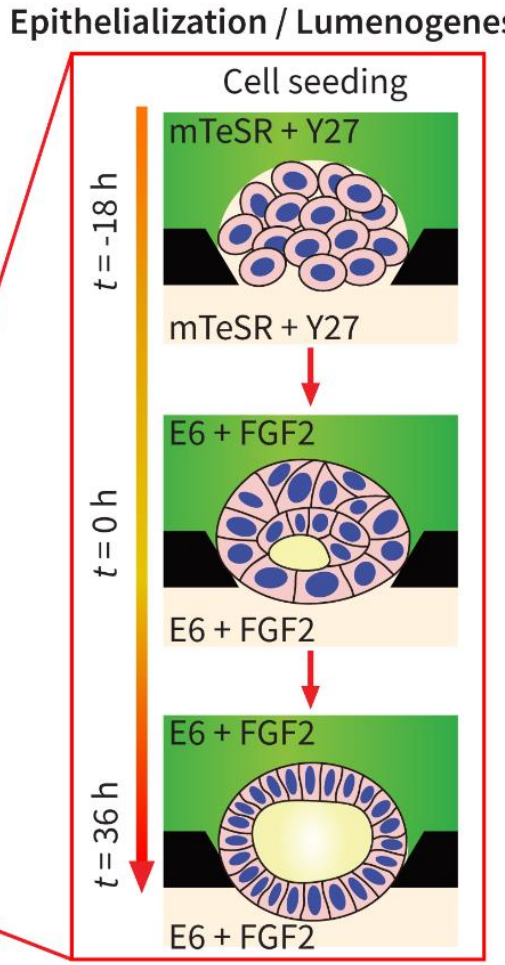


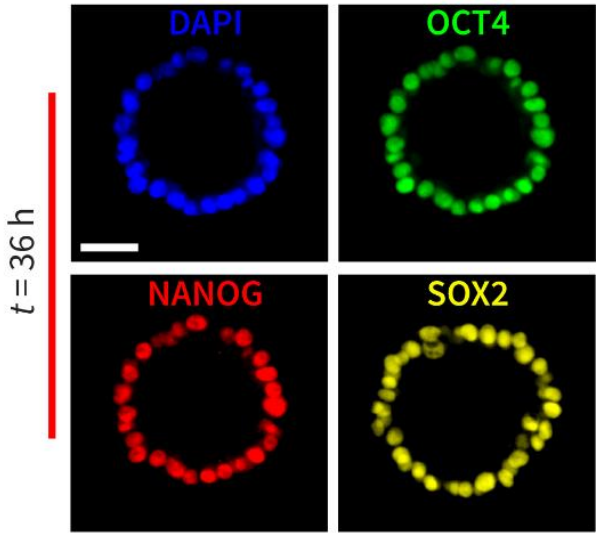
EPA, vEmbryo

Synthetic epiblast: *microphysiological system*

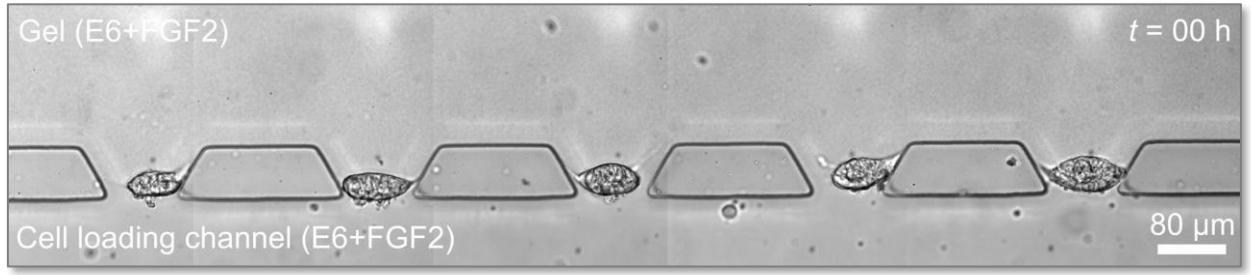






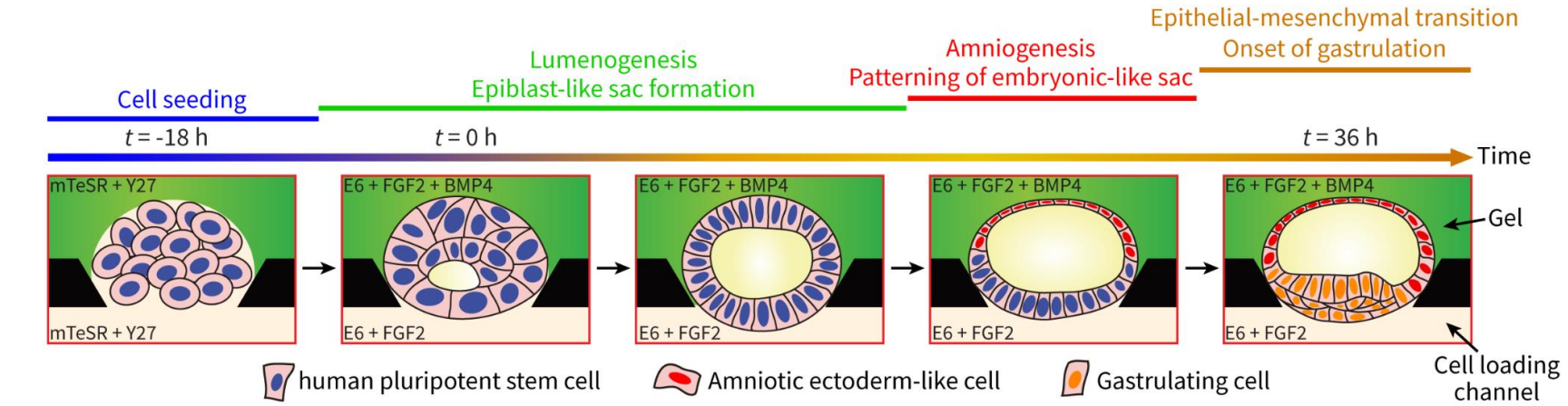


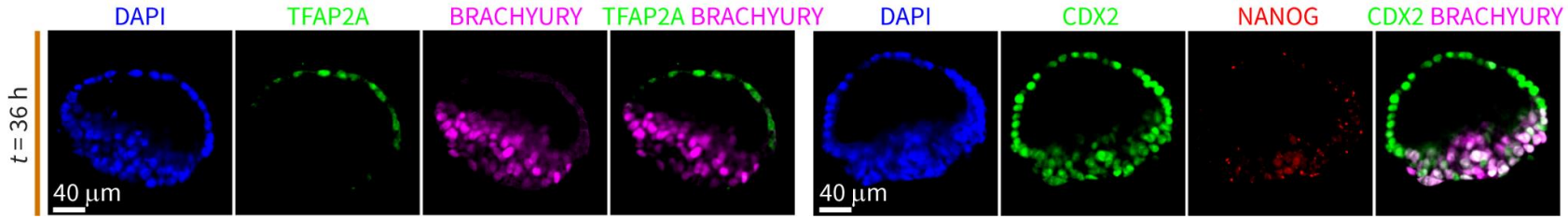
- FGF at 18h induces lumenization (36h):
- Pluripotency markers (OCT4/NANOG/SOX2);
- Intrinsic radial symmetry (spheroid).



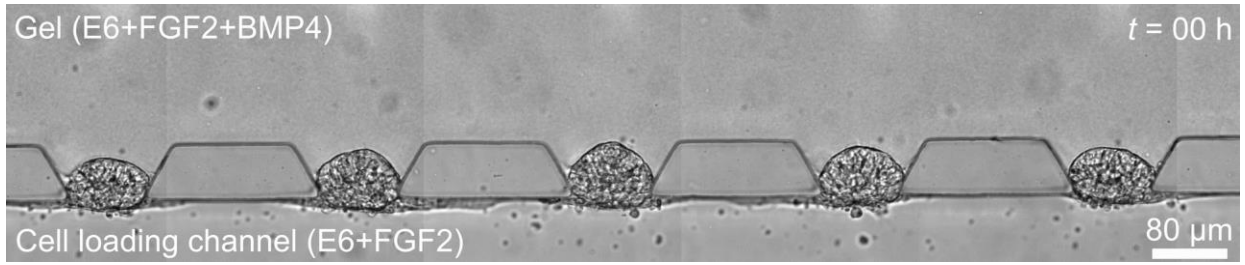
Shared by Jianping Fu: Zheng et al. Nature (2019)

Breaking the symmetry with BMP4





- BMP4 gradient breaks intrinsic symmetry.
- BMP4 primes posterior cell fate.
- Distinct axial domains emerge.
- Pluripotency advances to a determined state.
- But a bona fide primitive streak has not formed.

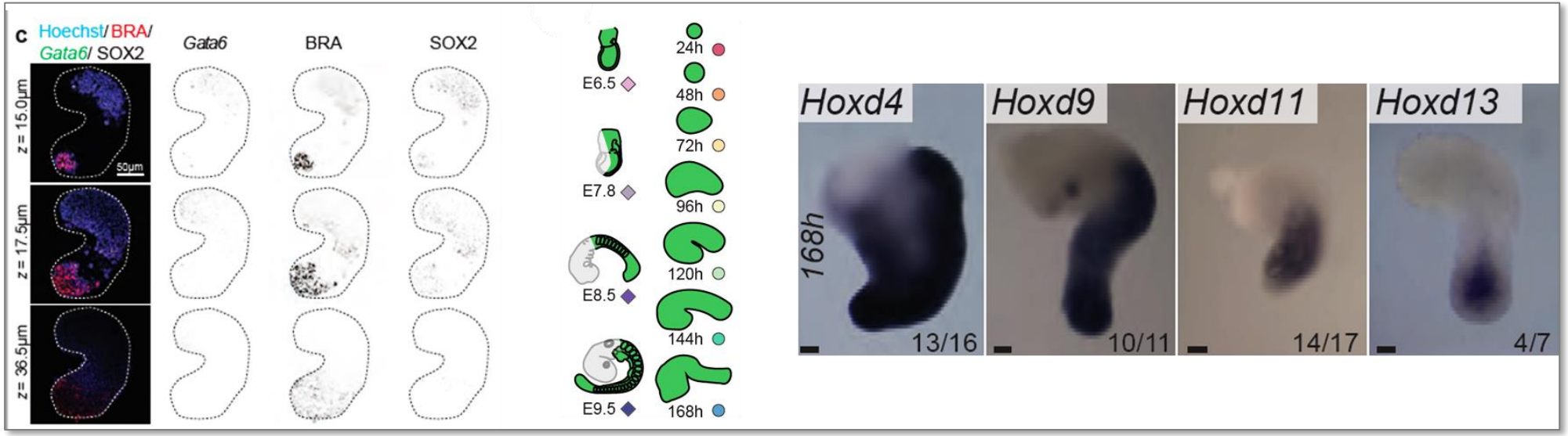


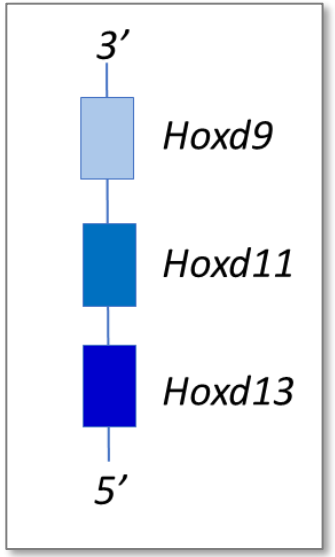
Shared by Jianping Fu: Zheng et al. Nature (2019)

Gastruloids

- mESCs aggregated with defined numbers of cells and induced with extrinsic BMP4 may under certain conditions spontaneously organize axial structures (gastruloids).

- These display hallmarks of postcranial axial gene regulatory systems such as colinear Hox expression along an extending antero-posterior axis.

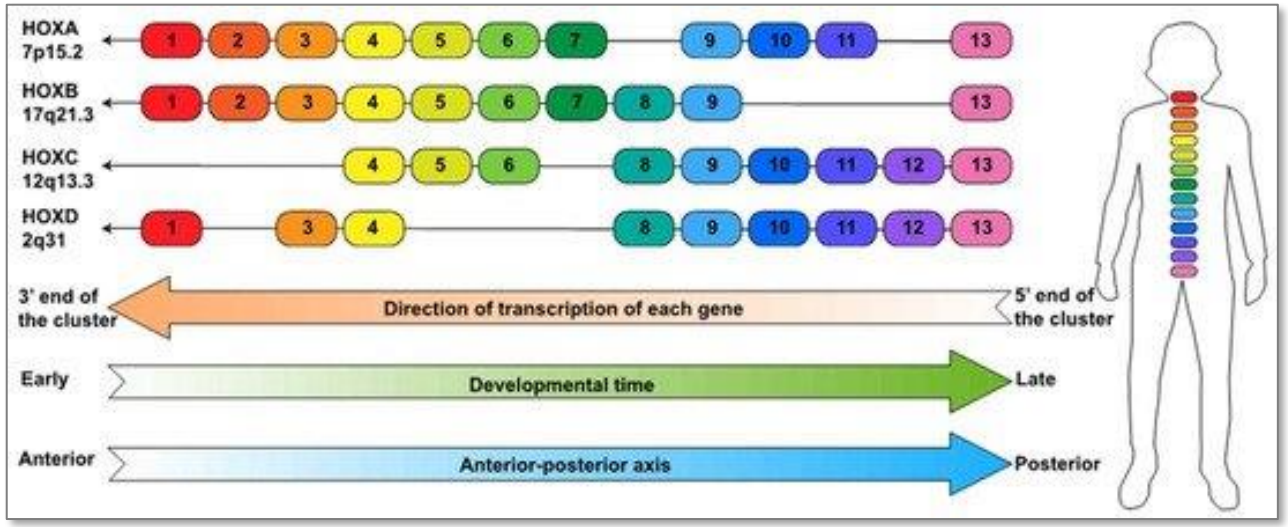




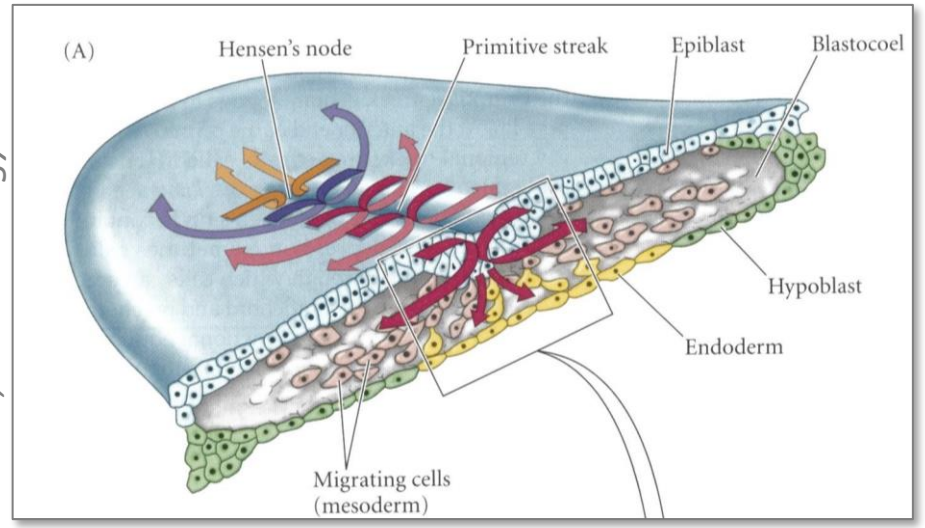
Beccari et al. (2018), Nature

Positional Information and mesoderm formation

- HOX pattern is determined as epiblast cells pass through the primitive streak; still other extrinsic signals needed to position a primitive streak (e.g., NODAL, LEFTY1, WNT3).



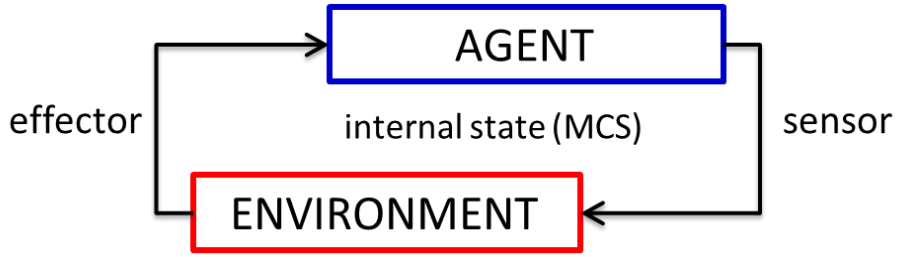
Luo et al. (2019)



Gilbert, Devel Biology

- A→P fate of a cell is based on epiblast position, which determines when and where it ingresses through the primitive streak and into the endomesodermal population.

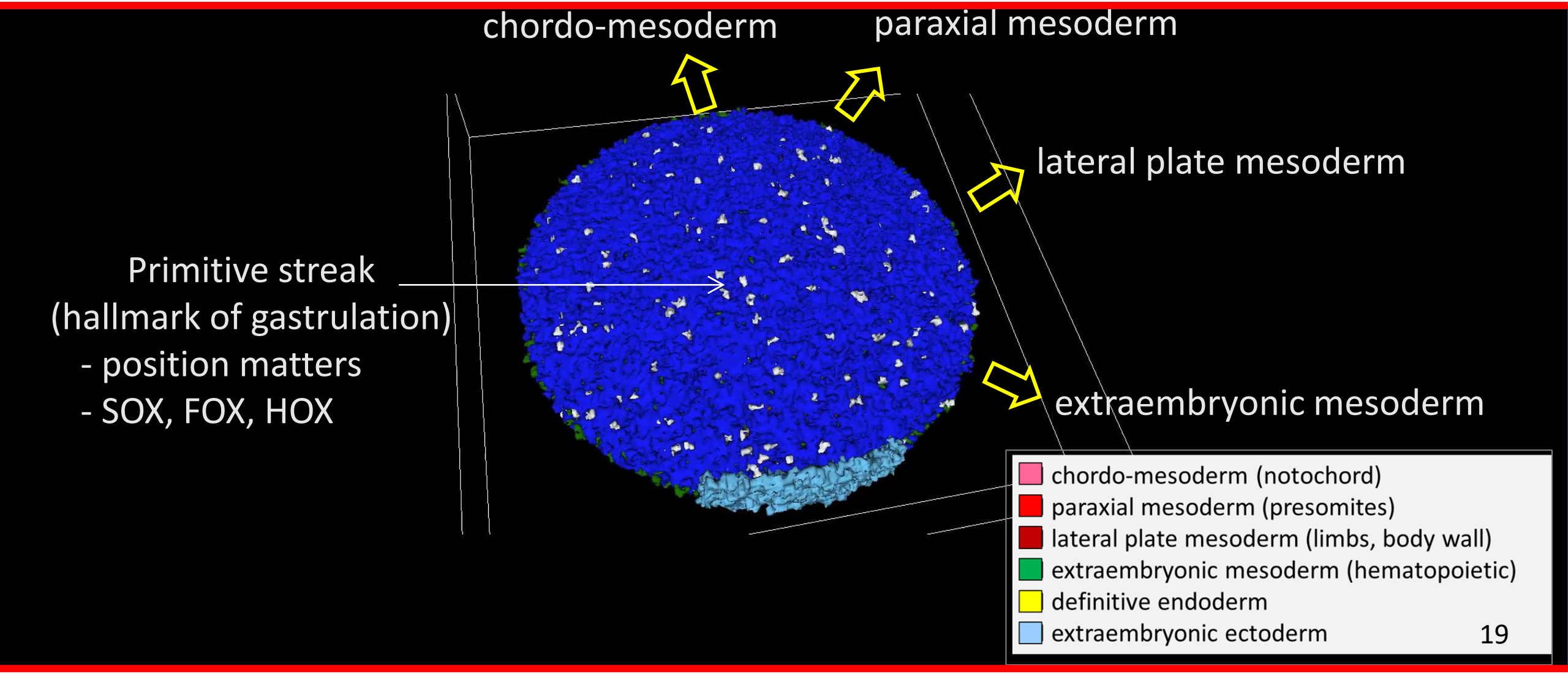
Agent-Based Models (ABMs)



- Nature-inspired *agents* (cells) and *rules* (behaviors) are set into motion as a self-organizing virtual system, using an open-source modeling environment (**CompuCell3d.org**).
- Soft-computing uses 'fuzzy logic' to simulate forces or properties governing cell fate and behavior where rules are inexact or knowledge incomplete (**computational intelligence**).
- Can change course in response to a particular situation or stimulus, such as genetic errors or biomolecular lesions fed to the model from real world data (**dynamic translation**).
- Probabilistic rendering of where, when and how a particular condition might lead to an adverse developmental outcome (**cybermorphs**).

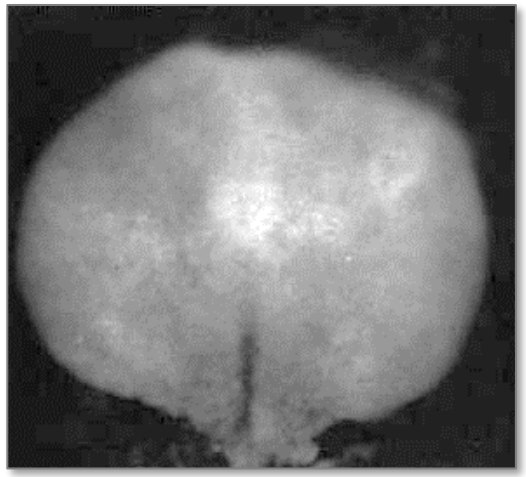
"Molecular biology took Humpty Dumpty apart … mathematical modeling is required to put him back together again." – *Schnell et al. (2007) Amer Scientist*

Quasi-gastrulation: *recoding the genomic blueprint of the fetal body plan?*

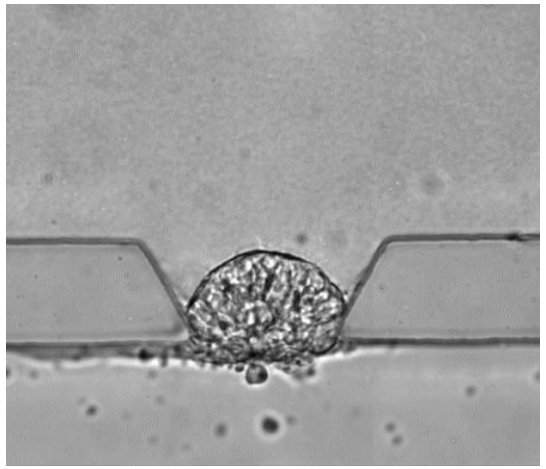


Practical use of a synoptic manifold

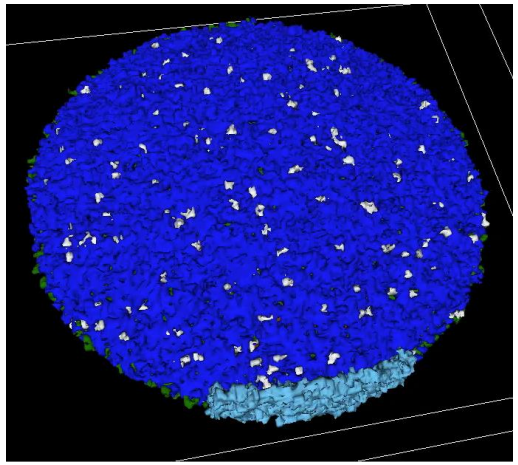
In vivo



In vitro

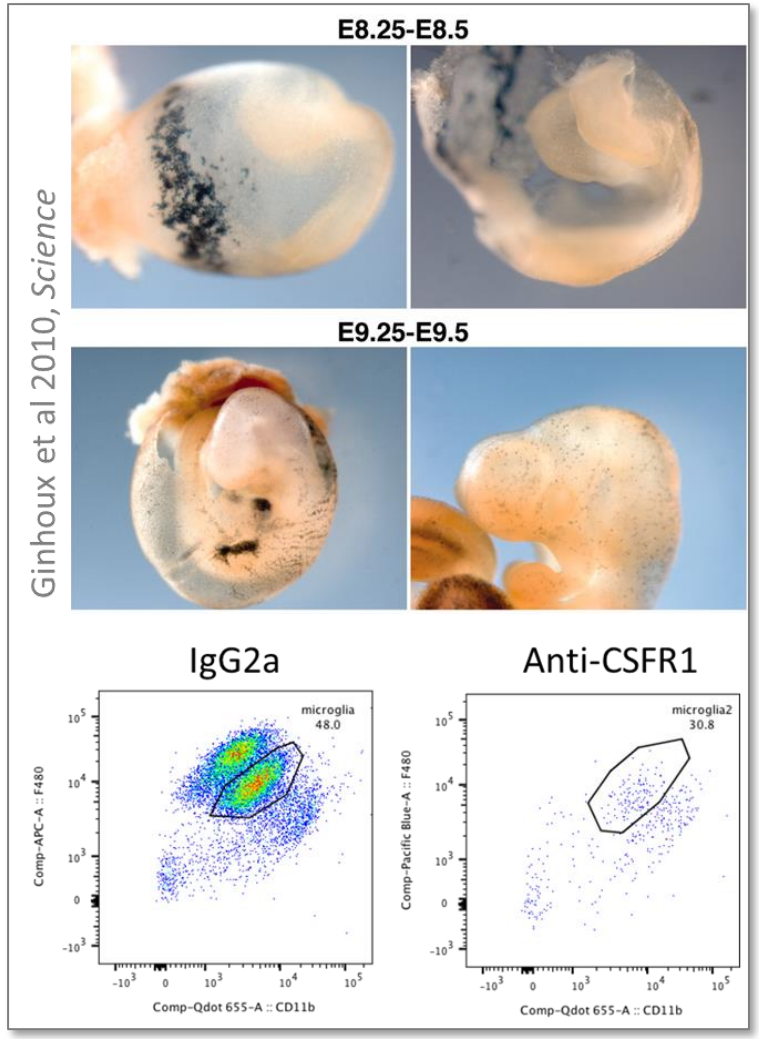


In silico



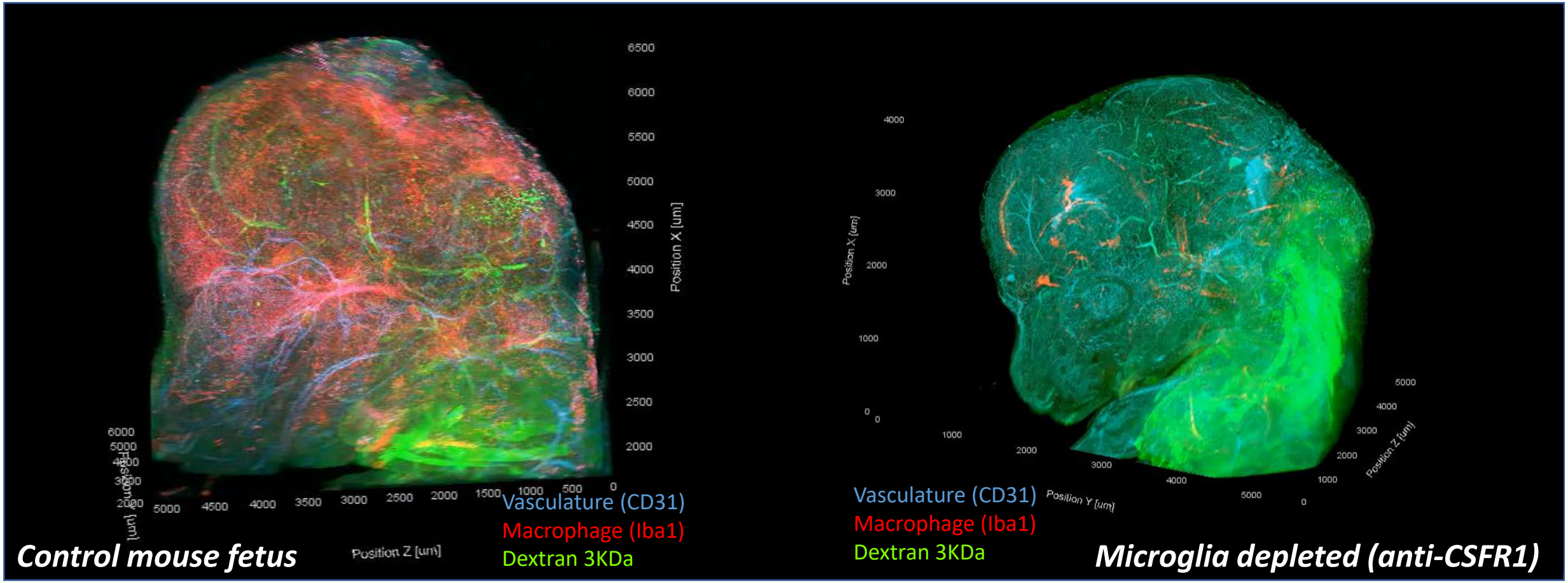
- MPS models can probe the interaction of physical geometry and cell signaling;
- FGF2 and BMP4 is a start, but still other signals needed to position a primitive streak;
- ABM adds positional information and tracks individual cell behaviors;
- computational intelligence can fill in for missing or incomplete knowledge;
- quantitatively simulate what chemical exposures could actuate at the cellular level;
- provide inferences on developmental effects in a human-relevant manner.

Microglia and neurovascular patterning



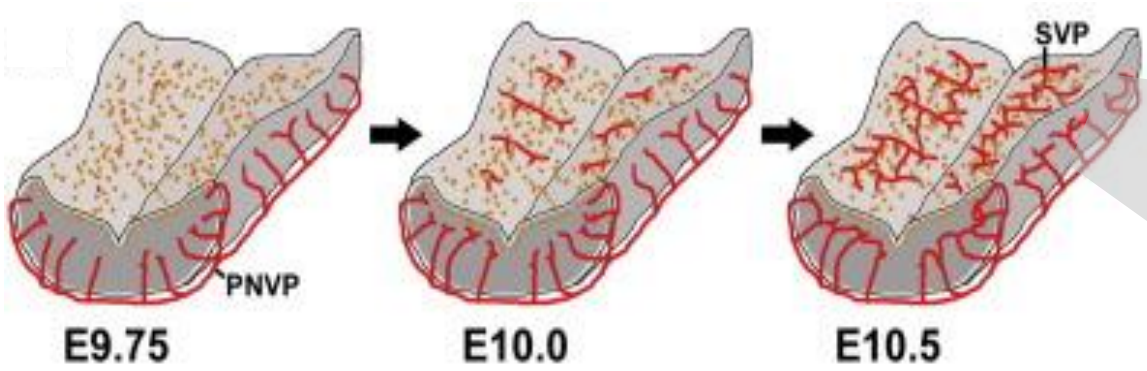
- Vascularization of the neural tube commences on E9-10 (mouse) with formation of blood-brain barrier by E11.
- Microglia from yolk sac blood islands form on E8 and circulate to colonize the neuroepithelium by E9.
- Anti-CSFR1 treatment on E6.5 -7.5 depletes 95-99% of the microglial population in the brain by E14.5.
- Microglia have 3 phenotypic states: M0 (resting), M1 (activated), M2 (protective).
- Microglia orchestrate neurovascular patterning, but when stress-activated → neuroinflammatory response.

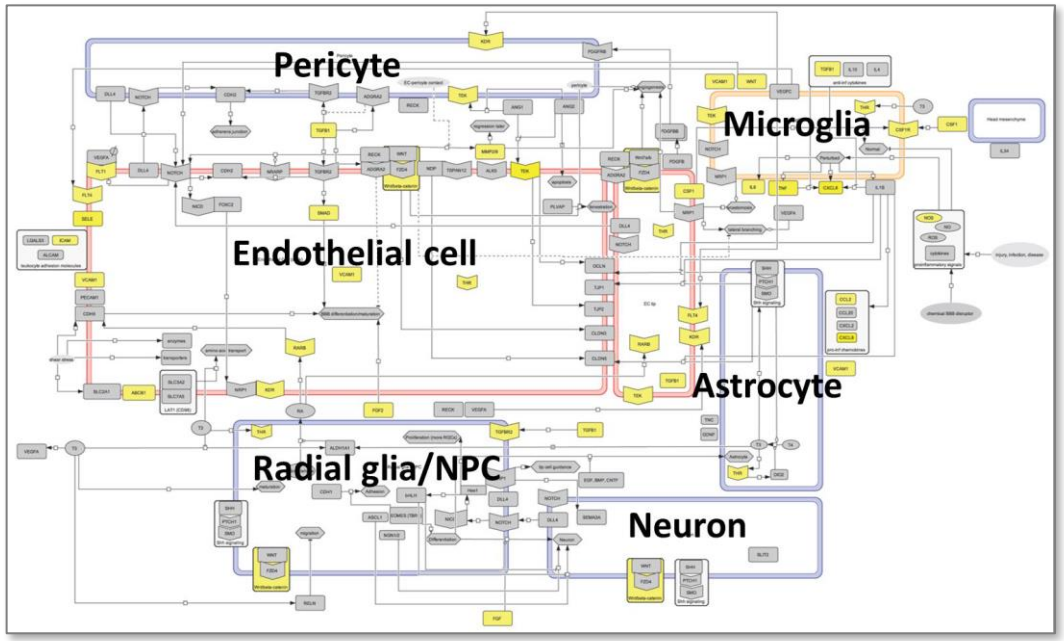
Microglia depletion: *reduces angiogenesis of the fetal brain and impairs or delays the development of barrier function of the microvasculature.*



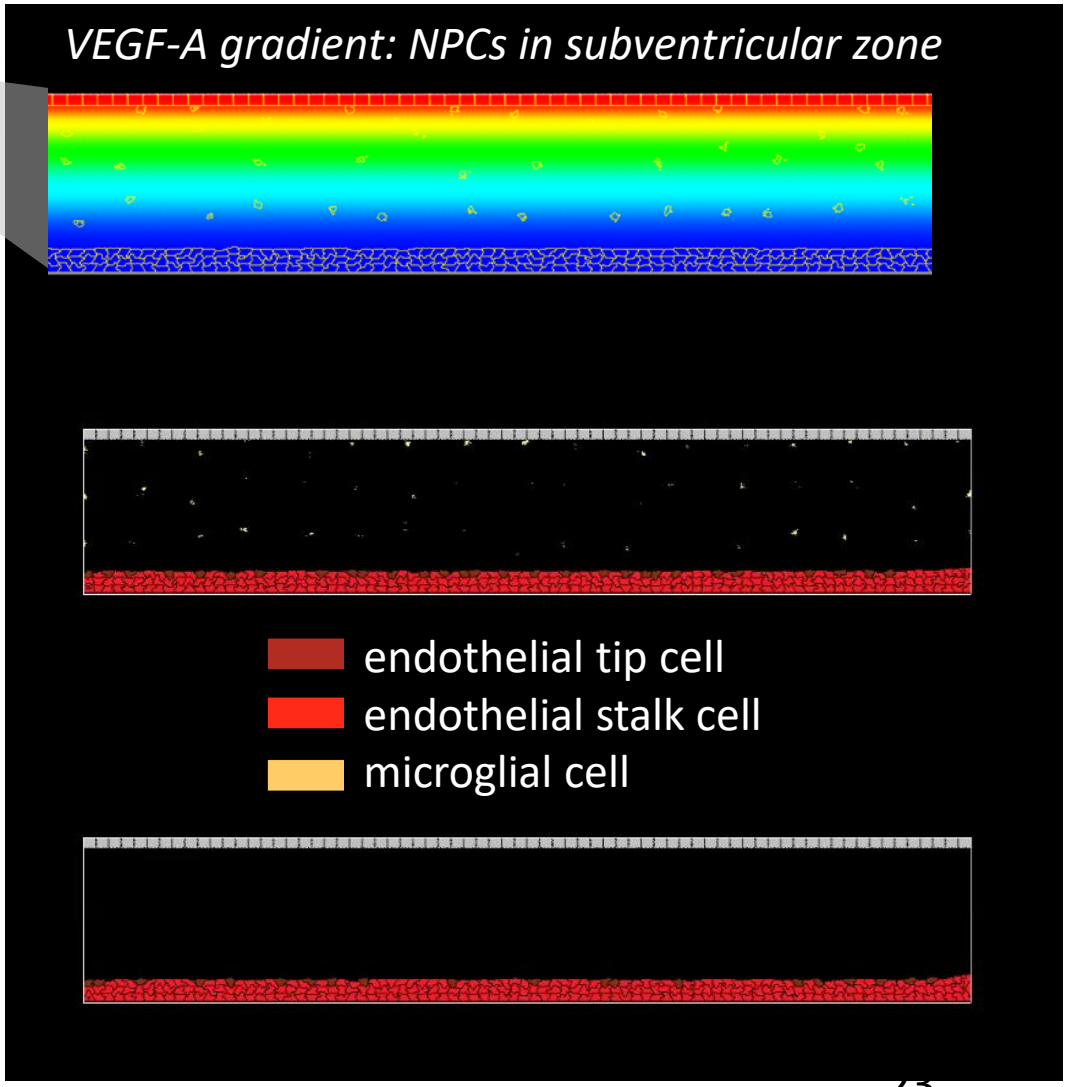
*EPA-A*STAR collaboration with A Silvin, F Ginhoux – A*STAR/SIgN*

Computational Systems Model

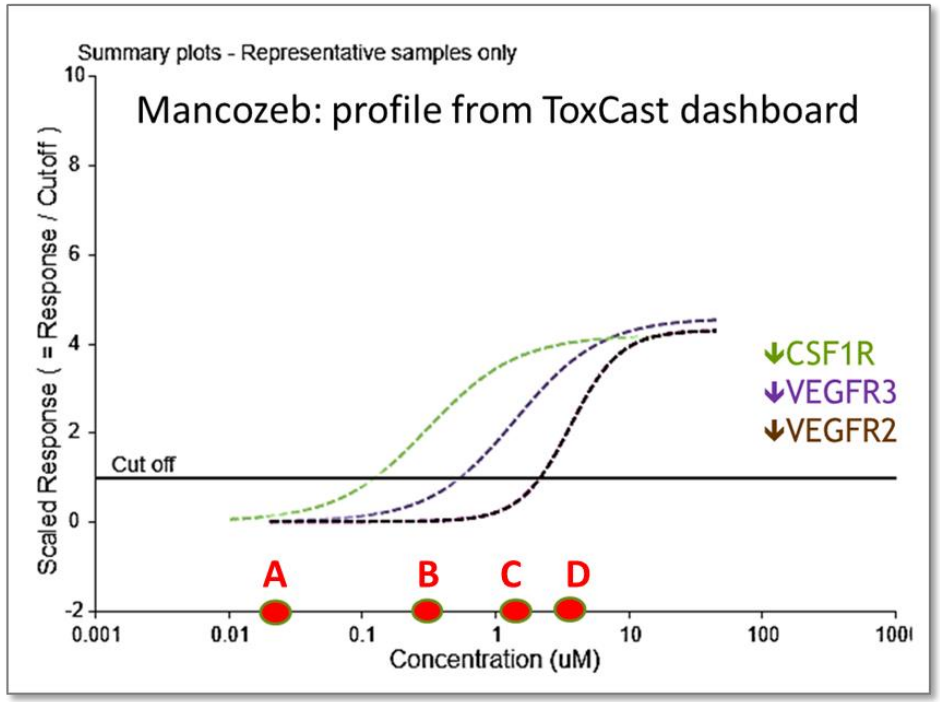


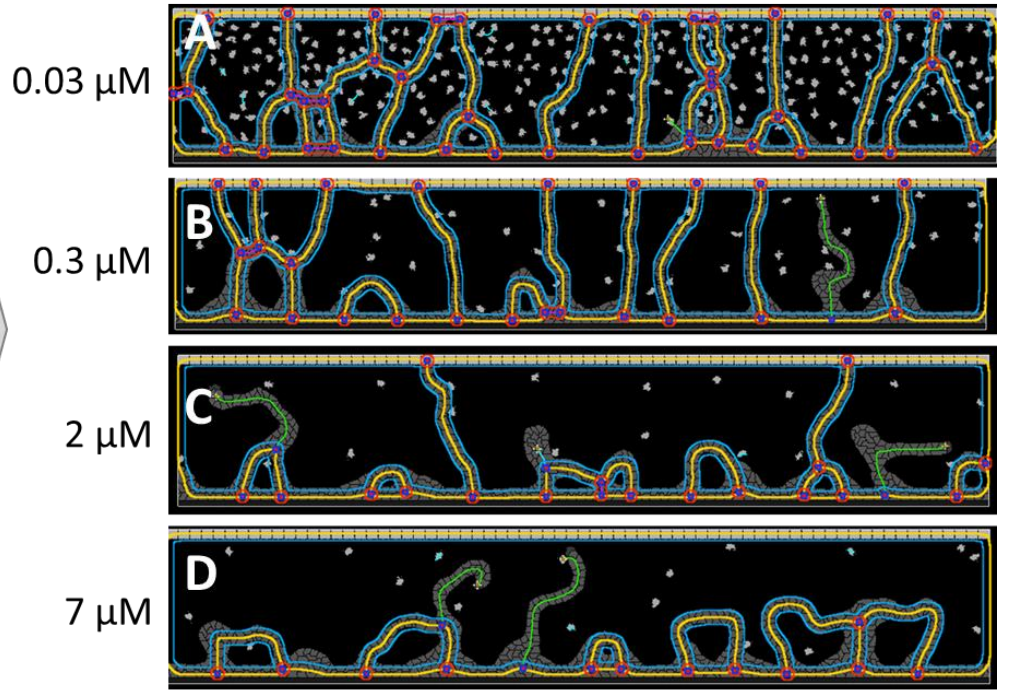


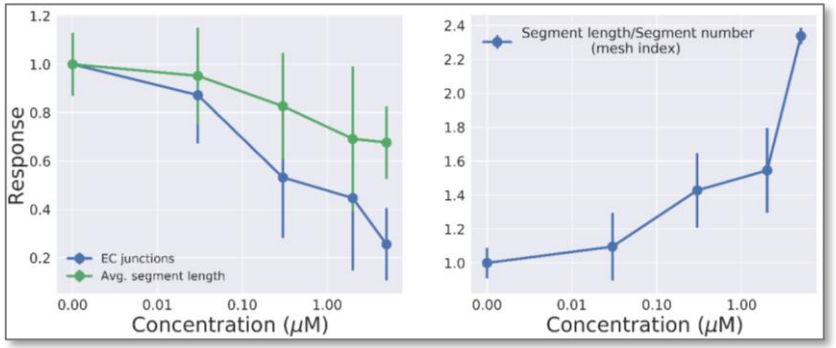
Produced in CellDesigner v4.4



Executing a simulated concentration-response

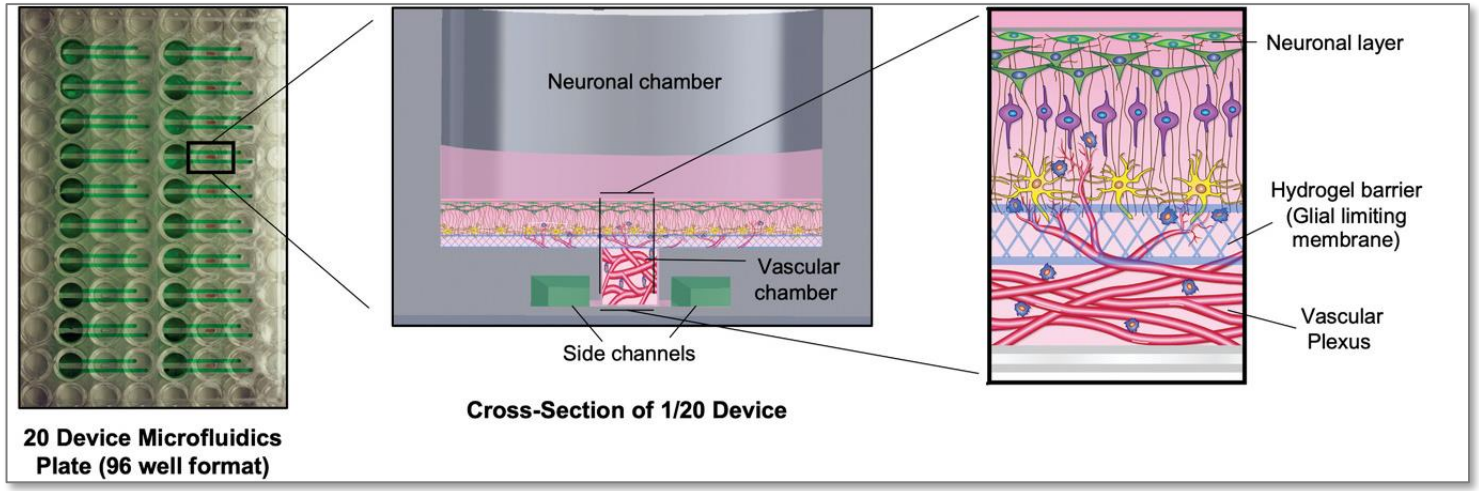






- Prediction: affects microglial-endothelial interaction (reduced tortuosity → deficiency of SVZ).
- Quantitative microvascular 'cybermorphs' predicts an AC50 for Mancozeb disruption at 0.5 µM.

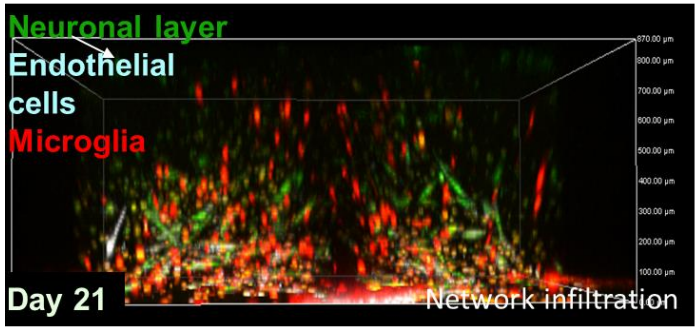
Checking the prediction: *microglial integration in a synthetic microsystem*



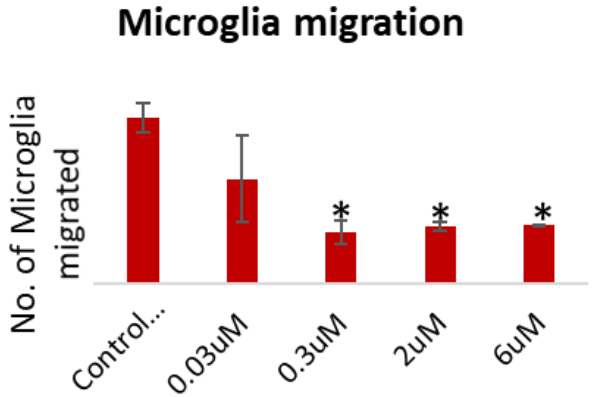
Engineered Perineural Vascular Plexus for Modeling Developmental Toxicity

Gaurav Kaushik, Kartik Gupta, Victoria Harms, Elizabeth Torr, Jonathan Evans, Hunter J. Johnson, Cheryl Soref, Suehelay Acevedo-Acevedo, Jessica Antosiewicz-Bourget, Daniel Mamott, Peyton Uhl, Brian P. Johnson, Sean P. Palecek, David J. Beebe, James A. Thomson, William T. Daly, and William L. Murphy**

Kaushik et al. (2020), Adv Hlthc Materials



Microglia Migration into Neural Layer



Critical concentration (PoD) for Mancozeb on neural tube vascularization:

- ***predicted*** *by in silico cNVU = 0.5 µM*
- ***observed*** *in organotypic culture = 0.3 µM.*

Microglial states may be an important sentinel for neurodevelopmental toxicity.

EPA STAR Center Co-operative grant #835737 , Univ Wisconsin (W Murphy)

Looking ahead …

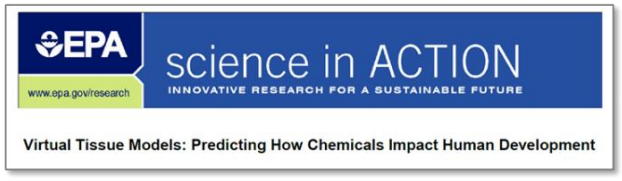
Translational: what do synthetic models of human development - both computational and organoids - bring to future of DART testing?

Investigational: how smart must these models be (A.I.) to support decision-making in the animal-free (3Rs) zone?

Operational: what best practices are needed to implement synthetic models into an integrative decision framework (eg, AOP-based IATAs)?

Communication: what are the practical considerations for science, engineering, and stakeholder engagement (academics, government, industry, NGOs, policy, …)?

Acknowledgements



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