SOT Hot Topic: Circulating Molecular and Cell-Derived Biomarkers for Translational Toxicology
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Extracellular microRNA as Biomarkers of Environmental Chemical Health Hazard Identification

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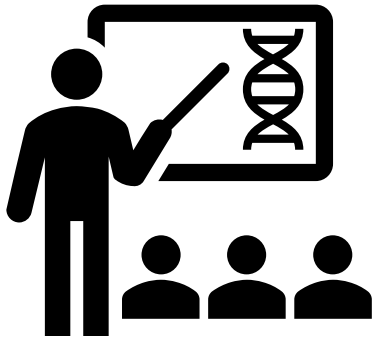
Presentation outline



1. Scientific drivers for transcriptomic biomarkers
   a) Regulatory drivers and our mission
   b) Why microRNA?

2. Background studies
   a) Biofluid-based indicators of liver disease in an PCB-exposed residential cohort
   b) Urinary miRNA as biomarkers of regional nephrotoxicity
   c) Dose-responsive microRNA biomarkers of chemical mode-of-action

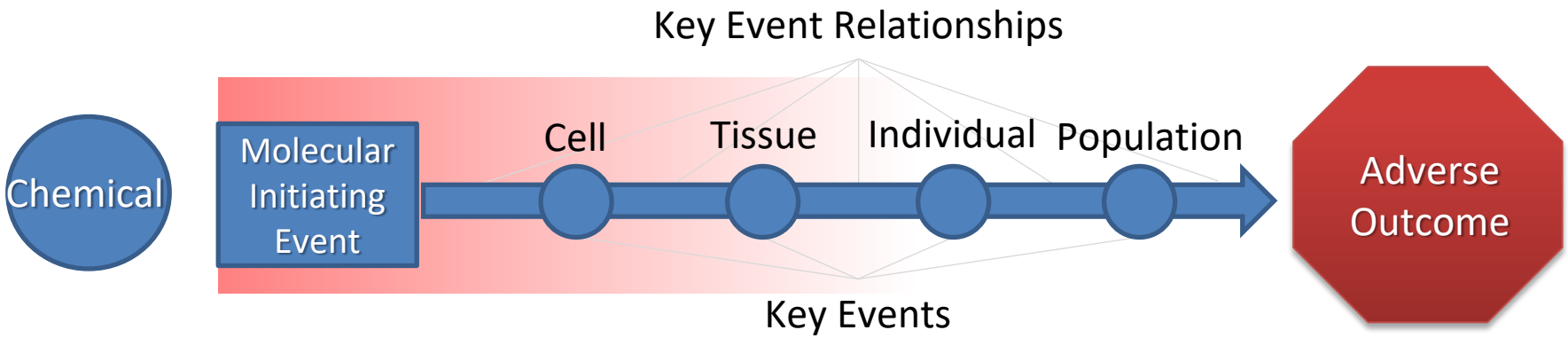
3. In vitro screening development using microRNA biomarkers
   a) Study design
   b) Identification of microRNAs in media with sequencing
   c) Chemical exposure study design and preliminary results

4. Conclusions/Future directions

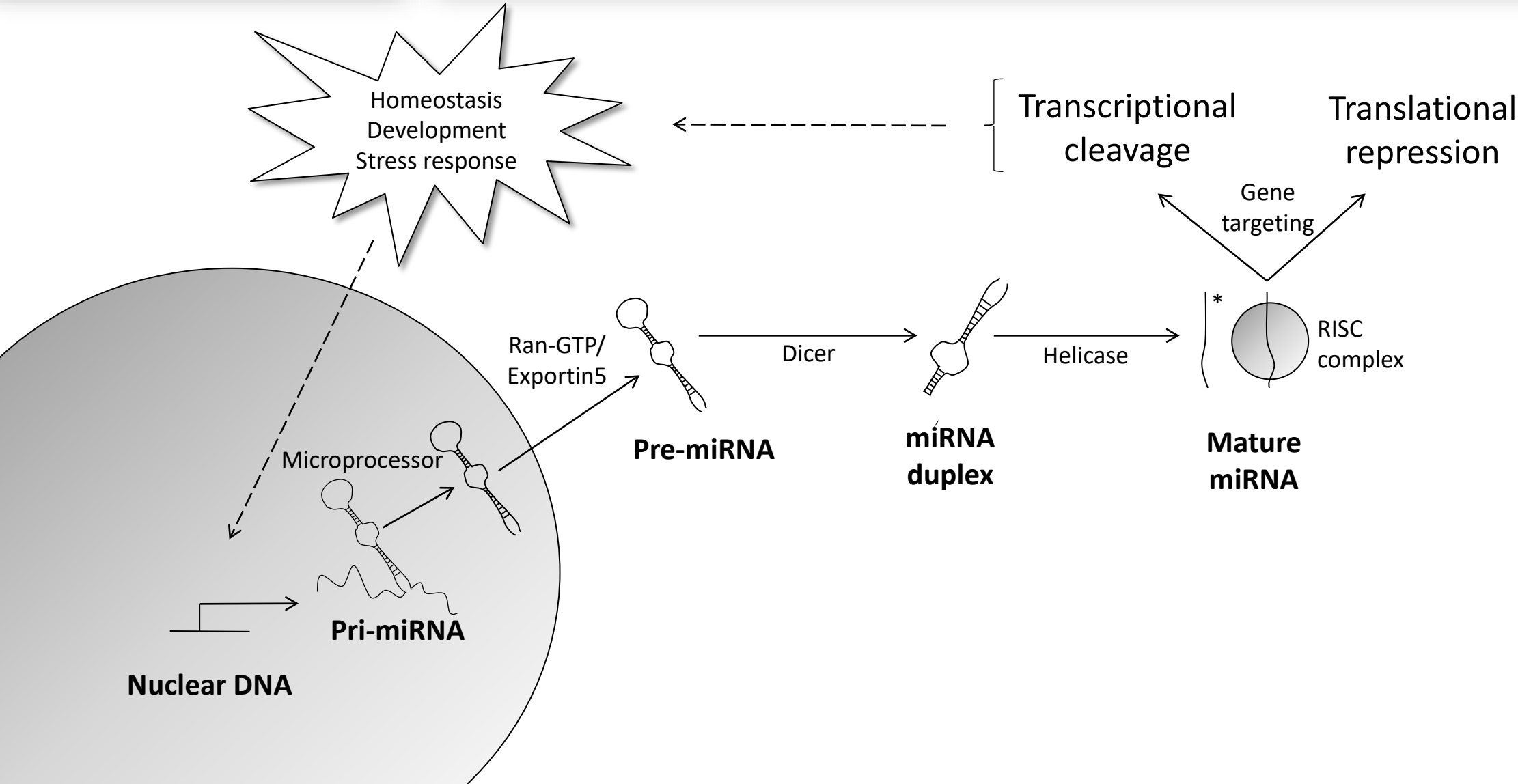
Transcriptomic biomarkers in toxicology

- Many thousands of chemicals without data to provide a reference value
- Costly and time consuming to generate apical data

- Early transcriptional biomarkers may be sensitive measure of chemical perturbation and link to mechanism of adverse outcome of regulatory interest



MicroRNAs as biomarkers in toxicology



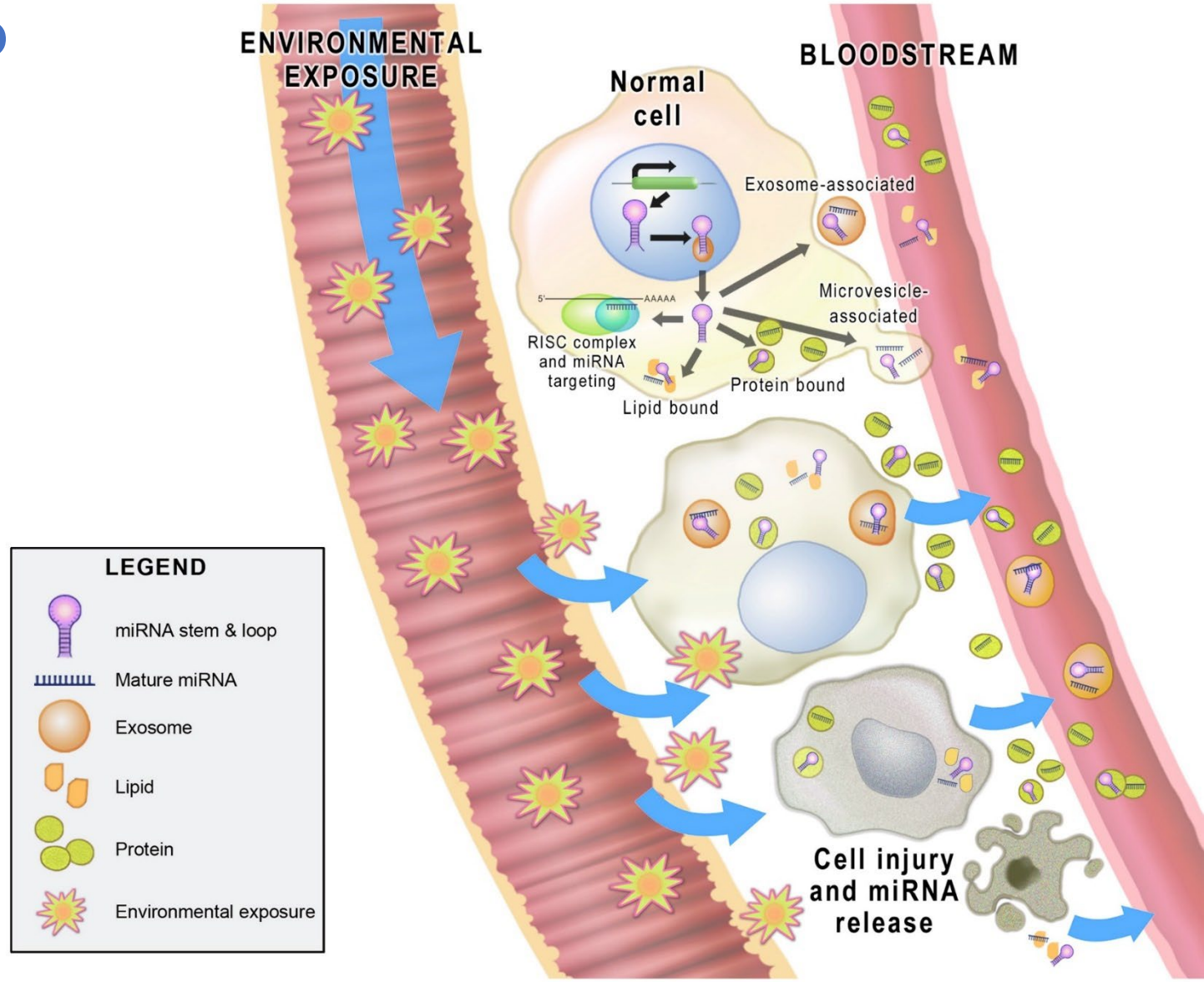
MicroRNAs as biomarkers in toxicology

- **MicroRNAs are responsive to exogenous exposures**
- **Regulatory nodes for transcriptional networks**
- **MicroRNAs present in biofluids**

MicroRNAs in Biofluids
Non-invasive biomarkers?

Predictive and non-invasive

- Passive secretion of microRNA
  - Associated with cell death and toxicity
- Active secretion of microRNA
  - Potentially vesicle-associated and involved in cell-to-cell signaling



Harrill *et al. Toxicological Sciences* 152(2):264-272, 2016

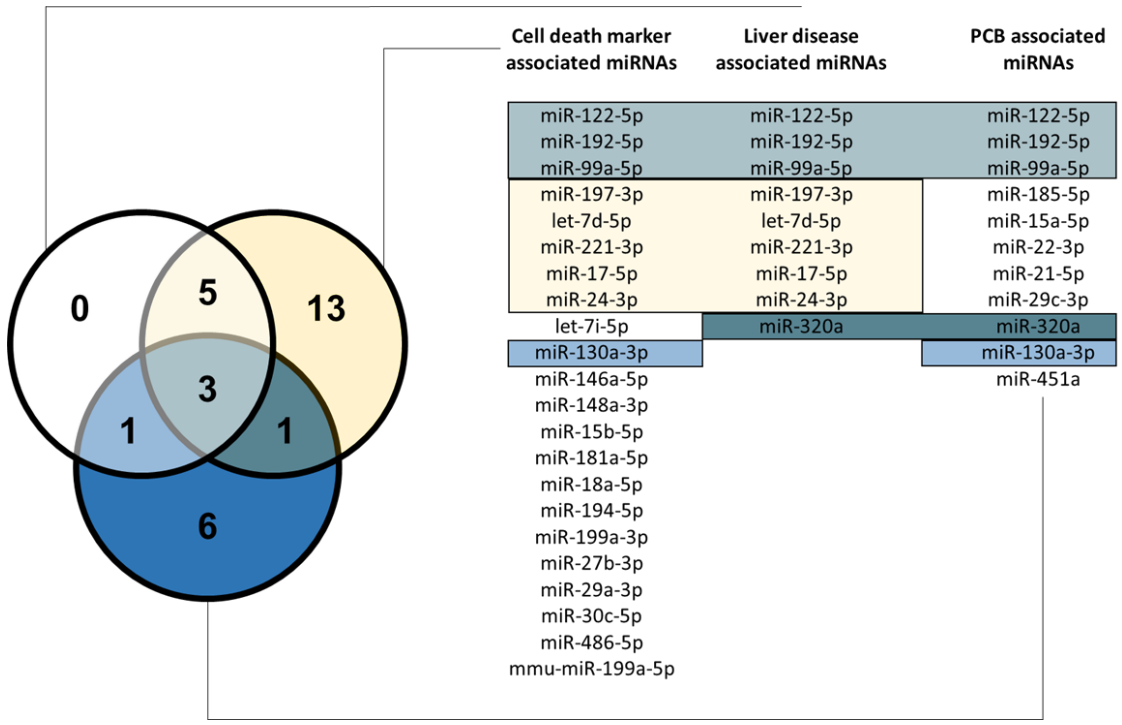
MicroRNAs as biomarkers in toxicology

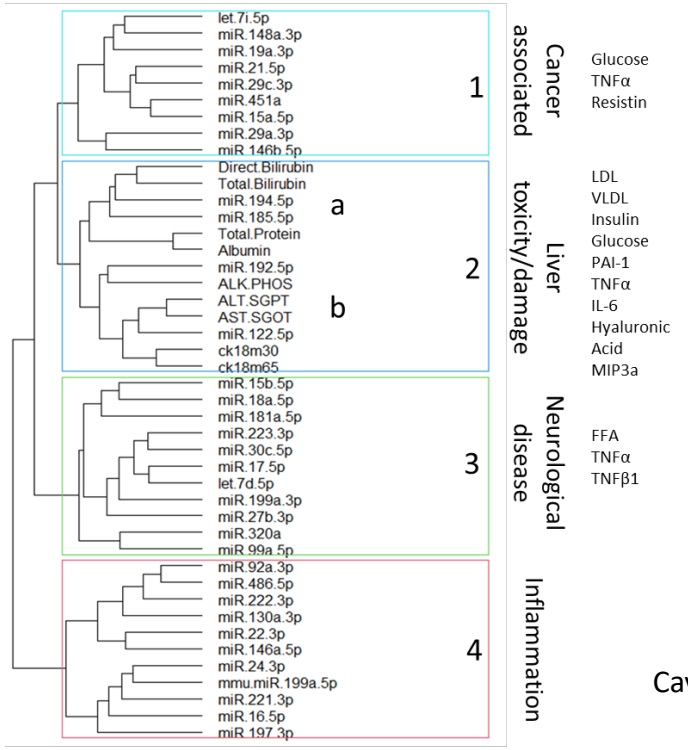
Are biofluid-based miRNA biomarkers informative for health effects due to environmental exposure?

Serum microRNA associated with liver disease

Hypothesis: Previously identified individuals with toxicant-associated fatty liver disease will exhibit an altered liver microRNA profile in serum.

Method: Use targeted panel to directly measure microRNA in archived serum and correlate with other liver toxicity and clin chem measures in cohort.





Cave MC, Pinkston CM, Rai SN, et al. *EHP* 2022

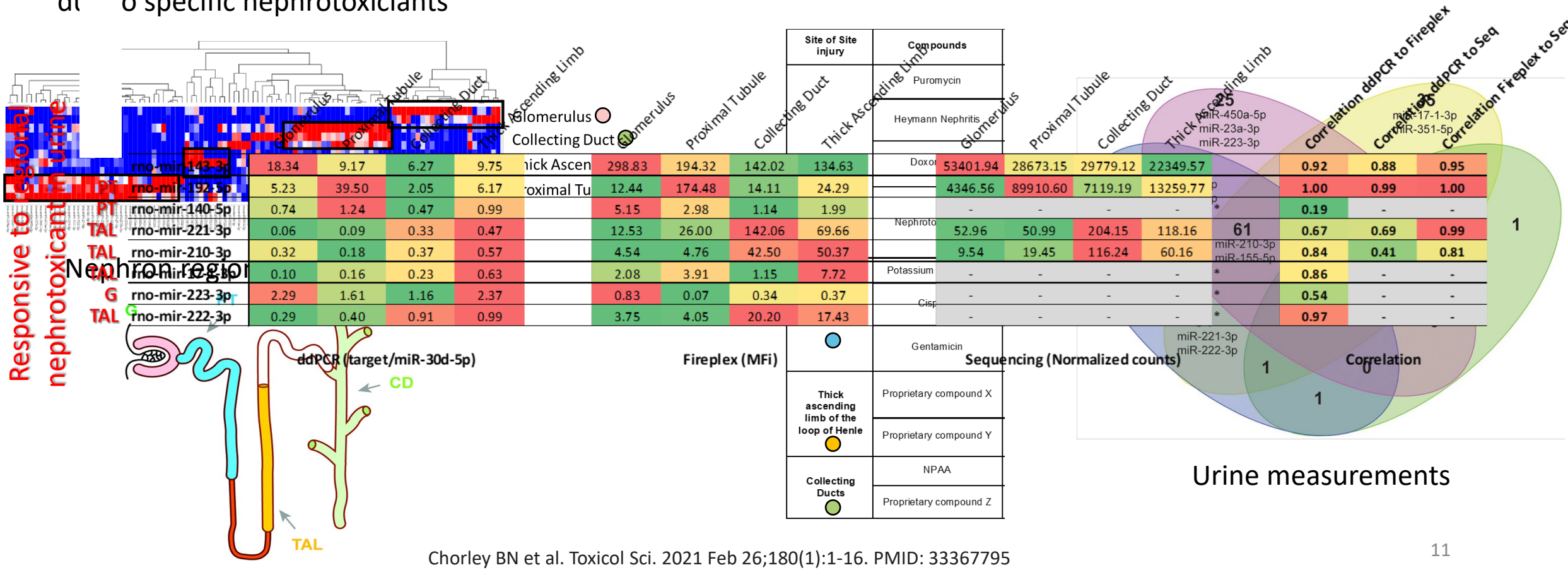
Project summary: Human serum miRNA

- Measured miRNA in biofluid correlated with specific liver injury biomarkers, but also indicated other adverse health processes
- *Are they indicative of adverse mechanisms beyond general toxicity?*
- *Can we link miRNA alterations to specific exposure-mediated mode-of-action?*

Urinary miRNA as Biomarkers of Nephrotoxicity

Hypothesis: Urinary miRNAs are subregion-specific bioindicators of chemical-induced nephrotoxicity

Method: Measure miRNA in laser-dissected regions of rat nephron and correlate with released miRNA in urine d[illegible]o specific nephrotoxiciants



	Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb
rno-mir-143-3p	18.34	9.17	6.27	9.75
rno-mir-192-5p	5.23	39.50	2.05	6.17
rno-mir-140-5p	0.74	1.24	0.47	0.99
rno-mir-221-3p	0.06	0.09	0.33	0.47
rno-mir-210-3p	0.32	0.18	0.37	0.57
[illegible]	0.10	0.16	0.23	0.63
rno-mir-223-3p	2.29	1.61	1.16	2.37
rno-mir-222-3p	0.29	0.40	0.91	0.99

	Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb
rno-mir-143-3p	298.83	194.32	142.02	134.63
rno-mir-192-5p	12.44	174.48	14.11	24.29
rno-mir-140-5p	5.15	2.98	1.14	1.99
rno-mir-221-3p	12.53	26.00	142.06	69.66
rno-mir-210-3p	4.54	4.76	42.50	50.37
[illegible]	2.08	3.91	1.15	7.72
rno-mir-223-3p	0.83	0.07	0.34	0.37
rno-mir-222-3p	3.75	4.05	20.20	17.43

	Glomerulus	Proximal Tubule	Collecting Duct	Thick Ascending Limb	Correlation ddPCR to Fireplex	Correlation ddPCR to Seq	Correlation Fireplex to Seq
Doxo[illegible]	53401.94	28673.15	29779.12	22349.57	0.92	0.88	0.95
	4346.56	89910.60	7119.19	13259.77	1.00	0.99	1.00
	-	-	-	-	0.19	-	-
	52.96	50.99	204.15	118.16	0.67	0.69	0.99
	9.54	19.45	116.24	60.16	0.84	0.41	0.81
	-	-	-	-	0.86	-	-
	-	-	-	-	0.54	-	-
	-	-	-	-	0.97	-	-

Chorley BN et al. Toxicol Sci. 2021 Feb 26;180(1):1-16. PMID: 33367795

Project summary: Urinary miRNA biomarkers of toxicity

- Measured miRNA in biofluid correlated with specific liver injury biomarkers, but miRNAs can be released by active mechanisms of response and signaling.

- *Are they indicative of adverse mechanisms beyond general toxicity?*

- *Can we link miRNA alterations to specific exposure-mediated mode-of-action?*

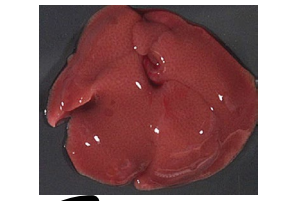
Dose Responsive miRNA Linked to Chemical Mode-of-Action

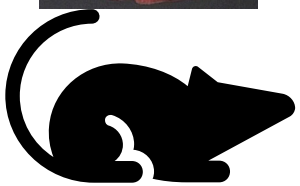
Hypothesis: Dose-responsive microRNAs correlates with gene expression and toxicology data in a PPARα mouse model of liver tumorigenesis

Method: Use microRNA profiling after short-term exposure of liver tumorigen

tumorigenic — di(2-ethylhexyl) phthalate (DEHP)

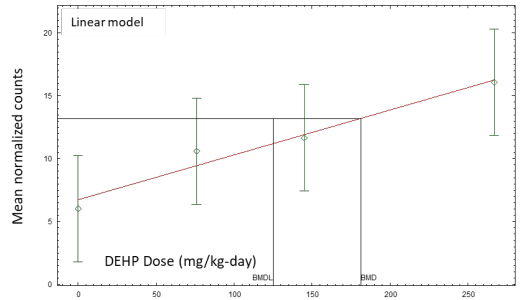
non-tumorigenic — di-n-octyl phthalate (DNOP)
n-butyl benzyl phthalate (BBP)





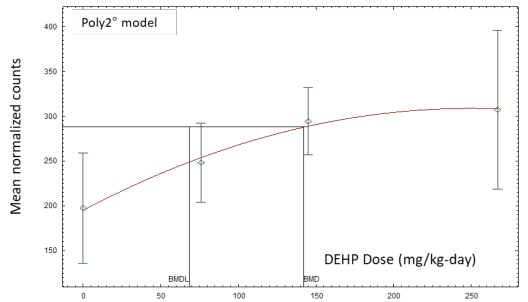
7 days (4 doses)

mmu-miR-182-5p



BMD_{miR} = 181mg/kg-day

mmu-miR-378a-3p



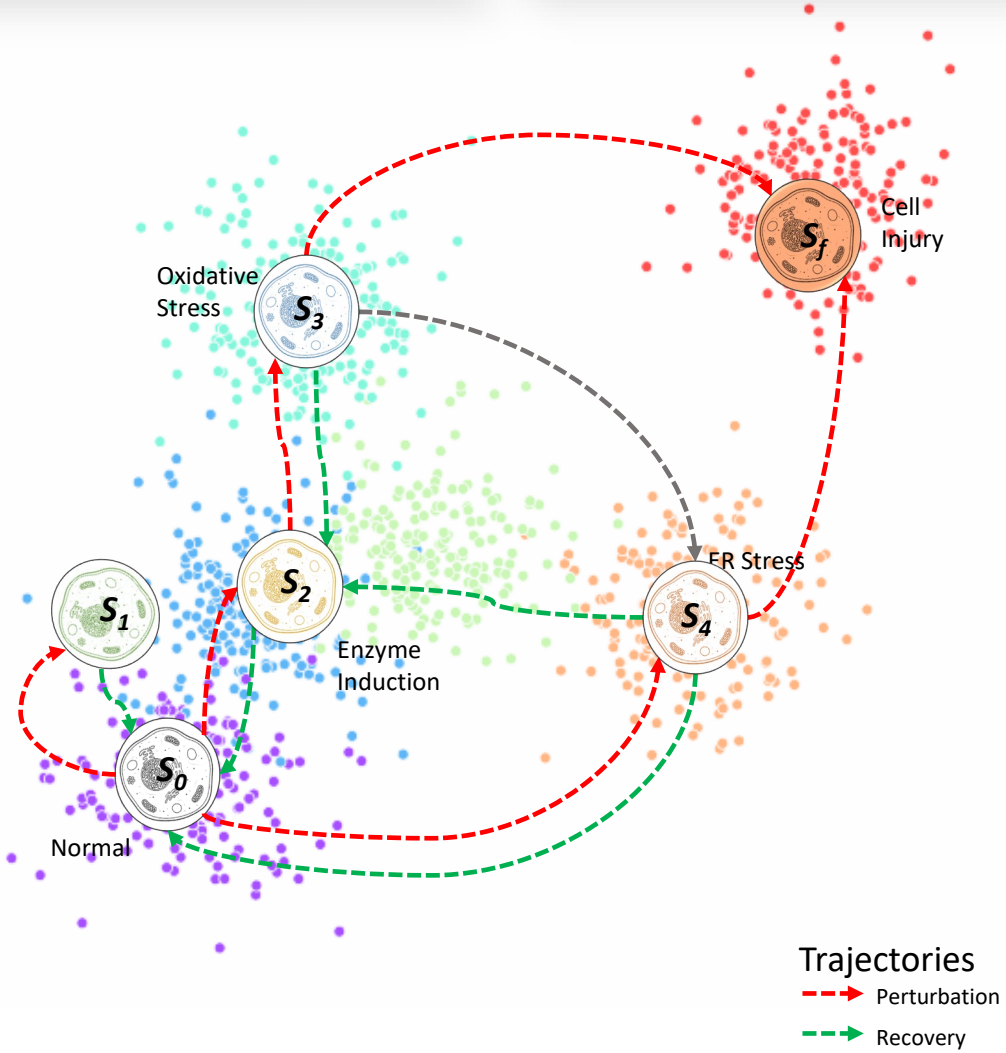
BMD_{miR} = 142mg/kg-day

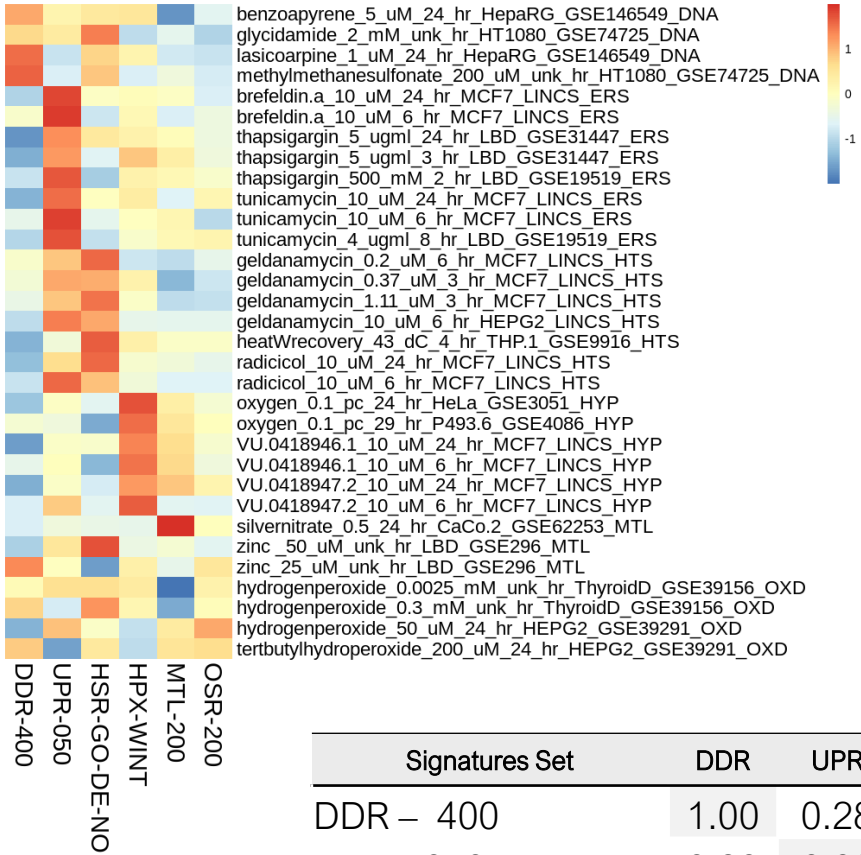
Chorley et al. *Toxicol Rep*. 2020 Jun 23;7:805-815.

Project summary: miRNA linked to MoA

- In this case study, dose-responsive miRNA are linked to the known primary mechanism of action (PPARα) for DEHP-mediated mouse HCC
- Indications these miRNAs may be leaked/transferred into circulation
- *Can these miRNA patterns enhance our chemical screening efforts?*

Transcriptomic signatures *in vitro* to identify cellular stress response





Signatures Set	DDR	UPR	HSR	HPX	MTL	OSR
DDR – 400	1.00	0.28	0.47	0.27	0.60	0.68
UPR – 050	0.23	0.89	0.70	0.38	0.21	0.16
HSR – GO_DE_NO	0.72	0.34	0.97	0.17	0.34	0.50
HPX – WINT	0.28	0.57	0.47	1.00	0.20	0.20
MTL – 200	0.12	0.63	0.33	0.85	0.66	0.28
OSR – 200	0.10	0.50	0.40	0.58	0.57	0.88

Shah I, et al. Environ Health Perspect. 2016 Jul;124(7):910-9. doi: 10.1289/ehp.1409029.

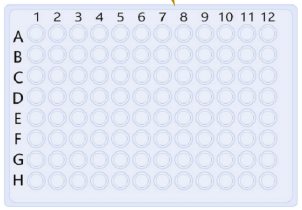
Slide courtesy of Imran Shah, Bryant Chambers, US EPA

Defining extracellular microRNAs signatures

- ***Non-destructive measurement of extracellular microRNA to define chemical mechanism-of-action***

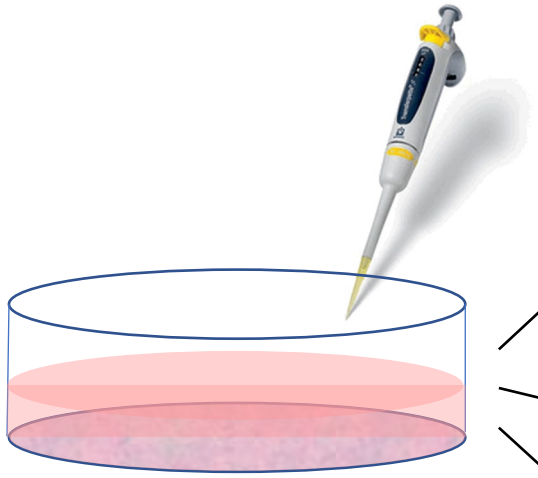
Reference chemicals for hepatotoxicant MoA

24-48 hr exposure



24 hr sample

48 hr sample



HepaRG
(human hepatocytes)

microRNA released in media

cytotoxicity and brightfield

cellular microRNA and mRNA (HTTr)

- Identify candidate miRNA measured in HepaRG media
- Distinguish active versus passive release of miRNA into media (toxicity vs. cellular response)
- Link to gene expression networks and link cellular microRNA
- Establish extracellular microRNA patterns linked to chemical MoA

Exposure | **Sampling** | **Measurements** | **Aims**

Defining microRNAs signatures of MoA

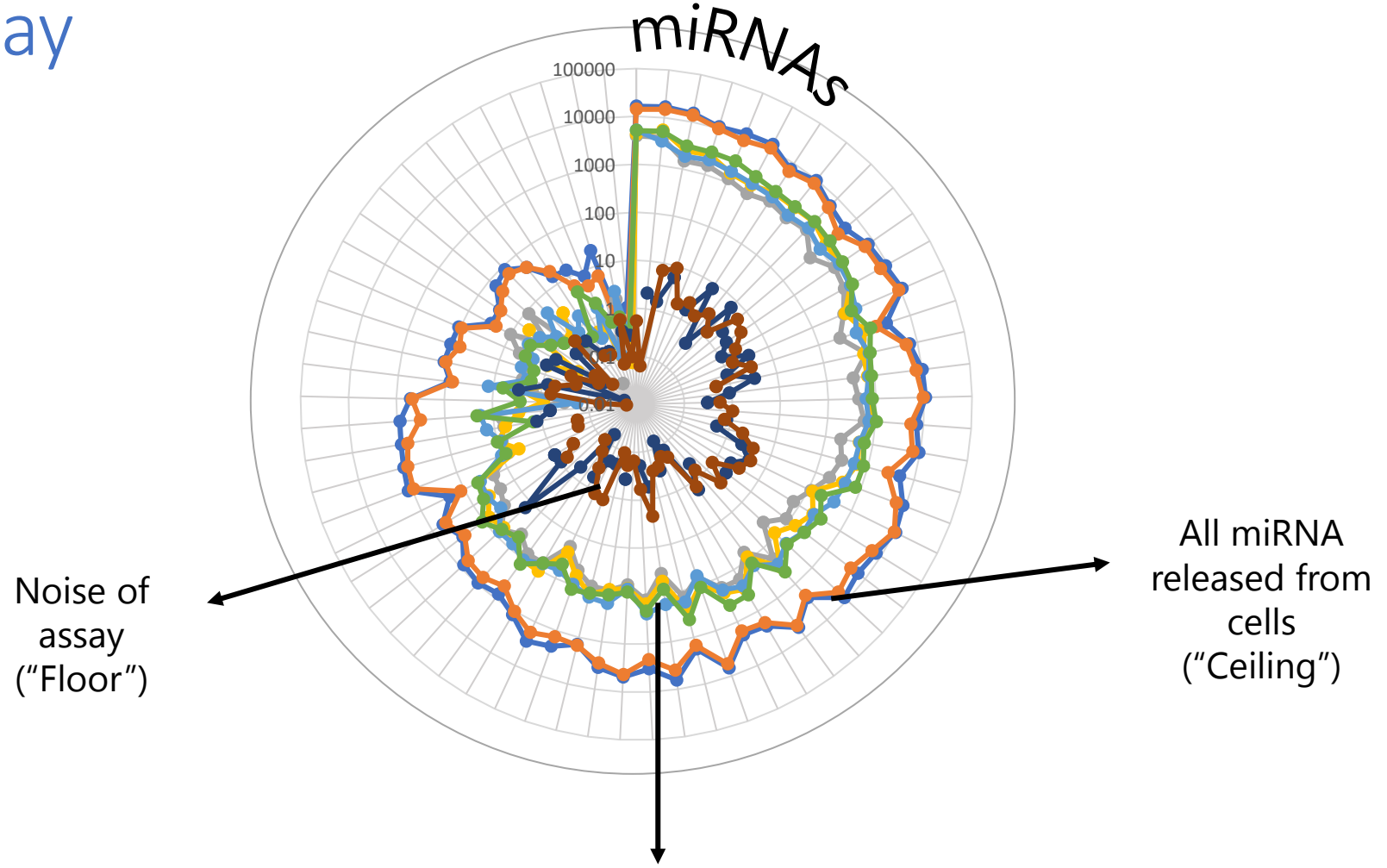
Phase I Chemicals

Benzo[a]pyrene – 10, 1, 0.25 uM	Aryl hydrocarbon receptor (AHR) agonist
Pirinixic acid (WY-14643) – 30, 3, 0.3 uM	Peroxisome proliferator-activated receptor (PPAR) α agonist
Menadione – 30, 15, 7.5 uM	Aldehyde oxidase-1 (AOX1) agonist
Ketoconazole – 10, 1, 0.1 uM	Cytochrome P450 3A4 (CYP3A4) antagonist
Retinoic acid – 10, 1, 0.1 uM	Retinoic acid receptor alpha (RAR-α) agonist
Chenodeoxycholic acid – 200, 100, 50 uM	Farnesoid X receptor (FXR) agonist
Trichostatin A – 3, 0.3, 0.03 uM	Histone deacetylase inhibitors (HDACi)
Rifampicin – 100, 50, 25 uM	Pregnane X receptor (PXR) agonist
Troglitazone – 100, 50, 5 uM	Peroxisome proliferator-activated receptor (PPAR) γ agonist
Atorvastatin – 10, 1, 0.1 uM	3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) inhibitor

Small RNA sequencing: candidate miRNA identification

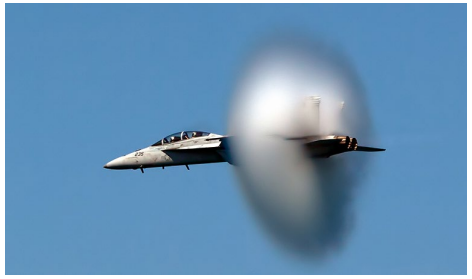
- 181 total miRNAs measured in media in small RNA-seq results
- 65 chosen for focused miRNA panel
- Candidates measured at 24h and 48h post exposure

Targeted miRNA data: The ceiling and the floor of the assay

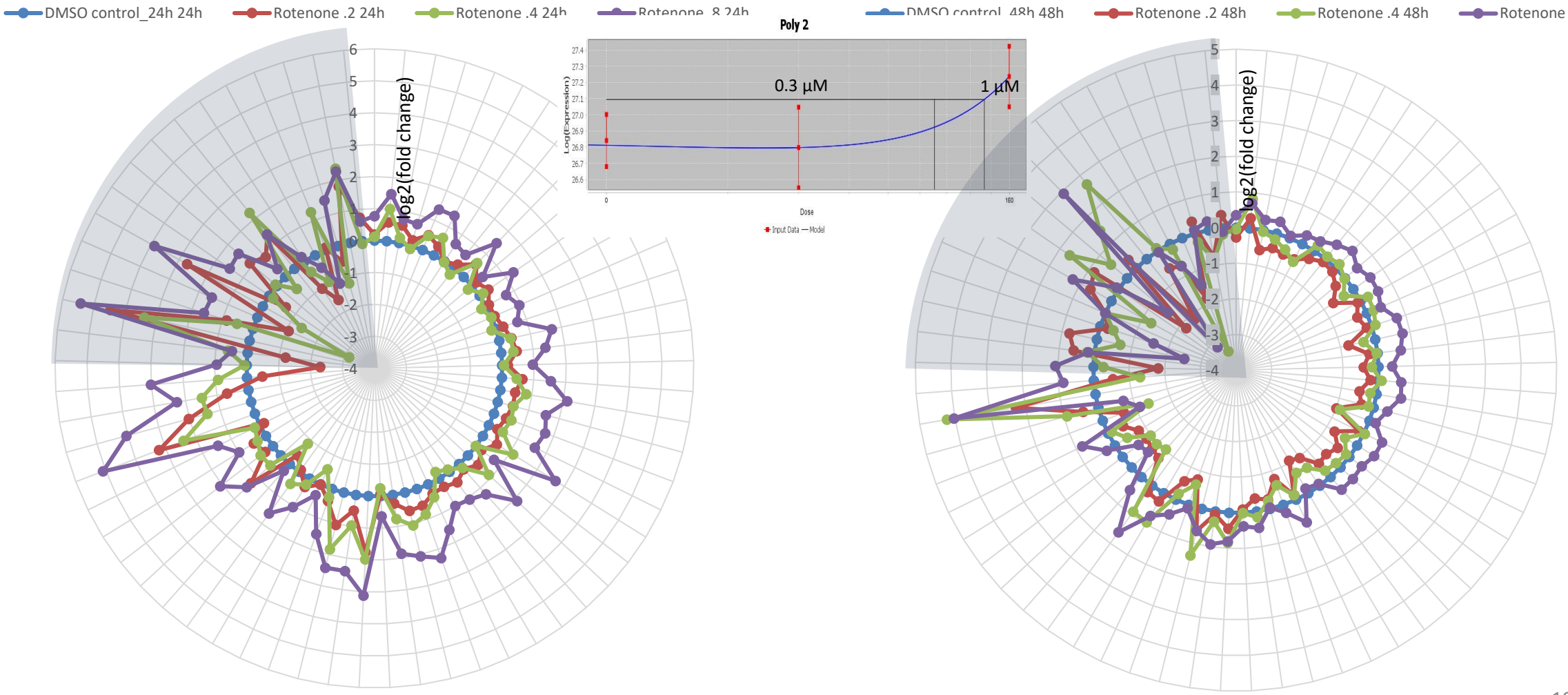


Unpublished results, please do not cite

Targeted miRNA data
Rotenone controls; “shockwave” toxicity indicator



24h

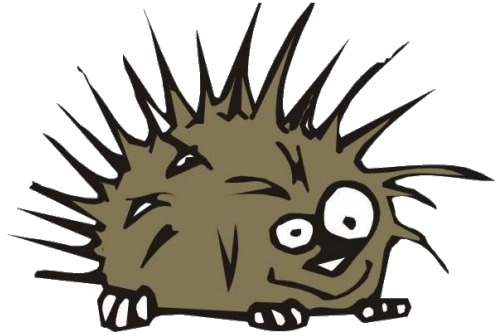


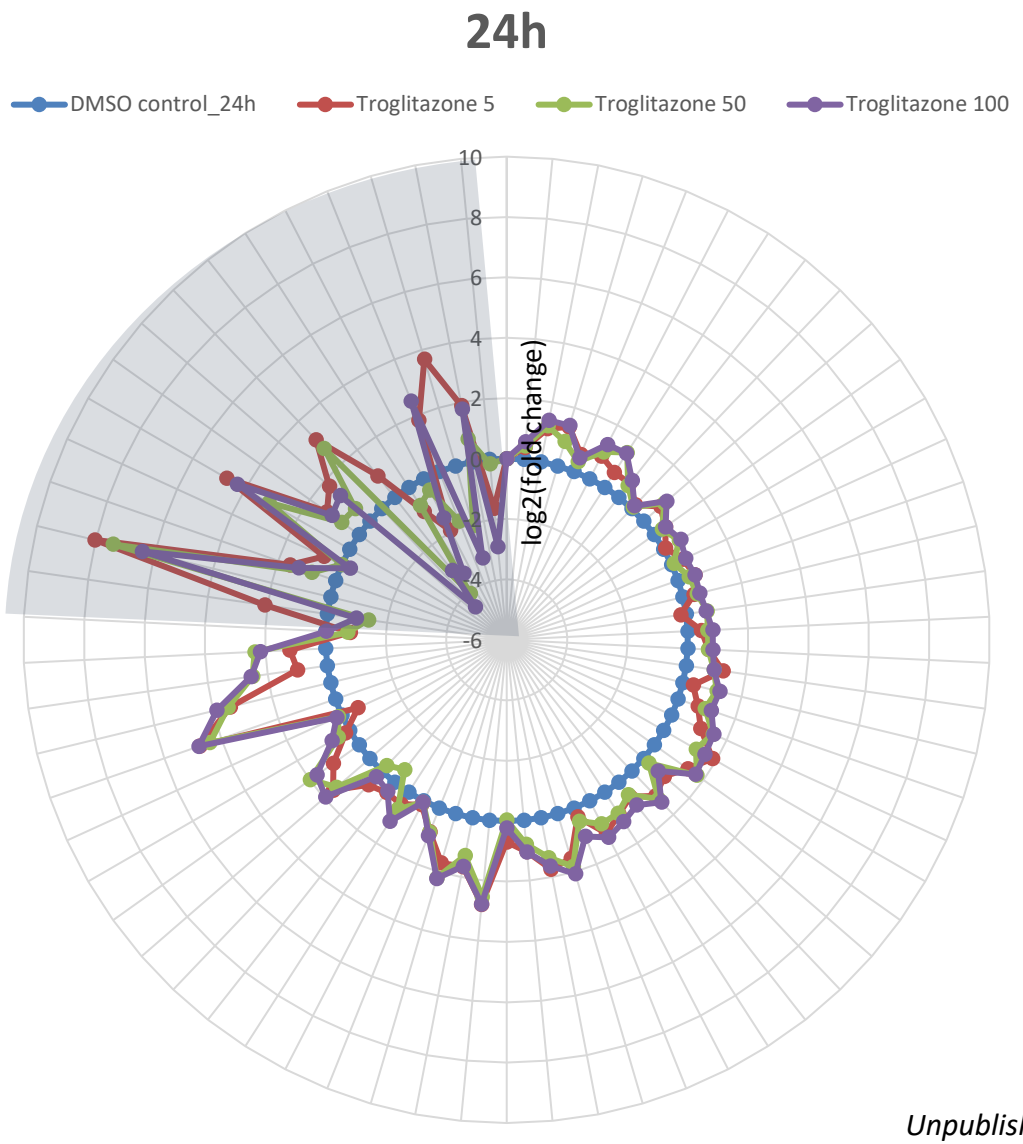
48h

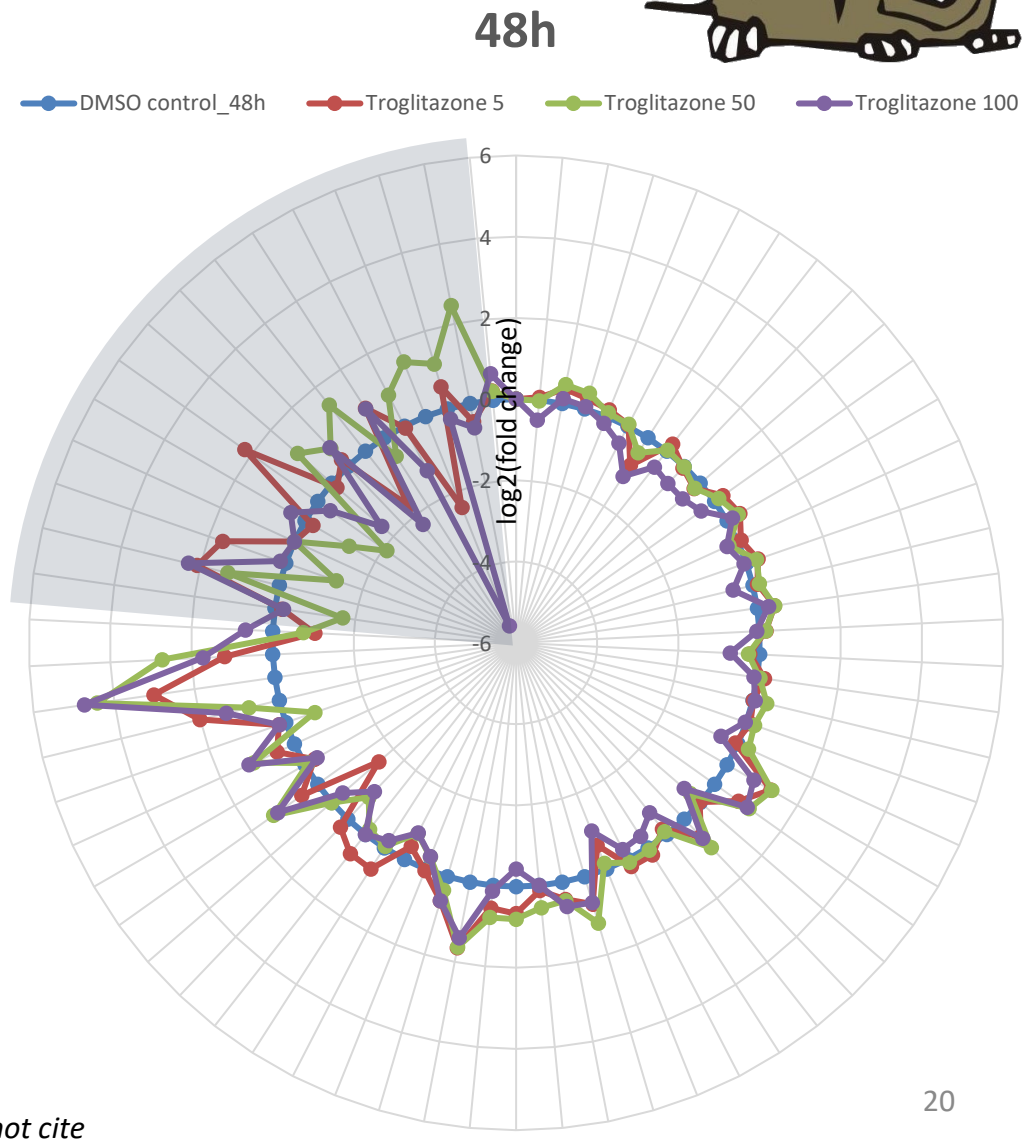
Unpublished results, please do not cite

Phase I Fireplex data

“Porcupines”; Potential signatures of MoA







Unpublished results, please do not cite

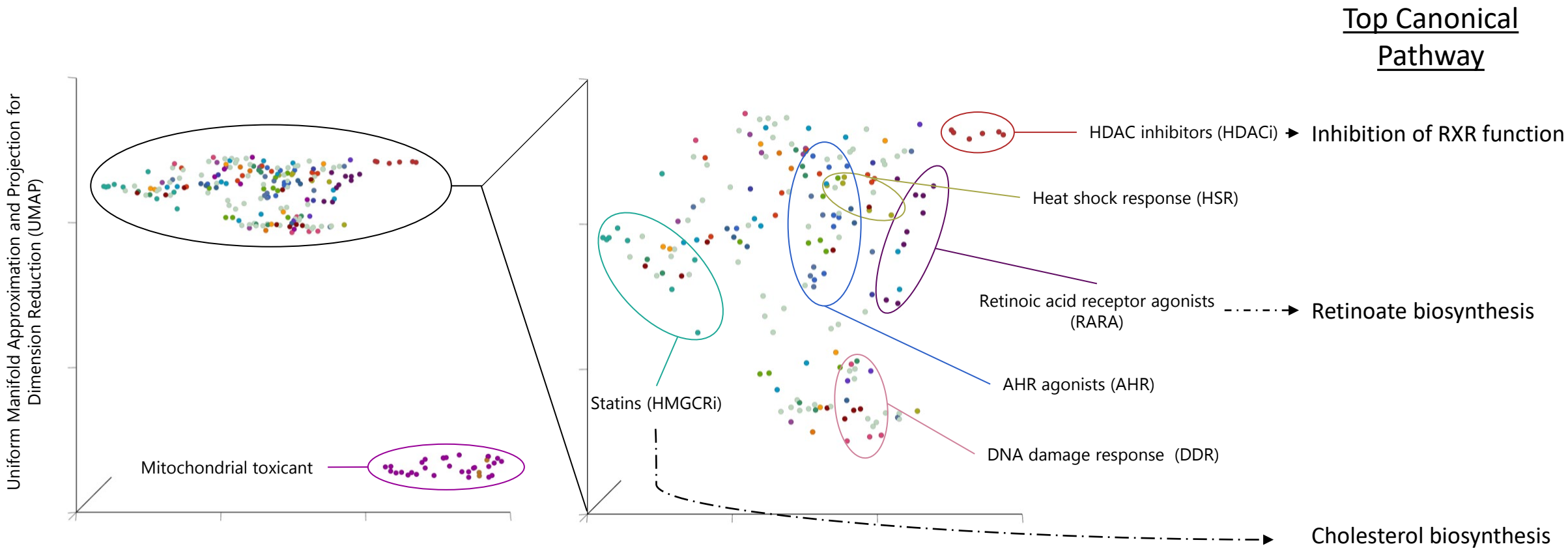
Can we replicate signatures? Adding more chemicals and doses

Omeprazole	Aryl hydrocarbon receptor (AHR) agonist	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
3,3'-diindolylmethane	Aryl hydrocarbon receptor (AHR) agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Isovanillin	Aldehyde oxidase-1 (AOX1) agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Hydralazine	Aldehyde oxidase-1 (AOX1) agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Amiodarone	Cytochrome P450 3A4 (CYP3A4) antagonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Itraconazole	Cytochrome P450 3A4 (CYP3A4) antagonist	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
GW4064	Farnesoid X receptor (FXR) agonist	10, 3, 1, 0.3, 0.1, 0.03, 0.01 uM
Obeticholic acid	Farnesoid X receptor (FXR) agonist	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
Suberohydroxamic acid	Histone deacetylase inhibitors (HDACi)	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
Vorinostat	Histone deacetylase inhibitors (HDACi)	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
Lovastatin	3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) inhibitor	10, 3, 1, 0.3, 0.1, 0.03, 0.01 uM
Simvastatin	3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) inhibitor	10, 3, 1, 0.3, 0.1, 0.03, 0.01 uM
Acetaminophen	Peroxisome proliferator-activated receptor (PPAR) α agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
MEHP	Peroxisome proliferator-activated receptor (PPAR) α agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Rosiglitazone	Peroxisome proliferator-activated receptor (PPAR) γ agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
Pioglitazone	Peroxisome proliferator-activated receptor (PPAR) γ agonist	100, 30, 10, 3, 1, 0.3, 0.1 uM
AM580	Retinoic acid receptor alpha (RAR-α) agonist	10, 3, 1, 0.3, 0.1, 0.03, 0.01 uM
Arotinoid acid	Retinoic acid receptor alpha (RAR-α) agonist	10, 3, 1, 0.3, 0.1, 0.03, 0.01 uM
Tunicamycin	Unfolded protein response (UPR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
Brefeldin A	Unfolded protein response (UPR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
Pyridaben	Unfolded protein response (UPR)/Hypoxia (HPX) response	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
1,10-Phenanthroline	Hypoxia (HPX) response	100, 30, 10, 3, 1, 0.3, 0.1 uM
Quercetin	Hypoxia (HPX) response	100, 30, 10, 3, 1, 0.3, 0.1 uM
Chlorothalonil	Heat shock response (HSR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
Cadmium Chloride	Heat shock response (HSR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
Piperine	Oxidative stress response (OSR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
Tert-butylhydroquinone	Oxidative stress response (OSR)	100, 30, 10, 3, 1, 0.3, 0.1 uM
1,4-Naphthoquinone	Oxidative stress response (OSR) /Hypoxia (HPX) response	100, 30, 10, 3, 1, 0.3, 0.1 uM
Etoposide	DNA damage response (DDR)	30, 10, 3, 1, 0.3, 0.1, 0.03 uM
5-Fluorouracil	DNA damage response (DDR)	100, 30, 10, 3, 1, 0.3, 0.1 uM

Chemical mechanism-of-action

Cellular stress response

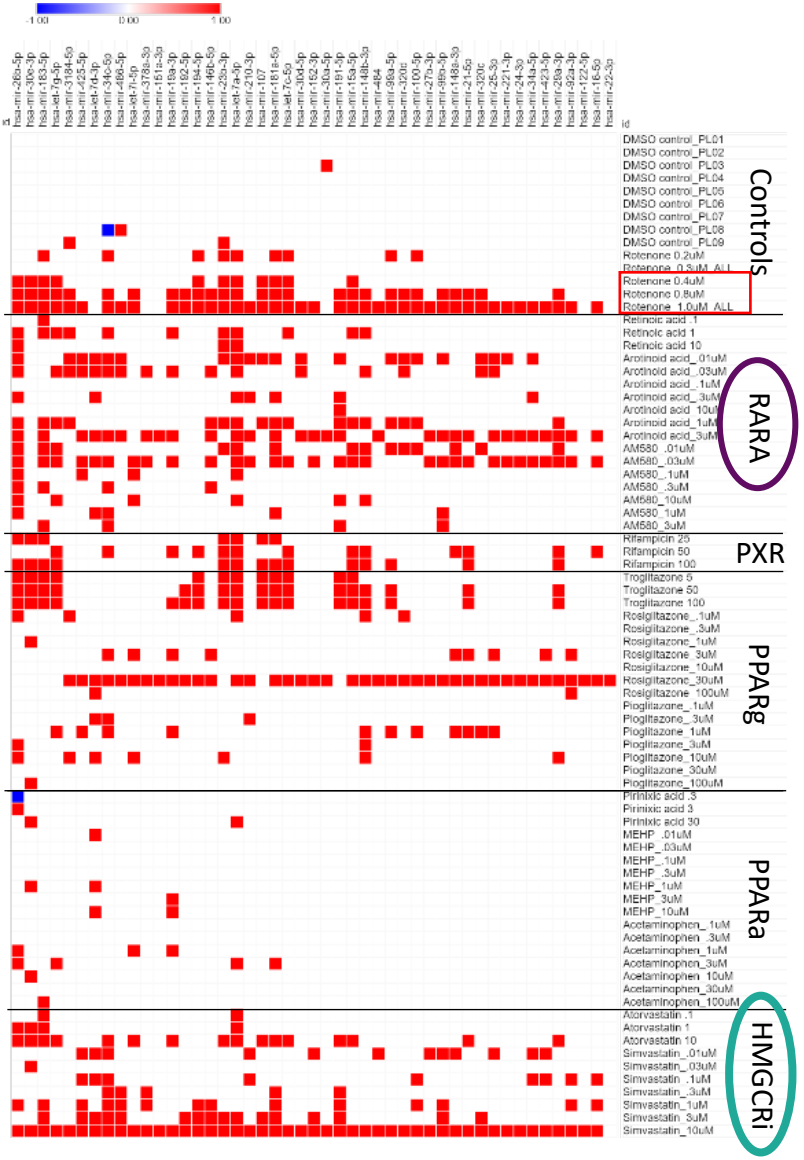
Intracellular gene expression indicated chemical group MoA

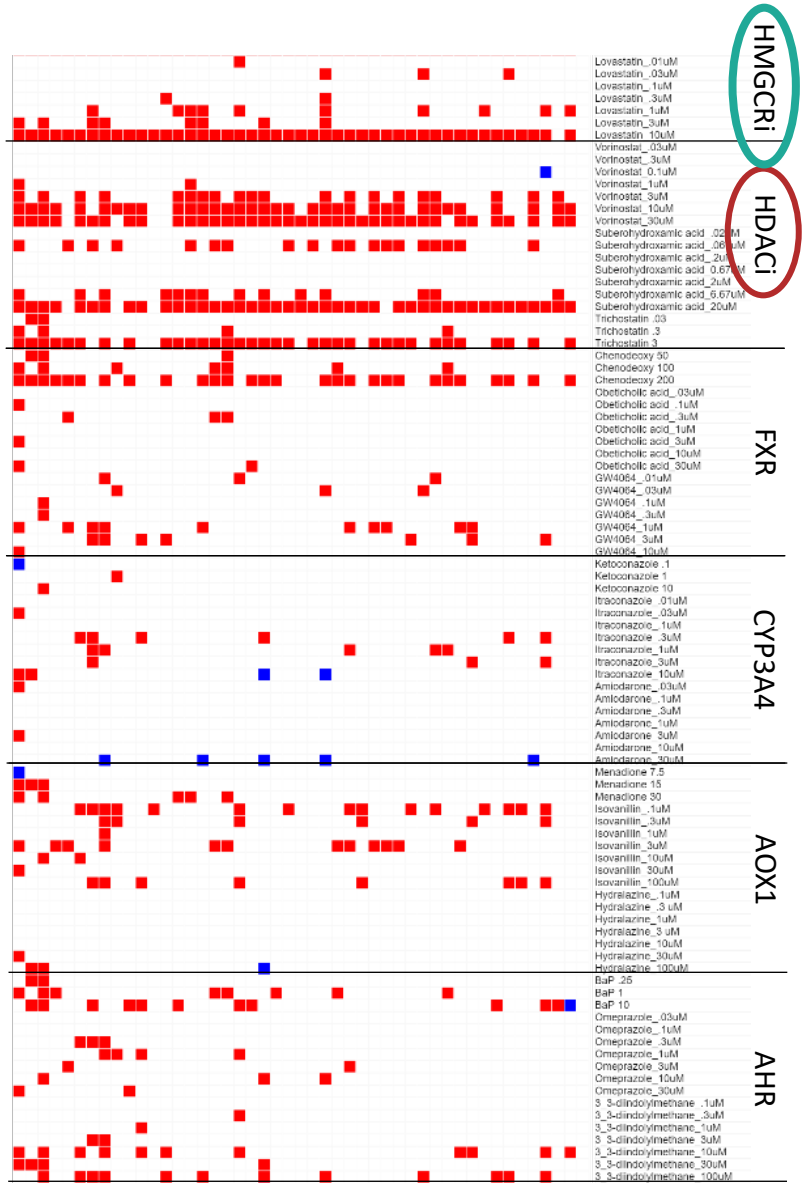


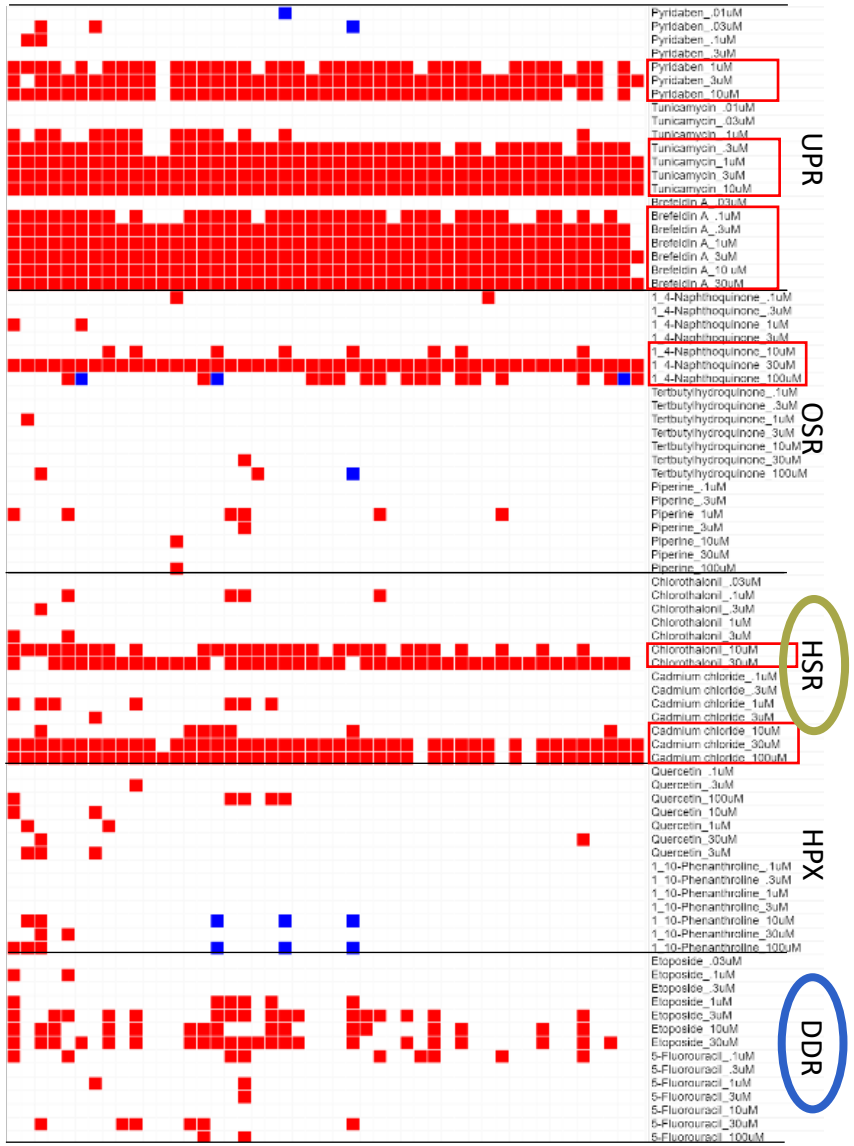
Extracellular miRNA measures: over/under baseline

under 3XBMAD

over 3XBMAD





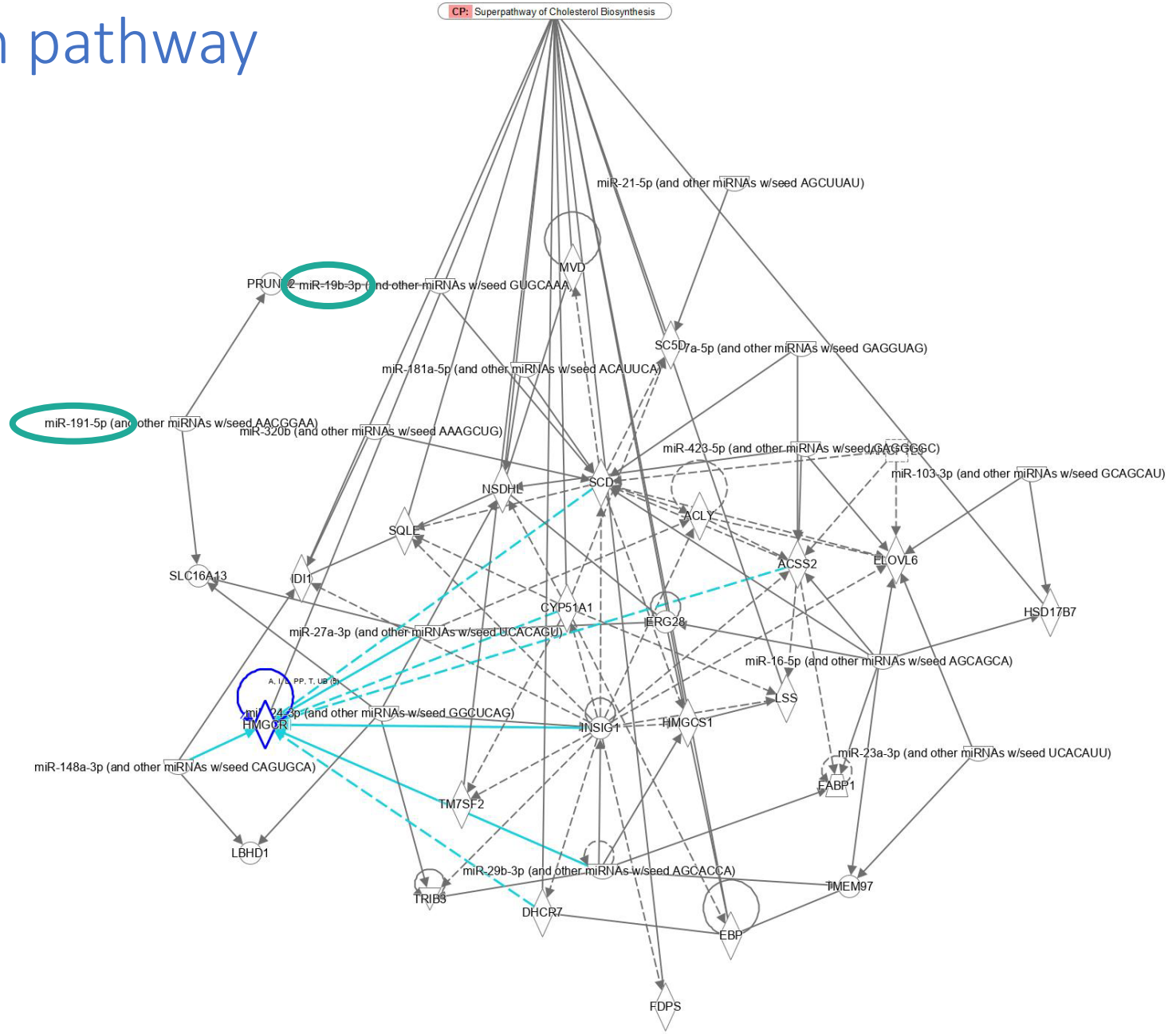


Unpublished results, please do not cite

Extracellular miRNAs above threshold: Statins (HMGCR inhibitors)

Unpublished results, please do not cite

Statin pathway



Summary: HepaRG media study

- Established extracellular microRNA patterns linked to chemical mechanism-of-action
  - Cellular toxicity due to chemical exposure is correlating highly with the “shockwave” toxicity pattern
  - However, some signatures seen with non-toxic doses. Does this link to a specific MoA? Does it link with more apical cellular effect?

- Will link to gene expression networks and cellular microRNA alterations
  - Message and small RNA sequencing are being performed for cell lysates
  - We will leverage this data and in silico prediction algorithms to identify correlations between miRNAs and gene expression regulation (node identification)

- Distinguish active versus passive release of miRNA into media (cellular response vs toxicity)

Conclusions

- **Overall, the evidence in these studies suggest microRNAs may serve as useful biomarkers for chemical screening and hazard identification in multiple toxicological contexts**

- In human populations, miRNAs in blood correlated with disease markers and exposure

- In short-term mouse studies of exposure, miRNAs linked to primary mechanism-of-action dose-dependently responded

- *In vitro*, non-destructive measurements of miRNA in media are indicative of mechanism-of-action

- Future studies will strengthen mechanistic relationship of miRNA alteration and cellular response

Acknowledgements

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Abcam

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Mike Tackett

University of Louisville

Matt Cave

Christina Pinkston

Shesh Rai

Kimberly Head

CDC/ATSDR

Marian Pavuk

NIEHS

Douglas Bell

Linda Birnbaum

Human liver disease cohort study

US EPA

CCTE, BCTD

Gail Nelson

Gleta Carswell

Charles Wood

(now at Boehringer Ingelheim)

CPHEA

Hongzu Ren (retired)

Beena Vallanat

Anna Fisher

NSF International

Virunya S. Bhat

Short-term in vivo biomarkers study

Extracellular miRNA and MoA

US EPA

Gail Nelson

Nyssa Tucker

Josh Harrill

Bryanna Vacca (UNC rotation)

Abcam

Mike Tackett

miRNA biomarkers of Nephrotoxicity

HESI Emerging Systems Toxicology for the Assessment of Risk (eSTAR)

Leadership

Jean-Charles Gautier (Sanofi; *retired*)

Ernie Harpur (Newcastle University)

HESI Staff: Connie Chen

Participant Affiliations

Abcam

Astellas

Bayer

Bristol-Myers Squibb

US Environmental Protection Agency

Harvard University

Janssen

National Institute of Environmental Health Sciences

Newcastle University

Pfizer

Sanofi

University of North Carolina

Thank you and any questions?

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