METABOLOMICS BASED COMPARISON OF HUMAN EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS TO PREDICT DEVELOPMENTAL TOXICITY

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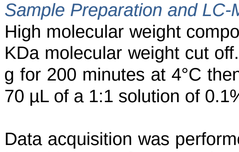
Introduction

Induced pluripotent stem (iPS) cells are derived from the genetic manipulation of somatic cells. These cells are being investigated for use in place of hES cells due to the moral, ethical and political controversies that surround them. Human iPS cells are phenotypically and genetically similar to hES cells in many respects (i.e. morphology, proliferation, gene expression). However, the metabolic similarity between hES cells and iPS cells is not known. It is vital to understand this information when considering using iPS cells in the same manner as hES cells, such as for human toxicity testing. Using a metabolomic approach, we evaluated 227 mass features by LC/MS that may represent secreted metabolites (secretome) across three hES cell lines and two iPS cell lines. Our data shows minimal differences exist in the secretome between cell types. Additionally, we exposed both cell types to a training set of pharmaceuticals with known teratogenicity (Table 1) and identified a cell line specific metabolic signature capable of predicting teratogenicity as assessed using a blinded test set of compounds of known teratogenicity. Pathway enrichment analysis of statistically significant, differentially metabolized putative compounds identified pathways that were disrupted in multiple teratogen treatments.

Figure 1. 96 Well Dosing Experimental Design



Symbol	Samples	Sample Name
	Media Controls	ST003H-90-057-1B-G
	Controls	ST003H-90-057-2B-G
	Carbamazepine Dosed Media Control	ST003H-90-057-3A/3H
	Carbamazepine ([70.9 ug/mL])	ST003H-90-057-3B-G
	Carbamazepine Dosed Media Control	ST003H-90-057-4A/H
	Carbamazepine ([7.09 ug/mL])	ST003H-90-057-4B-G
	Carbamazepine Dosed Media Control	ST003H-90-057-5A/H
	Carbamazepine ([0.709 ug/mL])	ST003H-90-057-5B-G
	Thalidomide Dosed Media Control	ST003H-90-057-6A/H
	Thalidomide ([0.62 ug/mL])	ST003H-90-057-6B-G
	Thalidomide Dosed Media Control	ST003H-90-057-7A/H
	Thalidomide ([0.062 ug/mL])	ST003H-90-057-7B-G
	Thalidomide Dosed Media Control	ST003H-90-057-8A/H
	Thalidomide ([0.0062 ug/mL])	ST003H-90-057-8B-G
	Cyclophosphamide Dosed Media Control	ST003H-90-057-9A/H
	Cyclophosphamide ([1540 ug/mL])	ST003H-90-057-9B-G
	Cyclophosphamide Dosed Media Control	ST003H-90-057-10A/H
	Cyclophosphamide ([154 ug/mL])	ST003H-90-057-10B-G
	Cyclophosphamide Dosed Media Control	ST003H-90-057-11A/H
	Cyclophosphamide ([15.4 ug/mL])	ST003H-90-057-11B-G

- 3 compounds per plate.
- 3 levels:
  - Circulating dose (CD)
  - 10 x CD
  - 0.1 x CD.
- 6 replicates per dose.
- Each plate contains control cells, dosed cells, media and dosed media controls.

Methods

Cell Dosing and Sample Collection

Three hES cell lines (WA01, WA07 and WA09) and two iPS cell lines (19-9-7T and 4-3-7T-A) were cultured in 6-well plates on Matrigel (BD Biosciences) in 2.5 mL/well mTeSR1 media (Stem Cell Technologies). Media was changed daily and cells were passaged using 2 mg/mL Dispase (Invitrogen). For dosing, cells were trypsinized (0.05% Trypsin, Invitrogen) and plated onto Matrigel-coated 96-well plates at a density of 250,000 cells per well in mTeSR1 supplemented with 10 µM Y027632 Rho-associated Coil Kinase (ROCK) inhibitor (Calbiochem) (Figure 1). One day following plating, the media was removed and dosing began. The media was replaced with mTeSR1 (200 µL/well) containing drug. Cells were treated for three days with known teratogens and non-teratogens (Table1). Media and compound were replaced daily. After 72 hours of compound exposure, the spent media was collected, quenched with 40% acetonitrile, and stored at -80°C until LC-MS analysis was performed.

Non-Teratogens	Doxylamine	**Teratogens**	Accutane
	Levothyroxine		Carbamazepine
	Penicillin G		Retinoic Acid
	Caffeine		Thalidomide
	Retinol		Valproate
	Diphenyhydramine		Methotrexate
	Ascorbate		Cytosine Arabinoside
	Isoniazid		5-Fluorouracil
	Saccharin		Busulfan
	Folic Acid		Hydoxyurea
	Thiamine		

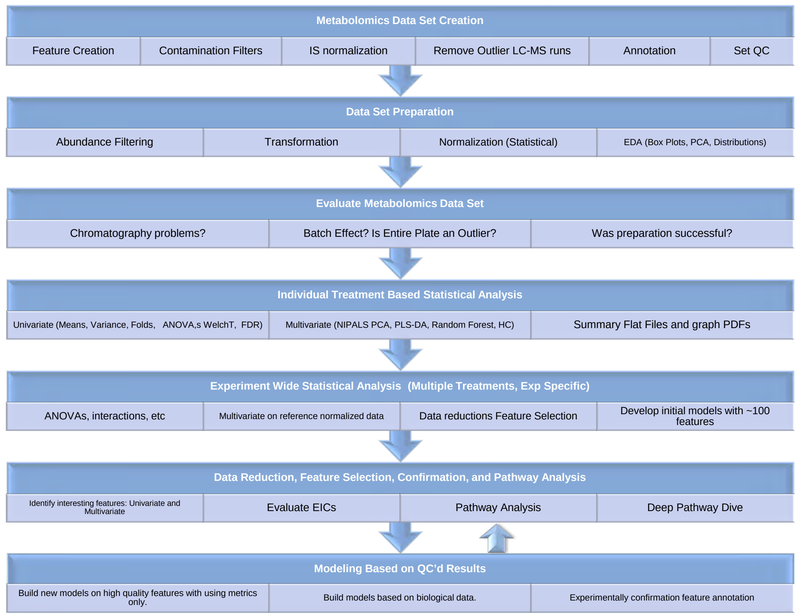
Table 1. Training Set.

Sample Preparation and LC-MS Method

High molecular weight compounds were removed using the Millipore Ultracel 96-well filter with a 10 KDa molecular weight cut off. Spent media samples were added to the plate, centrifuged at 2000 x g for 200 minutes at 4°C then dried overnight in a Savant SpeedVac. Samples were redissolved in 70 µL of a 1:1 solution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

Data acquisition was performed on an Agilent G6520A or G6530A QTOF mass spectrometer using MassHunter software under high-resolution exact mass conditions with a HILIC LC-MS method. This method was developed and optimized to provide a good compromise for the separation of both hydrophilic and hydrophobic compounds.

Metabolomic Data Analysis and Modeling



Results

A random forest model capable of classifying teratogenicity was trained using data from both lines 4.3.7TA and 19.9.7T. The features included in the final model were chosen by successively eliminating the least important variables and identifying features that consistently included chosen after 50 bootstrap iterations of the variable selection process.

Treatment	Line_4.3.7T.A	Line_19.9.7T
AscorbicAcid	0.67	0.80
Caffeine	0.80	0.67
Diphenhydramine	0.83	0.58
Doxylamine	0.53	0.74
FolicAcid	0.55	0.75
Isoniazid	0.68	0.44
Levothyroxine	0.72	0.83
PenicillinG	0.73	0.75
Retinol	0.79	0.62
Saccharin	0.66	0.64
Thiamine	0.82	0.77
5FU	0.83	0.93
Accutane	0.95	0.94
Busulfan	0.87	0.95
Carbamazepine	0.89	0.95
CytosineArabinoside	0.33	0.70
Hydroxyurea	0.59	0.54
Methotrexate	-0.27	0.26
RetinoicAcid	0.37	0.70
Thalidomide	0.98	0.70
VPA	0.58	0.40

Table 2 . Class probability prediction with IPS-devTOX96-well model. The difference in class predicted probability for the right class and the wrong class of *the training set* of compounds. High numbers suggest that the model was able to predict the right class of all the drugs for both IPS cell lines. Methotrexate in IPS cell line 4.3.7T.A was the only drug that was predicted incorrectly in this model.

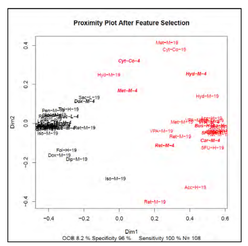
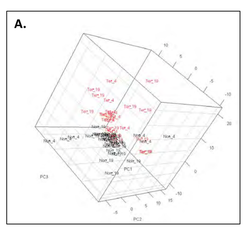


Figure 2. Multidimensional scaling plot of the random forest proximity matrix (similarity metric) from Stemina's IPS-devTOX 96-well IPS cell based developmental toxicity classification model. A clear separation of the toxicity classes is observed in both cell lines of IPS. Each drug treatment was replicated 2 to 6 times. Red=Teratogen, Black=Non-Teratogen, Points=Average drug treatment across replicates for a dose level for a cell line.



B.

Table 3. Confusion matrix

	Non	Ter	class.error
Non	32	1	0.03
Ter	4	24	0.14

Number of trees: 1000
No. of variables tried at each split: 10
OOB estimate of error rate: 8.2%

Figure 3. Statistical modeling to predict teratogenicity. A. PCA scores plot of the training set used for modeling colored by compound classification (**Red**: Teratogen **Black**: Non-Teratogen). B. Prediction of the training set on itself.

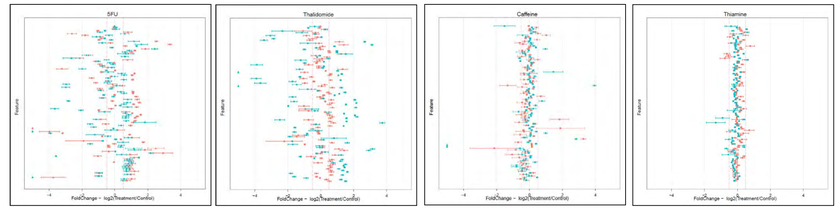


Figure4. Ratio Plots. Ratio plots for the 100 features selected by IPS-devTOX 96-well model. The toxic drugs impact the metabolites (features) more than the non toxic drugs. The response in IPS cell lines was very similar.(Blue – IPS cell line 19.9.7T, Red – IPS cell line 4.3.7T.A)

Figure 5. Comparison of the hES and iPS cell secretomes. 4 features were unique to hESC and 3 features were unique to iPSC. These features are unknown compounds with no putative annotation.

Pathway Description	hESC	iPSC
Alanine, aspartate and glutamate metabolism	◊	◊
Arginine and proline metabolism	◊	◊
Ascorbate and aldarate metabolism	◊	
Butanoate metabolism	◊	◊
Citrate cycle (TCA cycle)	◊	◊
D-Arginine and D-ornithine metabolism		◊
D-Glutamine and D-glutamate metabolism	◊	◊
Glutathione metabolism	◊	
Glyoxylate and dicarboxylate metabolism	◊	
Lysine biosynthesis	◊	
Lysine degradation	◊	◊
Pantothenate and CoA biosynthesis		◊
Pentose phosphate pathway		◊
Purine metabolism		◊
Pyrimidine metabolism		◊
Pyruvate metabolism	◊	
Sulfur metabolism	◊	
Tyrosine metabolism	◊	◊
Valine, leucine and isoleucine biosynthesis/degradation		◊
Vitamin B6 metabolism	◊	

Table 3. Comparison of pathways that are statistically enriched in one or more cell lines for each cell type. These pathways exhibit stronger perturbations in teratogens as compared to non-teratogens.

Conclusions

- There are **minimal** differences (7 of 227 features) in the secretome of hES and iPS cells.
- The 2 iPS cell lines examined in this study respond similarly to treatment.
  - Both lines are able to predict the right class for the compounds in the training set, except for methotrexate in line 4.3.7T.A
  - Features selected for model predictions show similar fold changes in both cell lines.
- iPS cells can be used as a model to predict teratogenicity of pharmaceutical compounds.
- ***Future Studies:***
  - Further elucidate the metabolic signature of teratogenicity by validation of significant small molecules.
  - Expand the training set to included more diverse sources of known teratogens such as environmental toxicants and biologics
  - Evaluate the predictive capacity of the model using a test set of blinded treatments from a cross section of known teratogens
  - Compare the metabolic signature of teratogenicity between iPS and hES cell lines and identify the common metabolites

Acknowledgements

We gratefully acknowledge the National Science Foundation (NSF SBIR Phase I Award IIP-0945105) for funding hES cell study and our collaborators at Agilent Technologies for providing technical assistance, software and instrumentation.