Capturing complex biology is critical for accurate *in vitro* prediction of developmental toxicity

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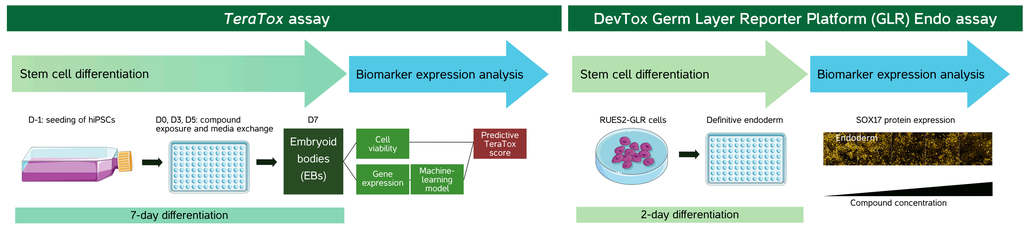
Introduction

- Exposure to teratogenic compounds during pregnancy can lead to significant birth defects. Given the considerable variation in drug responses across species, along with the financial and ethical challenges associated with animal testing, the development of advanced human cell-based assays is imperative for effectively identifying potential human teratogens.
- Over the past decades, several assays were developed that use directed differentiation of human induced pluripotent or embryonic stem cells (hiPSCs/hESCs) to assess chemical exposure effects on expression of early germ-layer genes as a surrogate for teratogenicity assessment. These assays (e.g. *TeraTox* and the DevTox Germ Layer Reporter Platform (DevTox GLR)) are generally fast (2-7 days) and can be applied for high-throughput screening. Despite their good reliability and applicability in early developmental toxicity screening, these assays have limited predictivity for detecting embryotoxic compounds (60-75%).
- ReproTracker follows the differentiation of hiPSCs into three germ layer cell lineages, ultimately leading to the development of specific cell lineages. This assay aims to capture the complex biology of early embryonic development.
- Here, we evaluated three hiPSC-based developmental toxicity assays for their ability to predict the teratogenic properties of compounds.

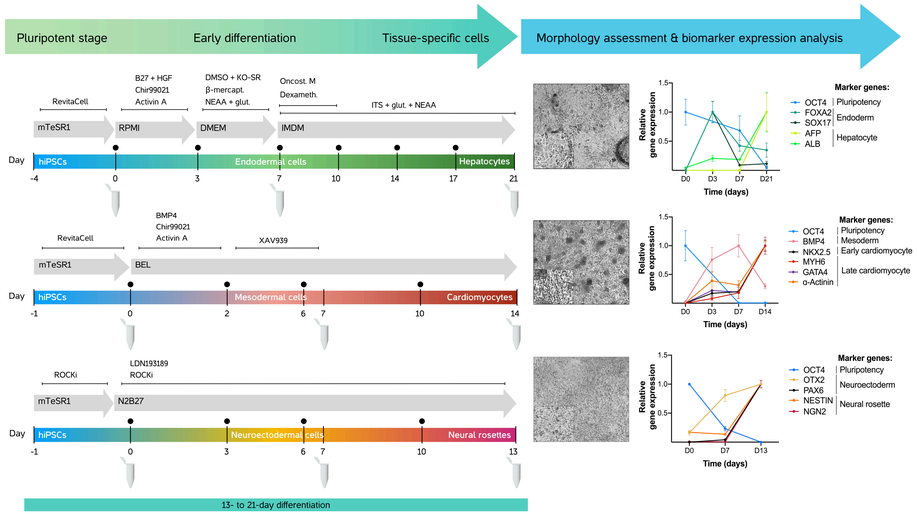
Overview of *in vitro* assays



Differentiation of hiPSCs towards embryoid bodies (EBs) over a 7-day course within the *TeraTox* assay. Following differentiation, a TeraTox score for predicting teratogenicity is calculated based on cell viability, gene expression data, and machine-learning model. Here, the expression of 87 early biomarkers is used for compound classification.

Differentiation of the transgenic RUES2-GLR cell line within the DevTox GLR Endo assay. Human iPSCs, expressing fluorescent reporter fusion protein biomarkers for SOX17 (endoderm marker), are differentiated towards definitive endoderm, after which protein expression is evaluated. The percentage of SOX17-positive cells is indicative for teratogenicity in the assay.

ReproTracker assay

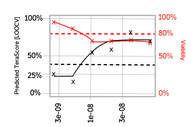


Trilineage differentiation of hiPSCs towards specialized cell types within the ReproTracker assay. Pluripotent stem cells are differentiated through mesoderm, endoderm, and ectoderm, into cardiomyocytes, hepatocytes and neural rosettes, respectively. Morphological profiling and assessment of time-dependent expression patterns of cell-specific biomarkers following compound exposure provides a measure of teratogenicity.

Developmental toxicity prediction for methotrexate

TeraTox assay	DevTox GLR Endo assay	ReproTracker assay	*In vivo*
Non-teratogen	Non-teratogen	Teratogen	Teratogen

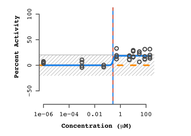
A)



Note: figure adapted from Jaklin et al. (2022)[1]

***TeraTox* assay results.** Exposure to methotrexate resulted in a positive teratogenicity response coinciding with a decrease in cell viability. With a *TeraTox* score of -0.23, methotrexate was misclassified as a non-teratogen in the *TeraTox* assay.

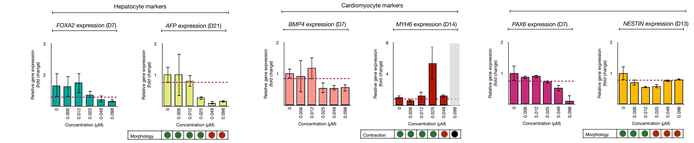
B)



Note: figure adapted from Gamble et al. (2022)[2]

DevTox GLR Endo assay results. Methotrexate exposure resulted in a minor increase in percentage activity relative to control for SOX17-positive cells in the DevTox GLR Endo assay. Since the observed effect did not exceed the baseline and noise band (gray striped box), methotrexate was misclassified as a non-teratogen in the assay.

C)

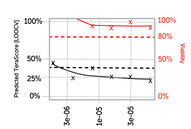


ReproTracker assay results. Methotrexate exposure during cell differentiation led to a concentration-dependent decrease in expression of multiple biomarkers, including late biomarker *AFP*, within the ReproTracker assay. Additionally, it disrupted the morphology of hepatocytes and neural rosettes and stopped the contraction ability of cardiomyocytes. Consequently, based on the assay criteria, methotrexate was correctly categorized as a teratogen in ReproTracker.

Developmental toxicity prediction for warfarin

TeraTox assay	DevTox GLR Endo assay	ReproTracker assay	*In vivo*
Non-teratogen	Teratogen	Teratogen	Teratogen

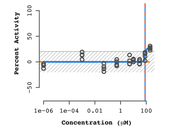
A)



Note: figure adapted from Jaklin et al. (2022)[1]

***TeraTox* assay results.** Exposure to warfarin resulted in a negative teratogenicity response coinciding with a minor decrease in cell viability. With a *TeraTox* score of 0.00, warfarin was misclassified as a non-teratogen in the *TeraTox* assay.

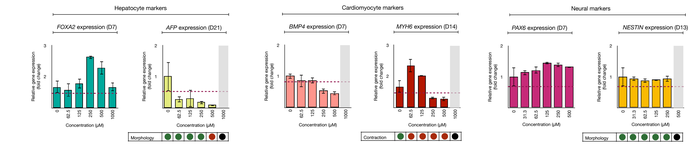
B)



Note: figure adapted from Gamble et al. (2022)[2]

DevTox GLR Endo assay results. Warfarin exposure resulted in an increase in percentage activity relative to control for SOX17-positive cells in the DevTox GLR Endo assay. Based on the obtained data, warfarin was correctly classified as a teratogen in the assay.

C)



ReproTracker assay results. Warfarin exposure during cell differentiation led to a concentration-dependent decrease in expression of cardiomyocyte-specific markers *BMP4* and *MYH6*, and hepatocyte-specific marker *AFP*. Additionally, warfarin disrupted the morphology of hepatocytes and stopped the contraction ability of cardiomyocytes. Consequently, based on the assay criteria, warfarin was correctly categorized as a teratogen in ReproTracker.

Summary of results

Teratogens

Compound	*Teratox* assay	DevTox GLR Endo assay	ReproTracker assay	*In vivo* classification
13-Cis Retinoic acid	Not tested	T	T	T
Acitretin	T	Not tested	T	T
Artesunate	T	Not tested	T	T
Boric acid	Not tested	NT	NT	T
Bosentan	T	Not tested	T	T
Busulfan	T	T	T	T
Carbamazepine	T	T	T	T
Dasatinib	T	T	T	T
Dexamethasone	T	NT	T	T
Hydroxyurea	T	NT	T	T
Imatinib	T	T	T	T
Isotretinoin	T	Not tested	T	T
Lenalidomide	Not tested	T	T	T
Methotrexate	NT	NT	T	T
Retinoic Acid	T	Not tested	T	T
Thalidomide	T	T	T	T
Valproic Acid	T	T	T	T
Warfarin	NT	T	T	T

Non-teratogens

Compound	*Teratox* assay	DevTox GLR Endo assay	ReproTracker assay	*In vivo* classification
Acrylamide	Not tested	NT	NT	NT
Amoxicillin	NT	Not tested	NT	NT
Ascorbic Acid	T	Not tested	NT	NT
Caffeine	Not tested	NT	NT	NT
Cetirizine	T	Not tested	NT	NT
D-Camphor	Not tested	NT	NT	NT
Dimethyl phthalate	Not tested	NT	NT	NT
Folic Acid	Not tested	NT	NT	NT
Metformin	NT	Not tested	NT	NT
Penicillin G	NT	NT	NT	NT
Progesterone	T	Not tested	NT	NT
Retinol	Not tested	T	T	NT
Saccharin	Not tested	NT	NT	NT
Sulfasalazine	Not tested	NT	NT	NT
1,2-Propylene glycol	Not tested	NT	NT	NT

T Teratogen
NT Non-teratogen

	Teratox assay	DevTox GLR Endo assay	ReproTracker assay
Accuracy	76%	78%	94%
Sensitivity	87%	69%	94%
Specificity	50%	90%	93%

Conclusions

- Here, we compared three human pluripotent stem cell-based *in vitro* assays of distinct assay duration for predicting developmental toxicity.
- Opposed to the DevTox GLR Endo and *TeraTox* assays, ReproTracker could correctly predict teratogenic properties of multiple compounds (e.g. methotrexate and warfarin). The latter assay demonstrated pronounced effects on tissue-specific markers, underscoring the importance of incorporating late biomarkers as assay endpoints in detecting a wider range of potential teratogens.
- The current data demonstrates that the trilineage differentiation in ReproTracker provides a broader biological coverage and could possibly enhance teratogenicity prediction.

References

1. Jaklin M, Zhang JD, Schäfer N, Clemann N, Barrow P, Küng E, Sach-Peltason L, McGinnis C, Leist M, Kustermann S. **Optimization of the *TeraTox* Assay for Preclinical Teratogenicity Assessment**. Toxicol Sci. 2022 Jun 28;188(1):17-33. doi: 10.1093/toxsci/kfac046. PMID: 35485993; PMCID: PMC9237991.
2. Gamble JT, Hopperstad K, Deisenroth C. The DevTox Germ Layer Reporter Platform: An Assay **Adaptation of the Human Pluripotent Stem Cell Test**. Toxics. 2022 Jul 13;10(7):392. doi: 10.3390/toxics10070392. PMID: 35878297; PMCID: PMC9321663.