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Current status of human pluripotent stem cell based *in vitro* toxicity tests

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1. ABSTRACT

The present review assesses the current status of *in vitro* tests based on human pluripotent stem cell-derived toxicologically relevant target cells. The majority of the evaluated test systems are in the phase of test development. In particular the success rates of differentiation protocols and their reproducibility are varying depending on different culture conditions but also on the assessed marker panel and the functional evaluation of the cells. However, the amount of differentiated cells decreases in relation to their maturation status. No harmonization has been achieved yet about the required maturation status of the cellular models to be used for toxicological applications. Even with an established cellular model, the selection of appropriate readouts is challenging. Some areas of toxicity, such as developmental toxicity, suffer from insufficient knowledge on predictive biomarkers which leads to difficulties in the selection of the most appropriate endpoints. In this heterogeneous context the rapidly increasing knowledge about 'omics' technologies, might lead to an improvement of the current situation and allow the establishment of more predictive human *in vitro* toxicity tests.

2. INTRODUCTION

The evolution of a predictive *in vitro* test based on human pluripotent stem cells (PSC) has to follow a sequence of steps until it can be considered for regulatory purposes. This includes several phases such as test development/optimization, (pre)validation and regulatory acceptance (Figure 1). Each of the different segments seeks to fulfil the criteria as required by the OECD guidance document on the "Validation and international acceptance of new or updated test methods for hazard assessment" and will – compiled as an entire dossier of the test method - form the basis for the evaluation of its validity for implementation in the regulatory decision making process (1).

The present review will discuss the current status of human pluripotent stem cell-based *in vitro* toxicity tests that have recently been published. Since none of the human stem cell based tests have entered into the prevalidation phase so far, the review will focus on test development requirements for stem cell-based *in vitro* tests which are the precondition for a test method to enter into prevalidation

Figure 1. Stepwise approach of an *in vitro* toxicity test to reach regulatory acceptance. During the research and development phase the tests seek to fulfill requirements for entering into the validation process. Most human stem cell based *in vitro* tests are currently in this phase of their evolution.

(2). In summary, this early phase of test development aims to describe the purpose as well as the mechanistic basis of the test and should deliver a sufficiently detailed protocol which might allow the transfer of the method from one laboratory to another. The selection of appropriate controls as well as an initial testing with a training set of chemicals are necessary steps to indicate the reproducibility and reliability of the proposed test (discussed in elsewhere in this issue).

When dealing with human embryonic stem cells (hESC), a careful consideration of the biological and toxicological relevance of the test and a review of existing alternatives to human embryonic stem cells is required. Generally, human health endpoints such as developmental toxicity, hepatoxicity, immunotoxiciy and cardiotoxicity (3) known for their variations between species might benefit from the development of new humanized *in vitro* tests (3). This is of special importance for other toxicological needs, where no satisfying *in vitro* model is currently available at all (e.g. male germ cell toxicity), where human pluripotent stem cells might significantly improve the present situation.

3. USE OF HUMAN PLURIPOTENT STEM CELLS IN TOXICITY TESTING

3.1. Cardiotoxicity testing

The development of cellular models relevant for the assessment of the cardiotoxic potential of a substance has recently demonstrated some progress due to the stabilization of the differentiation protocols of various hESC lines as well as of hiPSCs (human induced pluripotent stem cells) (4-6). Currently, the maximum reported differentiation rates towards the toxicologically relevant ventricular cells are of 20-30% (6-7). Emerging 3D culture conditions might improve the differentiation efficiency into nodal, atrial and ventricular cell types. New strategies leading to an upscaling of the cell types of particular interest, e.g. via the establishment of cell lines of precursor cells, might be another way forward to overcome the current shortcomings of a specific cell type.

The selection of an appropriate marker panel allowing the monitoring of the maturation status of cardiomyocytes is of high relevance since the degree of maturation of the derived cells might impact their toxicological responses. In the cardiac system, the marker expression differs between embryonic/fetal-like 12-day-old cardiomyocytes (7) and cardiomyocytes with a more adult profile that have been differentiated for 60 days in culture (6). A well-defined marker panel should serve as basis for acceptance criteria supporting/allowing a reduction of intra- and interlaboratory variability of the test system and is a prerequisite for toxicological *in vitro* tests entering into more formal validation efforts.

In vitro cardiotoxicity tests that are currently under development focus mainly on two adverse health effects: arrhythmias and drug-induced cardiomyopathies. The release of cardiac troponin T and the fatty acid binding protein 3 are clinically decisive cardiac biomarkers for the various forms of cardiomyopathies. In their system, Andersson *et al.*, have monitored the release of those two biomarkers after doxorubicin treatment by using biosensing methods (4). Nevertheless further studies will be necessary to fully prove the toxicological relevance of this system. Severe adverse effects such as Torsade de Pointes (TdP) are associated with chemical interactions with various ion channels of the heart (8). Two test systems analyze the extracellular field potentials of hESC-derived cardiomyocytes and recently a novel test system was developed that uses iPSCs from a patient with long QT syndrome (7, 9-10). One of the most advanced is the test system of Braam *et al.* that has performed a feasibility study with 12 compounds with well known effects on the different ion channels to demonstrate the biological and toxicological relevance of their test system (7). This and other currently available test systems are summarized in Table 1. Further efforts are now needed to define the intralaboratory variability as well as a robust procedure to interpret the data obtained by testing unknown compounds.

3.2. Tests for neurotoxicity

The development of *in vitro* tests for neurotoxicity and developmental neurotoxicity has always

Table 1. Differentiation protocols and test systems developed in the last years for toxicologically relevant cell models

	Cell type	Cellular system	Differentiation conditions	Markers (genomic and protein)	Differentiation success rate and/or functionality	Readout	Chemicals inc. control	Ref.
REPRODUCTIVE TOXICITY	Germ cells-haploid gamete formation	HSF1, HSF6, H1 and H9	Transfected with VASA-GFP reporter, diff. media with BMP4, BMP7 and BMP8b	VASA, DAZL, PRDM1, DPPA3 (STELLA), sperm markers TEKT1 and acrosin	5% VASA +	n.s.		(43)
	Primordial germ cells, Sertoli cells	HSF-6, H9	Spontaneus differentiation of small colonies, 10 days culture in diff. Media on laminin	CXCR4, PRDM-1, C-KIT, "germinal bodies", DPPA3, DAZL, VASA, acrosin, Sertoli cell markers MIS, FSHR, SOX-9, CLUSTERIN	25-30% CXCR4+ 40-45% VASA+	n.s.		(45)
	Germ cell-spermatids	Shef 1, 3, 4, 5, 6, H7	EB suspension culture for 14 days in media without additived or with RA or BMP4 or conditioned media beginning of flagellum development, selection of SSEA1+/C-KIT+, culture 14 days	Oct4, NANOG, DAZL, VASA, SCP3, C-KIT	0,5-5% spermatids	n.s.		(42)
	Primordial germ cells	H9, hES-NCL1	Differentiation +/- BMP4 in adherent culture for 3 weeks or EB differentiation in suspension for 16 days	SSEA 1+ , VASA, STELLA, OCT4, SCP3	5% SSEA 1+	n.s.		(44)
	Germ-like cells	BG01	Spontaneous differentiation on feeders or in feeder free culture up to 30 days	69% OCT4+ and VASA +, SYCP3, MLH1, Ifitm3, DPPA3, POU5F1, DAZL, NANOG, DDX4, PIWIL2, PUM2, MLH1, DAZ	90% MLH1+ and SYCP3+	n.s.		(46)
DEVELOPMENTAL TOXICITY	Undifferentiated cells	H9	Feeder-free culture of undifferentiated cells for 7 days		88% accuracy	Metabolomics, cytotox test, prediction model,	20 chemicals	(48)
	Undifferentiated cells/ neural progenitors	H1 and H9 undifferentiated, Cy203 differentiated in NP	Cy203 EBs were cultured in suspension for 7 days, adherent culture, rosette dissection, culture in neural induction media 7 days	Nestin+, SOX1-		Metabolomics, RT-PCR,	valproate	(47)
	Toxicity to neuronal precursors and neuron-like cells	H1	EB suspension culture for 2 days, diff. in neural induction media 12 days, rosette excision and suspension culture 2 days, dissociation and plating, terminal diff.	POU5F1, PAX6, Nestin, NCAM1, NEUROD1, NEFL, MAP2 and beta-tubulin III, TPH1, AChE, TH		Resazurin, RT-PCR,	MeHg	(49)
	Differentiating, undifferentiated hESC and human fibroblasts	ReliCell hES1, human foreskin fibroblasts	Hanging drop culture of Ebs for 4 days, 3days suspension culture,8days adherent culture, treatment for 15 days	POU5F1, NANOG, TDGF1, NES, NEFH, TUBB3, KRT, T, MSX1, ACTC1, CD34, AFP, FOXA2, GATA4, ALB, CCND1, CCRK, BAX, CASP3, DNMT3B, BCL2		cyQuant proliferation assay, rtPCR 21 genes,	Arsenic + inhibitor (Monoisoamyl dimercaptosuccinic acid) Busulfan, hydroxyurea, indomethacin, caffeine, penG, saccharin	(70) (71)
NEUROT	Neural crest cells	hESC, iPSC,	coculture system with MS5, diff. media with Noggin, SHH, ascorbic acid, FGF8, BDNF for 24days, replating of rosettes firther diff.	Peripheral neurons (pheripherin+, Mash1+, Brn3a+), Schwann cells (GFAP+, Sox10+), Mesenchymal Cell (CD73+)		n.s.		(11)

OXICITY	Cerebellar like cells	H1, H9	6 stage protocol with initial EB suspension culture and adherent culture with mix of factors (RA, BMP, FGF, WNT…)	NCAM, PAX6, TuJ1, PAX2, HOXA2, ZIC1, ZIPRO, WNT1	Electrophysiological properties, injected in mice, Na, K channels, functional receptors	n.s.		(15)
	Motoneurons	iPSC, HSF1 H1, H9, RUES1 -EGFP	Adherent culture, rosettes were isolated and cultured with RA and SHH for 1 week, 3-5weeks with BDNF, CNTF, GDNF, SHH Coculture with MS5, diff. with RA, SHH, ascorbic acid, BDNF	Brn2, Sox3, Pax6, Nkx6.1, Olig2 bIII-tubulin, ChAT, and Islet1+ (30%), Hb9 Pax6, Sox1, BF1, Otx2, Nkx6.1, Olig2, ChAT, HB9	Electrophysiological properties similar between iPSC and hESC motoneurons *In vitro*: AcH release *In vivo*: survive in chick embryo	n.s.		(21) (12)
	Dopaminergic neuron toxicity	BG01, I6,	differentiated on mouse PA6 cells for 3 weeks	80% TH+ colonies, C3 staining,		LDH activity, ROS formation,	MPP+	(14)
	Different toxicity to hESC and hESC derived dopaminergic neurons	I6, H9	EB suspension culture for 8 days, 2 days suspension culture in neural induction media, 2-3 days adherent culture, excision of rosettes and culture in PA6 conditioned media for 4 weeks	OCT4, NESTIN, TH, beta-III tubulin		ATP measurement;	720 chemicals from NINDS library (9 compounds selectively kill neurons: pirenzepine, amiodarone, selamectin, clofoctol, perhexilline, griseofulvin, chloroactoxyquinoline, menadione, hexetidine	(22)
	Inhibition of neurite outgrowth	H9 differentiated in neural hN2	Culture in differentiation media	bIII-tubulin, MAP2, nestin and panaxonal neurofilament SMI-312	Neurite outgrowth	Neurite length and no. neurite/ neuron,high-content image analysis, cell viability (ATP),	Brefeldin A, Bisindolylmaleimide, Na orthovanadate, LiCl, U0126	(23)
	Spinal muscular atrophy disease model	iPSC-differentiated in (motor) neurons	Grown in suspension for 2 weeks, media with RA 1 week, 1 week media with RA, SHH, dissociation and adherent culture for 2-6 weeks with RA, SHH, cAMP, ascorbic acid, GDNF, BDNF	Nestin+, Tuj1+, GFAP+, HOXB4,OLIG2, ISLET1, HB9, SMI-32 and ChAT	Neuronal axons	Number of nuclear "gems", increase in SMN protein,	Valproic acid, tobramycin	(18)
CARDIOTOXICITY	Cardiomyocytes	HES2	Coculture with END-2, culture up to 6 weeks	Atrial natriuretic factor, L-type Ca channel, Kv4.3 channel, MLC-2a, MLC-2v tropomyosin	Electrophysiological properties similar to human fetal cardiomyocytes, gap and adherens junctions	n.s.	Verapamil	(5)
	Cardiomyocytes	iPSC, H1, H9	EB suspension culture 3 days, 10 days adherent culture, culture in media with low FBS for up to 60 days	Oct4, NANOG, NKX2–5, TNNT2, MYH6, ACTN2, MYL7, MYL2, HPPA, PLN	Electrophysiological properties of nodal-, atrial-, and ventricular-like cells, 10% contracting EBs, functional beta-adrenergic receptors	n.s.	isoprotereno l	(6)

	Repolarization (QT screening), depolarization properties	H9.2	EB suspension culture for 7-10 days, adherent culture for 15-90 days, dissection of beating areas	MIRP, GAPDH, HERG	IKr channel	MEA, patch clamp;	E-4031, Sotalol, Quinidine, Procaineamide, Cisapride, Propafenone, 1-heptanol	(9)
	Na and Ca channel function	H9.2	Suspension culture of EB for 7-10 days, isolation of beating areas after 22-35 days	Expression of Na, Ca and HCN channel RNA	Electrophysiological recording of Na channel, Ca and HCN channel	MEA, patch clamp;	TTX, diltiazem, nifedipine	(72)
	QT prolongation	HES 3	Coculture with END-2 cells for 12 days, isolation of beating areas, culture for 7-14days		25% beating cardiomyocytes, functional Na, Ca and hERG K channels, electrophysiology of ventricular (93%), atrial and pacemaker cells	MEA, patch clamp;	Lidocaine, nifedipine, E-4031, cisapride, quinidine, verapamil, D,L-sotalol, sparfloxacin, sertindole, terfenadine, domperidone, ketoconazole	(7)
	Long QT model	iPSCs derived from patient with LQTS	Suspension culture of fragments for 10 days, EBs plated on gelatin for 30 days.	Troponin I, alpha actinin and connexion 43 staining, expression of NKX2-5, MYL2, MYH6, MYH7, KCNH2	Action potential generation, functional syncytium, chronotropic responses to isoproterenol	MEA, patch clamp	E-4031, cisapride, nifedipine, pinacidil, ranolazine	(10)
	Detection of biomarkers for cardiotoxicity detection of cardiomyopathies	SA002 differentiated in cardiomyocytes	EB suspension culture+ adherent culture for 10-12days, dissection of beating areas	NKX2.5, TNNT2 , HCN4,MYH7, KCNH2, cardiac troponin I		Surface Plasmon resonance assay for two clinically decisive cardiac biomarker: cTnT and cFABP; rtPCR for several genes, immunochemiluminescence for cTnT;	doxorubicin	(4) (73)
HEPATOTOXICITY	Hepatocytes	H1, H9	EB suspension culture for 4 days, adherent culture with addition of Na butyrate or DMSO or diff. in adherent culture with DMSO for 5 days, Na butyrate for 6-7 days, 4 days in media with Na butyrate and HGF	AAT+, CK8+, CK18+, CK19+, AFP, HNF4, ASGPR, C/EBP	10% diff. cells, 75-80% cell albumin+, Glycogen storage, inducible CYP1A2 activity	n.s.		(29)
	Hepatocytes	H1, H9	EB suspension culture for 7 days, adherent culture with FGF4 , HGF for 14 days	AFP, CK19, HNF-3beta, HNF-1, ASPGR1	Urea production, albumin secretion, ICG positive, CYP activity (phenobarbital induced)	n.s.		(33)
	Hepatocytes	SA002, SA002.5, SA167	Differentiated for 18-30 days with media change every 7-10 days, enriched by microdissection of hepatocyte- like cells	45 gene markers (CYP1A1, 1A2 and 2A6, CYP3A4 and 3A7), immunoblot for of CYP1A2, 1A1, 2A6, 2B6, 2C8/9/19, 2D6, 2E1, 3A4/7	Induction of CYP with rifampin, primidone, dexamethasone, EtOH, omeprazole and isoniazid, EROD assay	n.s.		(74)

	Hepatocytes	H1, H9	Adherent culture, media with Na butyrate and Activin A or Wnt3a and ActivinA for 3 days, media with Na butyrate and ActivinA for 2-3 days, culture in differentiation media with DMSO for 7 days, culture in media with HGF and OncostatinM	ALB, AFP, HNF4alfa, TAT, TO, APOF, CYP3A4, CYP7A1	70-90% albumin+ cells after 17 days; *In vitro*: urea production, gluconeogenesis, secretion of AFP, CYP1A2 activity, fibrinogen, TBPA, albumin *In vivo*: engraftment in mice	n.s.		(35) (75)
	Hepatocyte	H9, iPSC	3 days diff. with activin, FGF2, BMP 4, PI3-kinase inhibition, 5 days with FGF10, retinoic acid and inhibitor of activin/nodal receptors, 10 days with FGF4, HGF, and EGF	asialoglycoprotein receptor, tyrosine aminotransferase, alfa1-antitrypsin, Cyp7A1, hepatocyte nuclear factors 4 alfa and 6	*In vitro*: glycogen storage, cytochrome activity, and low-density lipoprotein uptake. *In vivo*: expressed human albumin and alfa1-antitrypsin	n.s.		(37)
	Hepatic endoderm	iPSC from XX and XY from 2 ethnic origins	Differentiation for 3 days with activin A and Wnt3a, 2 days with activin A, folloed by DMSO, maturation step with hepatic growth factor and oncostatin M	hepatic morphology, expression of albumin and E-cadherin, alpha-fetoprotein, hepatocyte nuclear factor-4a, Cyp 7A1	Secreted fibrinogen, fibronectin, transthyretin, alpha-fetoprotein, supported CYP1A2 and CYP3A4 metabolism	n.s.		(76)
	Hepatocytes	H9, iPSC	5 days activin A, 5 days BMP4, FGF2, 5 days HGF, 5 days Oncostatin M	80% of cells expressed albumin, expression of hepatocyte- specific genes	accumulation of glycogen, metabolism of indocyanine green, accumulation of lipid, active uptake of LDL, synthesis of urea, integrates into hepatic parenchyma *in vivo*	n.s.		(77)
	Hepatic progenitor cells	H1	3 days of Activin A treatment, fibroblast growth factor-4 and bone morphogenetic protein-2 for 5 days HGF and OSM to promote maturation into hepatocytes Activation of FGF and BMP pathways, purified N-cadherin+ cells (60%), co-culture with STO feeder cells	AFP, KRT7; AFP, ALB, HNF4A, CEBPA, FOXA2, GATA4 PEPCK, AAT, TAT, CYP3A7 CYP2A6, Hepatocytes ALB+, AAT+ cholangiocytes KRT7+	20-30% hepatocytes from progenitors; albumin secretion (439 ng/day/million), glycogen storage, uptake and release of indocyanine green, uptake of LDL, inducible P450 activity	n.s.		(78) (34)
	Hepatocytes	H9	Activin A induction for 2 days, differentiation for 10-14 days in media with FGF-4, HGF, BMP2, BMP4 and DMSO. Culture in hepatocyte media with FGF-4, HGF, Oncostatin M, Dexamethasone, and 0.5% DMSO	AFP, ALB, a1-AT, 60% positive for ASGPR, Phase I enzymes: CYP1A2, 3A4, 2D6, 2C9, 1A1, 1B1, 2A6, 2A7, 2B6, 2C8, 2C19, 2E1,7A1, Phase II enzymes: UDP-glucuronosyl-S-transferases and GST, Phase III proteins: MRP1, OATP2, nuclear receptors	Uptake and excretion of ICG, seven metabolic pathways of the drug bufuralol, metabolisation of phenacetin, midazolam, and bufuralol similar to primary hepatocytes, Metabolism of diclofenac lower.	n.s.		(39)
	Hepatocytes	HSF6	EB suspension culture for 9 days, adherent culture 10-14 days; Transfected with GFP vector with alfa-1-antitrypsin promoter; sorted GFP+ cells	AFP, ALB, a1-AT, CK18, G-6-P, ARG, CYP2B6, CYP2E1, CYP2C9, CYP3A4, CYP1A1, CYP1B1,	*In vitro*: glycogen accumulation, ICG uptake, CYP1A2 activity. *In vivo*: ALB, a1-AT, TF, CYP1B1, and GAPDH expression, human albumin in serum	n.s.		(79)

	Hepatocytes	IPSC : 3U1, 3U2, H1	Albumin fraction V, activin A for 1 day, 2 days with insulin-transferrin-selenium, FGF4, BMP2 for 4 days, HGF, KGF for 6 days, in HCM containing oncostatin-M, dexamethasone for 5 days, and in DMEM with supplements for 3 days	60% AFP+ and ALB+ ALB, CK8, CK18, CK19, PEPCK, HNF4alfa, HNF6, CEBPalfa, GATA4 and HEX	Glycogene storage, urea synthesis albumin secretion, Cyp4502B function	n.s.		(40)
OTHER CELL TYPES	Vascular cells	H9, H13	EB suspension culture for 10-15days, dissociation and sorting of CD31+/ CD34+ cells, culture for 3-4 weeks	CD34, CD31, VE-cad, von Willebrand factor, N-cad, LDL uptake	Formation of vessels in mice	n.s.		(80)
	Endothelial cells			CD31 and vascular endothelial cadherin, angiogenic growth factors	*In vivo* produced nitric oxide, migrated across a wound, and formed tubular structures, in mice neovasculisation	n.s.		(81)
	Lung epithelium	VUB03_DM1, VUB04_CF, VUB09_FSHD VUB07 VUB14,	growth on a porous membrane for 4 days, differentiation medium for 4 days; culture in air-liquid interface conditions for 20 days	CC16, NKX2.1, SP-C, Aquaporin 5, FOXJ1, tubulin IV	secretion of CC16	n.s.		(82)
	Alveolar epithelial type II	H9.2	Puromycin selection of cells transfected with plasmid with SPC	lamellar body formation, expression of surfactant proteins A, B, C, a-1-antitrypsin, cystic fibrosis transmembrane conductance receptor,	complement proteins C3, C5	n.s.		(83)
	Renal progenitors	HES 2-4	Spontaneous diff. in media with low serum conc. for 12 days	CD24+, podocaylxin+, GCTM2-, PAX2, LHX1, WT1		n.s.		(84)
	Ectodermal cells	H9, I3, I6	Coculture with PA6 stromal cells, induction with BMP4, FBS, epidermal growth factor	100% K18+, keratinocyte specific genes-, p63, K14, lama3, Oct-4-	No teratomas in nude mice, proliferate up to 60 doublings	n.s.		(85)
	T cells	H1	Coculture OP9 cellsor 1 f0-12days, coculture with OP9-DL1 monolayer for 7 days, transferred to OP9-DL1 for 4-6weeks	CD34+, CD43+, CD45+, c-kit After 30 days 50% CD3epsilon+, TCRalfabeta+ cells		n.s.		(86)

proven to be challenging. *In vivo* tests include the data about prenatal and postnatal development and can assess the effects of a chemical on both the morphology and functionality of the nervous system. At the moment, various protocols have been established to differentiate PSCs into neural precursor cells (11), and terminally differentiated cells (motoneurons (12), glia cells (11), dopaminergic neurons (13-14), cerebellar cells (15)).

The protocols based on hESCs usually yield a high percentage of neural precursors (more than 80%) (16) and a significant number of target cells (up to 60%) (17). Similar results can be obtained with iPSCs (16). iPSCs also give an opportunity to create patient- and/or disease-specific models for neurotoxicity testing as was done in the work of Ebert *et al.* who generated iPSCs from a patient with spinal muscular atrophy and differentiated them towards motoneurons (18).

General RNA and protein markers for neural precursor cells are quite well established and include among others NESTIN, SOX1, PAX6, NEUROD (19). However, the choice of markers for specific lineages of stem cell derived-neuronal cells can vary between the different authors, which makes the results hard to compare. In the case of murine ESCs, an extensive review of the markers for the different lineages was published recently (20) and a similar agreement in the field of human stem cell-derived neuronal cells might prove as useful for the comparison of differentiation protocols.

The major drawbacks of currently available protocols (Table 1) are the low yield of some cell types (19) and the long culture periods for the differentiation (up to two months) (11, 18, 21). However, in some systems it has been possible to produce a large stock of progenitor cells that could be differentiated at a later stage into the desired cell type. Furthermore, pluripotent stem cell-

derived neuroprogenitor cells are already commercially available (22-23) and can be differentiated by the end user. This could significantly shorten the time needed to obtain the target cells for toxicological applications.

Two tests have been developed in order to measure the effects of chemicals on dopaminergic neurons (14, 24). Han and co-workers have developed a screening platform for the toxicity testing of compounds on neural progenitors and differentiated neural cells. Their aim was to find compounds that would selectively kill the progenitor cells, leaving a pure population of differentiated cells that would be used for therapeutic purposes (22). This test system could be adapted also for the screening of compounds for potential neurotoxic effects on three stages of neuronal differentiation. The second test was developed by the US EPA. The system is based on the automated high content image analysis of the effects of chemicals on neurite outgrowth that allows for a medium to high throughput screening of chemicals (23).

The future challenge for test developers in the field of neurotoxicity is the establishment of test systems that could predict the effect of a chemical also on the functionality of the neuronal cells (e.g. electrophysiological properties, neurotransmitter activity, etc.). The described test systems are now offering the opportunity to determine the crucial biological pathways that following perturbation might lead to adverse effects *in vivo*. Highly standardized *in vitro* models are a prerequisite for a reliable pathway analysis.

3.3. Tests for hepatotoxicity

The lack of metabolic competence turned out to be a major bottleneck for the regulatory acceptance of *in vitro* toxicity tests (24). Furthermore, it is well known that the liver is one of the major targets of chemically induced adverse effects (25). The crucial characteristics of an *in vitro* liver model are the presence of a vast array of enzymes and a level of enzymatic activity that should mimic the *in vivo* situation. Major efforts are being currently undertaken to set up cell-based models allowing to address metabolism-mediated toxicity and hepatotoxicity. Of the currently available *in vitro* liver cellular systems, primary hepatocytes are problematic due to their rapid dedifferentiation during *in vitro* culture (26). Other models are mainly based on cells of carcinogenic origin. Malignant cells often show unwanted characteristics and, compared to primary cells, exhibit a lower functionality which could affect the toxicological and biological relevance of the *in vitro* system (27).

The first reports on hepatic cells derived from human pluripotent stem cells raised hopes to use human pluripotent stem cells as a basis for the generation of metabolically competent hepatocytes (28-30). In the last years several groups have established successful differentiation protocols into hepatocyte-like cells that express relevant markers that are found during liver development *in vivo* (31-33). Despite this progress, the differentiation protocols still vary in the combinations of growth factors used to drive the differentiation into various stages of hepatocyte development (see Table 1). In particular activin A, fibroblast growth factor 2, oncostatin M and BMP4 seem to be crucial for this process (34-36). Other factors such as LY294004, a phophatidylinositol 3-kinase inhibitor, provide support for further differentiation of cells along the hepatic lineage (37). Chiao *et al* pointed out signaling pathways that may be manipulated in order to direct more efficiently the differentiation of hESCs towards mature hepatocytes. Those pathways were revealed by the isolation and transcriptional profiling of purified hepatic cells derived from human embryonic stem cells (38).

Various liver-associated genes and transcriptional factors have been proposed for the close monitoring of the successful differentiation and maturation of hepatocyte-like cells when establishing the optimal culture conditions. On the other hand, the functionality of hESC-derived hepatocyte-like cells was proven by their capacity to produce urea and albumin, store glycogen and have functional metabolizing enzymes critical for toxicant biotransformation (39). The majority of published protocols are using the hESC lines H1 and H9 (WiCell Research Institute) but Song *et al.* demonstrated that also two iPSC lines can differentiate into hepatocyte-like cells to the same extent as H1 hESCs after stimulation with a series of growth factors (40). A comprehensive overview on the differentiation of stem cells into hepatic lineages and related markers was provided by Snykers *et al.* (41). Despite the advances in the differentiation protocols, a clear definition of the markers and functionalities a pluripotent stem cell-derived hepatocyte should have, to be acceptably used in toxicity testing, has not been achieved so far.

3.4. Tests for reproductive toxicity

The fields of reproductive and developmental toxicity testing are very complex as they include all phases from gamete formation up to birth, including fertilization and prenatal development. In the last years, efforts have been made to develop a series of test systems that use human pluripotent stem cells to detect the effects of chemicals on the reproductive system.

One of the most important targets in reproductive toxicity is the gametogenesis. Despite the fact that murine ESCs have been differentiated into early germinal cells and spermatozoa, the differentiation of human pluripotent stem cells into gametes is still under development. Efforts have been made to stabilize the differentiation of germ cells *in vitro*, in particular into male gametes (see Table 1). However, the amount of cells that can be obtained with the described protocols is very low (5%) (42-44). Different groups have reported the expression of markers of male primordial germ cells and in some cases even some meiotic markers (44-46). Two different groups have successfully differentiated hESCs into relatively mature haploid spermatids that were showing the first signs of flagellum development and acrosin expression (42-43). The development of protocols for the generation of female gametes has not been successful yet, even if the work done by Aflatoonian and colleagues has shown the increase of female gamete markers after *in vitro* differentiation (42).

Nevertheless it still remains unclear which would be the functional status of such *in vitro* derived gamete-like cells and whether their functionality would be relevant for toxicity studies.

3.5. Tests for developmental toxicity

Another important chemical target is embryonic development. The test systems for developmental toxicity that are currently being developed are mainly focused on the early phases of cell differentiation and maturation. Since developmental toxicity testing suffers from the fact that many mechanisms of developmental toxicants are unknown and predictive readouts are lacking, more research is required to define new biomarkers and a detailed understanding on the pathways that –if affected- will lead to pathologic findings. Emerging technologies such as "omics" and the automation of *in vitro* testing are important steps forward in order to define the toxicological information that is needed to make reliable forecast on the hazard of a chemical (47-48). The mechanisms leading to interspecies variations are not well known this is why clear concepts are required on how to validate novel *in vitro* models based on human cell types.

Stummann and colleagues have developed a test mimicking the early development of the human nervous system and have challenged it with the well-known developmental neurotoxicant methylmercury chloride (MeHg) (49). This system provides a tool to assess the different effects of chemicals specifically during the generation of neural precursors and mature neural cells. Nevertheless the application of this test for developmental toxicity testing would require a bigger number of chemicals to be assessed and also the establishment of a suitable data interpretation procedure. In any case, this test system only elucidates the chemical affects on ectodermal differentiation. Because of the complexity of embryonic development and the possible perturbations by chemicals of all three germ layers other test systems relying on cells originating from the mesodermal and endodermal layers should be established and be further analyzed. Currently it is not fully understood which cell types (or their precursors) exhibit crucial pathways and should get priority for assessing chemical effects. Special attention should be given to the windows of sensitivity during embryogenesis which means that not only terminally differentiated cells are of interest but also the correct temporal pattern of differentiation of their precursors (50-51).

Despite the progress that has been made in the last years, the methods that are currently in the phase of development still need to be refined regarding the amount of target cells as well as the readouts and data interpretation procedures. In a recently published draft report on alternative methods for cosmetics testing it was concluded that several years of test development will be still necessary before a test for developmental toxicity based on human pluripotent stem cells can enter into the validation process (52).

4. EMERGING TECHNOLOGIES IN TOXICITY STUDIES

Currently most of the efforts in stem cell based toxicity testing have been devoted to the standardization of the cellular models which is the one integer part of an *in vitro* test. However, another crucial parameter in the development of an *in vitro* test is the correct selection of the readout which reflects the toxicological endpoint one wants to assess with the *in vitro* test (e.g. the use of multielectrode arrays as readout for assessing Q-T prolongation). That the selection of the readout is not trivial becomes obvious when the exact mode of action of the chemical is not fully understood. In this case sophisticated technologies such as "omics" technologies are necessary to elucidate the chemical's mechanism of action. According to this information the selection of a readout for the *in vitro* system will be more focused. The experience with the validated murine embryonic stem cell test which relies on the appearance of beating cardiomyocytes after treatment with embryotoxicants (53), has demonstrated the high relevance of selecting predictive readout systems. This readout system must be associated with a detailed understanding of what is happening in the cell system. Good examples for the selection of predictive readouts are transcriptional and binding assays (54-55) that can provide information on the estrogenic and androgenic properties of chemicals. In the previous section we have shown that several toxicity tests based of stem cells are in the phase of development. The readouts differ from test to test and are sometimes difficult to compare. Some of the readouts are not adapted for high throughput screening which is becoming essential in toxicity testing where there is a need to understand the prevalence of a toxic effect in a universe of chemicals or to identify patterns of toxicity across various cellular models. The need for a higher throughput and identification of novel biomarkers of toxicity that would be useful in different test systems has fostered the application of new technologies in *in vitro* toxicology.

Emerging technologies now drive the establishment of novel readouts and biomarkers that lead to the definition of even more criteria to properly characterize and define the adequate functional ESC/iPSC-derived somatic cells required for toxicity testing purposes. Cell culture techniques should co-evolve with such techniques to apply them from the very beginning and to avoid later adaptations that might delay the test development process. Among the different high throughput (HT) technologies emerging right the "omics" technologies seems to be very promising for toxicity testing.

The recent advances in genomic research significantly contributed to the discovery of new toxicity pathways and the understanding of toxicological mechanisms on cellular and molecular level (reviewed in (56)). The technologies measuring the quantitative changes in molecules such as RNA, proteins and intermediary metabolites on a global level are often termed "-omics" technologies, encompassing toxicogenomics, toxicoproteomics, including high content imaging, and metabonomics. Global analyses of biological responses are based on the concept that the disturbed cellular and molecular functions do not only permit detection of toxicity, but can also help to understand mechanisms and pathways of toxicity. It is hoped that these technologies will explore possible common mechanisms of toxicity and

contribute identifying specific biomarkers of toxicity, independent of species or cellular model system used.

4.1. Toxicogenomics

Toxicogenomics measures the modulation of gene expression in response to exposure and explores the basic mechanisms of toxicity. Ideally, changes in gene expression levels correlate to traditional toxicological readouts such as changes in cellular response and organ structure induced by the toxicant. For measuring gene expression, DNA microarrays can be used to measure the differential expression of thousands of genes at the same time. A variety of array platforms such as cDNA arrays, high density oligonucleotide and oligonucleotide bead arrays have been used (57-58). For examining defined sets of genes, custom-made microarrays are also available (59).

The generation of high content microarray data is only the first step for understanding the mechanism of toxicological responses. Correlation of gene expression signatures to toxicological endpoints is essential for prediction of toxicity by expression profiling. Functional classification and assignment of transcripts regulated after exposure to toxicants will allow identification of pathways of toxicity (Toxicity of the 21st century) (59). As an example of this approach transcriptomics data were successfully used to classify toxicants according to their mechanism of action. Rat hepatocyte cultures were treated with 15 known hepatotoxicants. The trancscriptomics analysis of the gene expression profiles of the different toxicants showed that, despite the fact that each toxicant has a specific signature, they could be clustered according to their similar toxicological mechanisms. The clustering of these compounds was concordant with previously published *in vivo* reports (60).

Huang and co-workers compared cisplatin-induced nephro- and hepatotoxicity *in vitro* and *in vivo* using focussed microarrays. The microarray studies succeeded in identifying pathways of cisplatin-induced nephrotoxicity demonstrating discrepancies between *in vivo* and *in vitro* findings. Whereas *in vivo*, the kidney was the primary target organ, hepatic toxicity was more pronounced in vitro (61). Also Jagtab et al. propose a transcriptomic approach in hESCs for monitoring specific toxic effects in early embryonic development (62). Cytosine arabinoside (Ara-C) was used as reference compound and demonstrated a specific induction of neural genes such MAP2, TUBB III, PAX6, TH and NESTIN whereas mesodermal markers (HAND2, PITX2, GATA5, MYL4, TNNT2, COL1A1) were inhibited at day 14 of differentiation.

The relevance of toxicogenomic approaches in safety testing is widely recognized. In a retrospective study of non-clinical safety studies, Foster et al. concluded that significantly regulated transcripts can serve as robust biomarkers of toxicity. While correlation with histopathological data was limited, transcriptional changes often preceded traditional endpoints, demonstrating the sensitivity of transcriptomics. The major challenges of evaluating toxicogenomics data are the integration with traditional histological data, the agreement on data analysis methods and procedures for biomarker identification (63).

4.2. Metabonomics

The term metabonomics refers to the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification (64).

Metabonomics-based toxicity studies are mainly performed using samples from *in vivo* studies, including blood plasma, urine, saliva or cells. After preparation, samples are analysed by either nuclear magnetic resonances (NMR) or mass spectroscopy (MS) technologies. The resulting metabolic profiles are then evaluated by a multivariate statistical analysis to identify significant differences in metabolite repertoires and to propose candidate markers, as it was recently suggested (65-66).

In 2007, Cezar and colleagues were the first to analyze hESCs with metabonomics approaches for developmental toxicity testing. Their platform was capable to identify alterations in the metabolic profile of hESCs exposed to valproate, revealing novel biochemical pathways of toxicity. The study highlights the possibility of using metabonomics in hESCs and hESC-derived cells as a novel tool to identify predictive biomarkers for efficacy and safety assessment of pharmaceuticals (47). In 2010 the same group provided a more comprehensive dataset in which 7 out of 8 substances were correctly classified (48).

However, many challenges remain before the full potential of metabonomics in *in vitro* toxicology will be developed. One major challenge is the identification of masses: approximately fifty percent of the measured masses are not annotated in public databases. Deciphering chemical structures to identify small molecules is labor-intensive and time-consuming, requiring costly NMR spectroscopy and/or MS-MS setups that are not generally available to most biomedical research laboratories.

4.3. Toxicoproteomics

Another approach to identify biomarkers for developmental toxicity is based on proteomics technology. Toxicoproteomics integrates the traditional toxicology and pathology with differential protein and gene expression analysis and systems biology. It focuses mainly on the assessment of the proteome in organs and biofluids, such as liver and blood. Toxicoproteomics is mainly used to discover key modified proteins as early biomarkers for the prediction of an adverse effect. In addition, the knowledge of the perturbed pathway will provide the necessary mechanistic support when interpreting the data of animal studies and their relevance for humans (67). The identification of novel biomarkers and their mechanistic interpretation is still time consuming, however, using high density LC-MS/MS platforms and its complementary methods can accelerate the toxicoproteomics analysis.

Beside the wide use of toxicoproteomics for diagnosis in clinical chemistry, or in the identification of biomarkers for specific environmental exposures or stressors, it can also play a role in *in vitro* toxicology studies. Groebe *et al.* have challenged the validated murine embryonic stem cell test with two known embryotoxicants

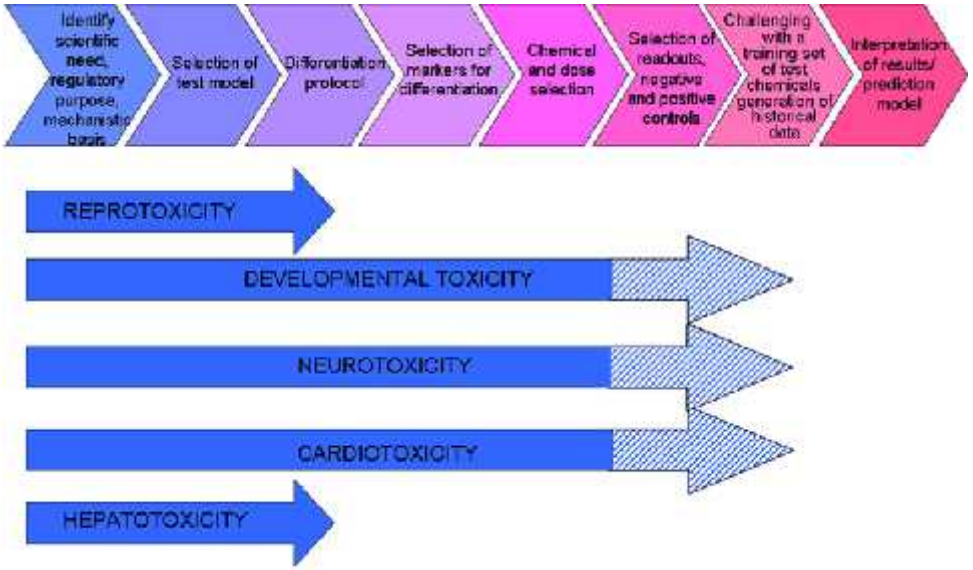

Figure 2. Simplified graph demonstrating the current situation of the most advanced protocols for human health endpoints.

(warfarin and lovastatin) in order to further evaluate the mode of action which seems to be related to the Ras signalling pathway (68). However, further studies are necessary to confirm the relevance of identified proteins as biomarkers. Because toxicoproteomics have the potential to establish a relationship between adverse effects and protein markers they have the great potential to contribute to an understanding of toxicological mechanisms.

5. PERSPECTIVE

Some human endpoints in toxicity testing are suffering from low concordance between animal studies and humans (e.g. cardiotoxicity, hepatoxicity, developmental toxicity, metabolism-mediated toxicity). For this reason toxicity tests that are based on human stem cells have been developed in the recent years to overcome the interspecies variations. Another challenge in toxicity testing is the need for medium to high throughput which can be facilitated by the unlimited supply by stem cells. Human stem cells have some other advantages because they are of non-malignant origin and can generate several types of cells of toxicological relevance.

However, the major bottlenecks in the development of such *in vitro* toxicity tests remain the differentiation protocols, leading to an insufficient number of toxicologically relevant target cells. In the past, several EU-funded projects have addressed the development of stem cell-based *in vitro* tests e.g. ReProTect, InVitroHeart and Vitrocellomics. ReProTect allowed the identification of new valuable *in vitro* assays that can partially mimic the complexity of the mammalian reproductive cycle. Several of the developed tests are now considered for the entry into prevalidation. The aim of the InVitroheart project was to establishing relevant hES cell derived cardiomyocytes cultures whereas the Vitrocellomics project focused on stem cell derived hepatocytes to be used for drug discovery and toxicity testing. Additional support might come from other international R & D collaborations in the field of regenerative medicine aiming to develop cell-based therapies, since some target cells for toxicological testing are also of therapeutic interest. In addition, basic research activities may contribute to the optimization of differentiation conditions (69). It is also expected that emerging technologies in cell culture such as 3D models, supporting biomaterials, etc. will contribute to the upscaling of toxicological relevant cells.

Nevertheless, scientific consensus on the degree of functionality of the *in vitro* system and/or on the expression levels of a panel of marker genes of the relevant target cells is urgently required in order to define the maturation stage of the cell types of interest as well as the level of purity/homogeneity of cultures used for toxicity assessments. This information will underline the correlated biological relevance of cellular models based on stem cells or their derivates. Induced pluripotent stem cell technology might be able to provide an unlimited cell supply and may have other advantages such as the possibility to generate cells for different genetic backgrounds and disease models. At a later stage in the development of a test, the use of iPSCs and their derivates may gain international acceptance in relevant test guidelines for hazard identification. However, the technologies based on iPSCs are still in their infancy and need to be further developed. Nevertheless, these activities require a link to well-characterized standard models which can currently only be provided by hESCs.

The selection of predictive readout systems depends mainly on the purpose of the tests. While in the area of cardiotoxicity and neurotoxicity multielectrode arrays are well established, and promising (with feasibility studies ongoing); other areas such as the area of developmental toxicity still suffer from the lack of suitable predictive biomarkers, which is clearly related to the complexity of the field (Figure 2). Emerging technologies, such as the ones discussed in this review, will hopefully

provide input to this scientific challenge. In addition, several strategies are currently being defined combining various stem cell-based *in vitro* tests with genomic readouts. These strategies aim at improving the developmental toxicity predictions by identifying new relevant biomarkers. This strategy will be further complemented by metabolic mediated toxicity systems and PBPK modeling in order to provide a better *in vitro* toxicity-based hazard identification and characterization for this particular application.

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Abbreviations: a1-AT- alfa 1 antitrypsin, AAT- alfa 1 antitrypsin, AcH- acetylcholine, AcHE- acetylcholine esterase, ACTC- alpha cardiac actin, ACTN2- alpha actinin 2, AFP- alfafetoprotein, ALB- albumin, APOF- apolipoprotein F, ARG- arginase, ASGPR- asialoglycoprotein receptor, BAX- Bcl- associated X protein, BCL2- B cell lymphoma 2, BDNF- brain- derived neurotrophic factor, BF- brain factor, BMP- bone morphogenetic protein, BRN- POUF, C/EBP- CCAAT/enhancer binding protein, C3, C5- complement component 3, 5, cAMP- cyclic AMP, CASP3- caspase 3, CC16- secretoglobim, CCDN1- cyclin D1, CCRK- cyclin dependent kinase, CEBPA- CCAAT/enhancer binding protein, CFABP- fatty acid binding protein, CK- keratin, C-KIT- cellular homolog of the feline sarcoma viral oncogene v-kit**,** CNTF-ciliary neurotrophic factor, CXCR- chemokine (C-X-C motif) receptor, CYP- cytochrome P450, DAZ- deleted in azoospermia, DAZL- deleted in azoospermia-like, DMEM- Dulbecco's modified Eagle's media, DMSO- dimethylsulfoxide, DNMT3B- DNA (cytosine-5-)-methyltransferase 3 beta, DPPA (STELLA)- developmental pluripotency associated, EB- embryoid body, EGF- epidermal growth factor, EPA- Environmental Protection Agency, EROD- ethoxyresorufin-O-deethylase, EtOH- ethanol, FBS- fetal bovine serum, FOX- forkhead box, FSHR- follicle stimulating hormone receptor, GAPDH- glyceraldehyde-3-phosphate dehydrogenase, GATA4- GATA binding protein 4, GCTM2- cell surface keratan sulfate, GDNF- glial cell derived neurotrophic factor, GFAP- glial fibrillary acidic protein, GFP- green fluorescent protein, GST- glutathion- S- transferase, HB9- motor neuron and pancreas homeobox 1, HCN- hyperpolarization activated cyclic nucleotide-gated potassium channel , HERG- potassium voltage-gated channel, HEX- hematopoietically expressed homeobox, HGF- hepatocyte growth factor, HNF4- hepatocyte nuclear factor 4, HOX- homeobox, HPPA- atrial natriuretic factor, ICG- indocyanin green, IFITM3- interferon induced transmembrane protein 3, Ikr- potassium voltage-gated channel, ISLET- islet amyloid polypeptid, K18- keratin 18, KCNH2- potassium voltage-gated channel H2, KRT- keratin, lama3- laminin alpha 3, LDH- lactate dehydrogenase, LDL- low density lipoprotein, LHX- LIM homeobox, MAP- mitogen activated protein, MASH1- achaete-scute complex homolog 1, MEA- multielectrode array, MIRP- potassium voltage-gated channel, Isk-related family, MIS- Mullerian Inhibiting Substance , MLC- megalencephalic leukoencephalopathy with subcortical cysts, MLH1- mutL homolog 1, MPP+- 1-methyl-4-phenylpyridinium, MRP- multidrug resistance-associated protein, MSX1- msh homeobox 1, MYH6- myosin heavy chain 6, MYL- myosin light chain, NANOG- nanog homeobox, N-cad- neuronal cadherin, NCAM- neural cell adhesion molecule, NEFH- neurofilament heavy polypeptide, NEFL- neurofilament light polypeptide, NES- nestin, NEUROD- neurogenic differentiation, NINDS- National Institute of Neurological Disorders and Stroke, NKX- NK homeobox, NP- neural precursors, OATP2- solute carrier organic anion transporter family, OCT4- POU class 5 homeobox, OLIG- oligodendrocyte transcription factor, OSM- oncostatin M, PAX- paired box, penG- penicillin G, PEPCK- phosphoenolpyruvate carboxykinase, PI3- phosphoinositide-3-kinase, PIWIL2- piwi-like 2, PLN- phospholamban, POU5F1- POU class 5 homeobox 1, PRDM- PR domain containing, PUM2- pumilio homolog 2, RA- retinoic acid, ROS- reactive oxygene species, RT-PCR- real time PCR, SCP- sterol carrier protein, SHH- sonic hedgehog, SMI- hypophosphorylated neurofilament, SMN- survival of motor neuron, SOX- sex determining region Y-box, SPC- surfactant protein C, SSEA- stage specific embryonic antigen, SYCP- synaptonemal complex protein, T- T bracyury homolog, TAT- tyrosine aminotransferase, TBPA- thyroxine-binding prealbumin, TCR- T cell antigen receptor, TDGF- teratocarcinoma-derived growth facto, TEKT- tektin, TF- tranferrin, TH- tyrosine hydroxylase, TNNT2- troponin T type 2, TO- tryptophan dioxygenase, TPH- tryptophan hydroxylase, TTX- tetrodotoxin, TUBB3- tubulin beta 3, TUJ1- Neuron-specific class III beta-tubulin, UDP- uridine diphospho, VASA- DEAD box protein, VE-cad- vascular endothelial cadherin, WNT- wingless-type MMTV integration site family, WT1- Wilms tumor 1, ZIC1- zinc finger protein, ZIPRO- zinc finger and SCAN domain-containing protein

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