Markers of murine embryonic and neural stem cells, neurons and astrocytes: reference points for developmental neurotoxicity testing

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Markers of Murine Embryonic and Neural Stem Cells, Neurons and Astrocytes: Reference Points for Developmental Neurotoxicity Testing*

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Summary

Developmental neurotoxicity (DNT) is a serious concern for environmental chemicals, as well as for food and drug constituents. Animal-based DNT models have relatively low sensitivity, and they are burdened by high work-load, cost and animal ethics. Murine embryonic stem cells (mESC) recapitulate several critical processes involved in the development of the nervous system if they are induced to differentiate into neural cells. They therefore represent an alternative toxicological model to predict human hazard. In this review, we discuss how mESC can be used for DNT assays. We have compiled a list of mRNA markers that define undifferentiated mESC (n = 42), neural stem cells (n = 73), astrocytes (n = 25) and the pattern of different neuronal and non-neuronal cell types generated (n = 57). We propose that transcriptional profiling can be used as a sensitive endpoint in toxicity assays to distinguish neural differentiation states during normal and disturbed development. Importantly, we believe that it can be scaled up to relatively high throughput whilst still providing rich information on disturbances affecting small cell subpopulations. Moreover, this approach can provide insight into underlying mechanisms and pathways of toxicity. We broadly discuss the methodological basis of marker lists and DNT assay design. The discussion is put in the context of a new generation of alternative assays (embryonic stem cell based DNT testing = ESDNT V2.0), that may later include human induced pluripotent stem cells, and that are not designed for 1:1 replacement of animal experiments, but are rather intended to improve human risk assessment by using independent scientific principles.

Keywords: stem cell, development, neurotoxicity, gene ontology, astrocyte, systems biology

1 Introduction

Embryonic stem cell (ESC)-based novel test systems are amongst the most dynamic areas of *in vitro* toxicology and biomedicine, and their development is funded e.g. by a large scale EU project (ESNATS → http://www.esnats.eu/). They may become future alternatives to animal testing and a key element of modern risk assessment approaches (Pellizer et al., 2005). At the start of such a paradigm shift in toxicology it is essential to define the new test systems and their performance to the maximum possible degree. Therefore this review undertakes a first attempt to define markers for mESC and derived cell types as a starting point for an intense scientific discussion and further improvements in this area.

* a report of t4 – the transatlantic think tank for toxicology, reviewed by T. Hartung and A. Goldberg (Baltimore, MD, USA)

This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Murine embryonic stem cells (mESC) are pluripotent cells able to differentiate into all cell types in the mouse, including functional germ cells. Under appropriate conditions, mESC can be kept as *in vitro* cultures with an indefinite capacity for self-renewal (Evans and Kaufman, 1981; Martin, 1981). The derivation, use and properties of murine and human embryonic stem cells (ESC) have been reviewed earlier (Leist et al., 2008a), also with the perspective of generating induced pluripotent stem cells (iPSC) by reprogramming of somatic cells from various species, including humans (Baker, 2010; Nagy and Nagy, 2010; Lee and Studer, 2010). Pluripotent cells are suitable for molecular biological manipulations, such as homologous recombinations with exogenous DNA to alter sequences of their genome. These properties have been used successfully for the generation of knock-out and knock-in mice from modified mESC (Capecchi, Martin and Smithies, Nobel Prize 2007). Such mice stand as *in vivo* proof that every stage and every cell of the nervous system can develop from mESC under appropriate conditions, and that the produced cells display different phenotypes according to the genotype of the mESC used initially for generation of the mice. It has also been demonstrated, that mESC can differentiate *in vitro* to different neuronal or glial subtypes (Wobus and Boheler, 2005). In theory, this offers the possibility to study all steps – in detail, in real time and at the resolution of individual cells – that lead from the multipotent mESC to the formation of neuroectoderm tissue, and further to the generation of neural stem cells (NSC), neuroblasts and various intermediate and mature types of neural cells (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Ying and Smith, 2003; Conti et al., 2005). The *in vitro* differentiation of mESC or human ESC (hESC), as well as of murine or human iPSC or neural precursor cells (NPC) to neurons or other defined cell types is of high interest to the understanding of developmental biology, but also its disturbances. Thus, such test systems appear useful for the examination of teratogenicity and the wide field of reproductive toxicity (RT). Moreover, introduction of neural endpoints relevant for developmental neurotoxicity (DNT) at different stages of development and development of more predictive and more sensitive model systems may significantly improve this testing strategy (Breier et al., 2009; Moors et al., 2009; Coecke et al., 2007; Lein et al., 2007).

2 Towards new test systems for developmental neurotoxicity

2.1 Lessons from the history of developmental neurotoxicity (DNT) testing

The area of developmental toxicology (DT) came into public focus 50 years ago. At that time, the drug thalidomide caused severe birth defects, while the metal-organic contaminant methylmercury caused Minamata disease (Harada, 1995). The latter also includes a congenital form, which is triggered by exposure of the unborn fetus to the toxicant. It has been shown that the mercury concentration in umbilical cord blood can be significantly higher than in the maternal blood (Sakamoto et al., 2004). Decades later, but still in consequence of this miniepidemic of methylmercury intoxication, autopsy studies revealed that this compound targeted the fetal neural system (e.g. Eto et al., 1992), and thereby, Minamata disease contributed significantly to the identification of developmental neurotoxicity (DNT) as an important endpoint in toxicology.

At the same time, the problem of developmental ecotoxicology (e.g. reduced reproductive success of birds due to pesticides in their food chain) was introduced by Rachel Carson in her book "silent spring". The above mentioned examples provide insights into the specific problems of DT. Another example for specific DNT issues is thalidomide that has a defined "window of sensitivity". It did not cause problems when taken by pregnant women earlier than about 20 days after conception or later than about 35 days after conception. However, within this window it caused different effects, such as facial paralysis, when taken rather early, malformations of arms and legs in the middle and e.g. deformities of the intestine when only taken late during the window of sensitivity. Notably, although thalidomide acted as a sedative in rats and mice (just as in humans), it had no teratogenic effects in these rodent species most frequently used for toxicity testing.

In Minamata, Chisso Corporation was found responsible for having caused the disease by introducing mercury waste into the Minamata bay. However, it was much more difficult in the case of the victims of the congenital disease (who had never eaten contaminated fish, but had been exposed in utero) to prove a causal relationship between their disease and the methylmercury contamination. The situation was similar with other environmental contaminants, where a cause-effect relationship was disputed until R. Carson's book became one of the key triggers for a wave of public concern that resulted in the ban of dichlordiphenyltrichlorethane (DDT). These examples illustrate the specific problems of the discipline of developmental toxicology, i.e. the difficulty to provide evidence for cause-effect relationships, and to identify suitable test systems. This fundamental weakness is also evident from less dramatic and more prevalent human poisonings that have reached the pandemic scale. The most prominent example of such an omnipresent contaminant is lead. It causes human developmental neurotoxicity, associated with a reduction of intelligence estimated to have resulted in an economic cost of > 100 billion $/year for each birth cohort born between 1960 and 1990 (Grandjean and Landrigan, 2006). The average lead blood levels in children fell by 90% after the eventual ban of lead additives to gasoline (Grandjean and Landrigan, 2006). However, those exposed earlier may keep suffering from lead neurotoxicity due to its long biological half-life in addition to the DNT effects (Cory-Slechta, 1990). In the case of the developmental toxicity of lead, the overwhelming epidemiological evidence finally helped to convince regulators to reduce acceptable thresholds, and the availability of trustworthy human reference data helped to optimise a suitable experimental system to improve the toxicity evaluation. There are still many other wide-spread contaminants with effects below the threshold of a pandemic, but with the potential to affect a large population.

For most of these hazardous compounds evidence from human epidemiology is not available. Therefore, standardised test systems, mainly rodent-based bioassays, are used to derive points of departure (POD) for human health risk assess-

ment in regulatory toxicology. In the 1960s, it became evident that developmental exposure to chemicals and drugs can alter behavioural function in young and adult animals (e.g. Werboff and Dembicki, 1962). As an indirect measure of neurotoxicity, behavioural readouts have been used and validated since the 1960s. These behavioural alterations are considered as an observable expression of effects on nervous system function (Reiter, 1978). Therefore, guidelines and test batteries have been developed (Moser and MacPhail, 1990, 1992) and validated for use in behavioural toxicology. In the 1980s, the U.S. Environmental Protection Agency (U.S. EPA) developed the first DNT guidelines and initiated the standardisation of this testing strategy by the Organisation of Economic Co-operation and Development (OECD). The development of the pertinent OECD test guideline 426, which was finally accepted in 2007 (Makris et al., 2009), was guided by two ideas: first, the methods need to yield reproducible results within and across laboratories, and second, they must be sensitive to the effects of a range of neurotoxic agents (Middaugh et al., 2003). A recent review (Makris et al., 2009) revealed that just over 100 compounds have been tested in studies using the OECD 426 draft guideline. Most of these compounds were pesticides (66%) and only 8 industrial chemicals were included. Another review identified about 174 compounds for which neurobehavioural risk assessment had been performed, in many cases also on the offspring of the exposed animals (F1 generation). Only 1% of these compounds were industrial chemicals (Middaugh et al., 2003). The available data for this relatively new area of toxicology of industrial chemicals is therefore rather limited. Some of the studies indicate that compounds exist for which DNT testing is the most sensitive of all toxicity endpoints in a broad safety evaluation battery. Therefore inclusion of DNT testing in compound safety evaluation programmes such as REACH is likely to add important information for regulatory decisions (Makris et al., 2009; Middaugh et al., 2003). At present the available data is insufficient to predict how representative these findings are.

In summary, the historical development of DNT testing strategies was strongly based on the statistical concepts of reliability and sensitivity, and biological modes of action played a relatively minor role. In addition to the relatively low numbers of animal studies, few human reference data are available. Thus, the predictive value of traditional DNT testing for human health is hard to estimate. Establishment of alternative and additional approaches remains a huge scientific challenge requiring new strategies.

2.2 The road to a mechanism-based developmental toxicology

The number of chemicals with potential for environmental exposure is large. The new European law entitled REACH triggered an administrative procedure aiming at registration, evaluation and authorisation of all chemicals produced in the EU at > 1 t/year and not tested under the chemical safety law of 1982. It is expected that at least 30,000 chemicals will be registered, amongst these several thousand that are produced or used at > 100 t/year (Rovida and Hartung, 2009). A considerable percentage of these chemicals is found in the environment or at work places, where human exposure could potentially trigger DT. As these substances should all be evaluated for their reproductive toxicity, experiments involving millions of animals would be performed to satisfy the legal requirements (Hartung and Rovida, 2009). However, these tests of individual chemicals constitute only the tip of the iceberg. Practically, and scientifically, we should also consider mixtures of compounds that humans and the environment are exposed to. Already a dozen compounds can form thousands of different mixtures, which would be impossible to test by classical toxicological approaches based on animal experiments. Even though some of the most relevant chemicals will be tested for their effects on reproduction, these tests will most likely leave open the safety questions concerning low dose effects on DNT. As indicated above, testing for DT in the low-dose range and basing legal decisions on these data has proven very difficult, if not impossible, in most cases. This is even more an issue for the subarea of DNT. Within the REACH testing requirements, DNT is only addressed in exceptional cases triggered by positive findings from other studies. Dedicated studies are otherwise not required. Thus, the concern remains that subtle, and predominantly functional, DNT effects triggered by chemicals might remain undiscovered. A comprehensive safety assessment will therefore require alternative approaches. Technical (limited test capacities), ethical (reduction of animal testing) and scientific reasons call for new strategies in toxicology testing (Leist et al., 2008b; Hartung, 2009a; Stingl et al., 2009; Bottini et al., 2007). One such strategy was suggested by the National Research Council (NRC, 2007). This milestone publication has been described in many reviews (Collins et al., 2008; Leist et al., 2008c; Hartung and Leist, 2008; Hartung, 2009b), and the strategy is now often summarised under the heading "tox21c" (**tox**icology for the **21**st **c**entury). Two changes are particularly important: first, novel test systems would be based on cell cultures (human, where possible) and simple model organisms (e.g. worms and flies) instead of rodents and other higher vertebrates; second, the essential primary endpoints should cover disturbances of cellular (e.g. signalling, metabolic, homeostatic, proliferation, differentiation) pathways, and the overall resulting toxicological effect on humans would be predicted by systems biology-based approaches from these mechanistic data. The vision is that the new test systems would allow a much higher throughput of compounds and would work better in the low-dose range relevant for human exposure. The use of a systems-based approach (e.g. omics data, quantitative models linking cellular processes to adverse effects) is expected to be more predictive of human toxicity (see above issue of rodent testing of thalidomide). Added value may come from the possibility to use and to compare cells of different species, including humans.

For this vision to become reality, the new methods must be trusted and accepted globally (Bottini and Hartung, 2009; Bottini et al., 2007). For instance, technical/scientific barriers are linked to the problem of validation (Hartung, 2007), as detailed for the areas of food safety and cosmetics safety (Hartung, 2008b; Hartung and Koëter, 2008; Vogel, 2009). New technologies and ideas can be imported and developed with specialists of other disciplines (e.g. Mitterhauser and Toegel, 2008; Schrattenholz and Klemm, 2007), and teaching of alternative approaches may be achieved in different ways (Jukes, 2008; Jukes, 2009; Leist,

2006; Hartung et al., 2009). However, much research in the 3R field addresses technical problems within already established concepts (e.g. Rothen-Rutishauser, 2008; Heindl, 2008; Wanner, 2008; Li, 2008a,b; Hagelschuer et al., 2009; Bahramsoltani et al., 2009; Manzer et al., 2009; Hartung and Hoffmann, 2009; Sauer et al., 2009). The next generation of methods (see chapter below on ESDNT V2.0) should set its own standards instead of aiming at a 1:1 substitution of existing animal protocols with their own set of problems (Hartung, 2008a; Pelkonen et al., 2009; Vedani et al., 2009; Sauer, 2009).

3 Markers for DNT testing

3.1 Challenges for an *in vitro* DNT test system

A number of questions arise when one considers developing mESC, iPSC or hESC as potential test systems for DNT. These involve species, source, genotype, developmental status, throughput and endpoints of the model system. At the present stage, all different options and their combinations require testing, standardisation of protocols and exploratory activities, and a large variety of different approaches should be promoted and explored for a sufficiently long time before a rational selection process can be initiated with the goal of identifying a smaller set of assays that may be used for regulatory decisions. Therefore only some general considerations are highlighted here:

3.1.1 Species

For human predictivity, hESC may appear more promising than rodent systems. However, for comparison with already existing murine and rat *in vivo* databases, mESC may be more suitable. In general, mESC presently represent a system with higher throughput and robustness: neurons are generated much faster and with higher yield than in the human system. As many more laboratories have worked with mESC compared to hESC, there is more experience in using the murine cultures. They are easier to handle, and the tools to genetically modify these cells are more advanced, while hESC show considerable variability *in vivo* and *in vitro* (Parsons et al., 2009; Wu et al., 2007; Osafune et al., 2008; Abeyta et al., 2004). It is also evident that hESC

Tab. 1: Marker genes for mESC

name	accession number	full name	comment	ref
Bat1a	NM_019693	HLA-B-assoc. transcript 1A		[1]
Cd9	NM_007657	Tspan29	cell migration and adhesion	[2]
Cxxc1	NM_028868	Cgbp, Cxxc finger 1 (PHD domain)	Cgbp knock-out cells are viable but unable to differentiate upon removal of LIF	[3]
Myc	NM_010849	C-myc, myelocytomatosis oncog.		[4]
Dppa2	NM_028615	dev. plurip.-assoc. 2	expressed in human pluripotent stem and germ cells	[5], [6]
Dppa3	NM_139218	Stella, dev. plurip. Assoc. 3		[6]
Dppa4	NM_028610[a)]	dev. plurip. assoc. 4	inner cell mass	[6], [5]
Dppa5a	NM_025274	Esg1, dev. plurip. assoc. 5		[6], [23]
2410004A20-RIK	NM_025890	Ecat1, ES cell assoc. transcript 1	also called Oeep 48	[7]
Eras	NM_181548	Ecat5, ES cell-expressed Ras	involved in the control of ES cell proliferation	[8], [9], [10]
Esrrb	NM_011934[b)]	estrogen receptor, beta	activates Oct4 transcript., sustains self-renewal and plurip.	[11], [12]
Fbxo15	NM_015798	ecat3, F-box only protein 15	target of Oct4/Sox2	[8], [13], [14]
Fgf4	NM_010202	fibroblast growth factor 4	target of Oct4/Sox2, activates Erk	[15], [14]
Gab1	NM_021356	GRB2-assoc. binding protein 1	expressed in blastocyst	[16], [17]
Gjb3	NM_008126[c)]	Cx31, Connexin 31	gap junction protein, specific for mESC	[18]
Gnl3	NM_178846[d)]	Nucleostemin	low in EB, but also expressed in NPC	[19], [20]
Khdc1a	NM_183322	KH domain containing 1A	member of the Khdc1/Dppa5/Ecat1/Oeep family	[7], [21]
Khdc1b	XR_031927[e)]	Khdc1c, KH domain cont. 1C	member of the Khdc1/Dppa5/Ecat1/Oeep family	[21]
Klf4	NM_010637	Kruppel-like factor 4	inhibits cell differentiation, target of Oct4/Nanog	[22], [23]
Klf5	NM_009769	Kruppel-like factor 5	related to Klf4	[24]
Lefty2	NM_177099	left-right determination factor 2	antagonistic Tgfbeta ligand, sometimes called Leftb	[25]
Lefty1	NM_010094	Left-right det. factor 1	target of Klf4/Oct4/Sox2	[26], [27]
Lin28	NM_145833	ln-28 homolog	reprogramming factor, RNA-binding protein	[28], [29]
Msh2	NM_008628	mutS homolog 2	DNA repair protein, downregulated during diff.	[30]
Msh6	NM_010830	mutS homolog 6	DNA repair protein, downregulated during diff.	[30], [31]
Nanog	NM_028016[f)]	Nanog homeobox		[8], [32]
Phc1	NM_007905[g)]	polyhomeotic-like 1	regulation of Hox genes via Polycomb	[33]
Phf17	NM_172303[h, i, j)]	Phd finger protein 17		[34]
Pou5f1	NM_013633	Oct4, POU domain, class 5, transcription factor 1	transcription factor regulating plurip.	[14], [32]

behave differently from mESC concerning the pathways that control stemness. It has been suggested that they correspond to epiblast stem cells rather than to inner cell mass-derived cells, as do mESC, and they may not be able to form chimeras and an organism (Li and Ding, 2009). Continuing basic research on robust and more rapid hESC protocols is still needed to eventually provide a model system that avoids the species differences and the necessity for an interspecies extrapolation.

3.1.2 Type of cells used as starting material

Different cell types have been used to study aspects of DNT. ESC are derived from the inner cell mass of blastocysts (Martin, 1981; Evans and Kaufman, 1981; reviewed in Leist et al., 2008a), and, using ESC-based models, all developmental steps are accessible for examination (Winkler et al., 2009). The downside of this approach is that the cells need to be directed through all differentiation steps, preferably in a synchronised way, even under circumstances when only information on the last step is of interest. To avoid this problem, various other cell types have been used to study particular stages of DNT. For instance primary neurons or certain neuroblastoma, phaeochromocytoma or teratoma cells can differentiate to a partially neuronal phenotype (e.g. axonal elongation and maturation), and this forms the basis for many test systems, which are of more limited scope but often of high reproducibility and throughput (Radio and Mundy, 2008; Radio et al., 2008, 2009; Hogberg et al., 2009, 2010). An intermediate solution would be the use of neural stem cells or neuroblast-like cells, which may be developed from ESC and that do not necessitate the initial differentiation steps required for ESC but still have the potential to develop into a number of different, morphologically and functionally mature neuronal and glial cell types (Buzanska et al., 2009; Breier et al., 2008; Wang et al., 2007). The advantages and disadvantages of such systems illustrate an important issue of DNT testing. The downside is that such NSC-based systems cannot model the initial phase of neuroectoderm specification and formation. Thus, the effect of compounds on this developmental period, associated with an important coordinated wave of gene transcription, cannot be tested. The upside of the use of NSC is that other phases, e.g. the step from NSC or neuroblasts, can be examined with

name	accession number	full name	comment	ref
Rest	NM_011263	RE1-silencing transcription factor	maintains self-renewal and plurip., (also NSC), discussed	[35-39]
Sox2	NM_011443	SRY-box containing gene 2	transcription factor regulating plurip., (also NSC)	[14], [32]
Stat3	NM_213660[k, l)]	signal transducer and activator of transcription 3	involved in LIF signaling	[40], [23]
Stip1	NM_016737	stress-ind. phosphoprot.	role in plurip. signaling	[41]
Tcfcp2l1	NM_023755	transcription factor CP2-like 1		[2], [23]
Tdgf1	NM_011562	Cripto, teratocarcinoma-derived growth factor 1	target of nanog, Oct4, SMAD	[8], [42]
Tdh	NM_021480	L-threonine dehydrogenase		[43]
Tead4	NM_011567	TEA domain family member 4, TEF-1-related factor 1	expressed from 2 cell stage on to blastocyst	[44]
Tert	NM_009354	telomerase (RT)	reverse transcriptase	[45]
Tex19.1	NM_028602	Nuclear protein	also germ line	[46]
Timp1	NM_011593[m)]	tissue inhibitor of metallo-proteinase 1		[2], [47]
Utf1	NM_009482	undifferentiated embryonic cell transcription factor 1	target of Oct4/Sox2	[8], [14], [48-50]
Zfp42	NM_009556	Rex1, zinc finger protein 42		[8]
Zic3	NM_009575	zinc finger protein of the cerebellum 3	required for maintenance of plurip. in ES cells and neural crest development	[51], [52]

Additional accession numbers:
a) NM_001018002, b) NM_001159500, c) NM_001160012, d) NM_153547, e) NM_001033904, f) NM_001080945, g) NM_001042623, h) NM_001130184, i) NM_001130185, j) NM_001130186, k) NM_213659, l) NM_213660, m) NM_001044384

1. Sharov et al., 2003; 2: Abranches et al., 2009; 3. Carlone et al., 2005; 4. Lewitzky and Yamanaka, 2007; 5. Maldonado-Saldivia et al., 2007; 6. Bortvin et al., 2003; 7. Imamura et al., 2006; 8. Mitsui et al., 2003; 9. Takahashi et al., 2003; 10. Sorrentino et al., 2007; 11. Zhang et al., 2008; 12. Feng et al., 2009; 13. Tokuzawa et al., 2003; 14. Okumura-Nakanishi et al., 2005; 15. Kunath et al., 2007; 16. Schaeper et al., 2007; 17. Xie et al., 2005; 18. Worsdorfer et al., 2008; 19. Tsai and McKay, 2002; 20. Beekman et al., 2006; 21. Pierre et al., 2007; 22. Li et al., 2005; 23. Wei et al., 2005; 24. Ema et al., 2008; 25. Hamada et al., 2001; 26. Farthing et al., 2008; 27. Nakatake et al., 2006; 28. Hagan et al., 2009; 29. Hanna et al., 2009; 30. Roos et al., 2007; 31. Mason et al., 2009; 32. Chambers and Tomlinson, 2009; 33. Isono et al., 2005; 34. Tzouanacou et al., 2003; 35. Singh et al., 2008; 36. Canzonetta et al., 2008; 37. Johnson et al., 2008; 38. Buckley et al., 2009; 39. Jørgensen et al., 2009; 40. Kues et al., 2005; 41. Longshaw et al., 2009; 42. Liu et al., 2005; 43. Wang et al., 2009; 44. Nishioka et al., 2009; 45. Armstrong et al., 2005; 46. Kuntz et al., 2008; 47. Singla and McDonald, 2007; 48. van den Boom et al., 2007; 49. Nishimoto et al., 2005; 50. Okuda et al., 1998; 51. Lim et al., 2007; 52. Nakata et al., 1998

highly synchronised cells and therefore less experimental noise. Ideally, many different test systems will be used to optimally test potential DNT during all important phases of nervous system development with high sensitivity and specificity.

3.1.3 Culture quality

The particular setup of the cultures is a major factor for the success of a DNT test system, independent of the endpoint chosen. Therefore we will just briefly list some of the factors that may be important for transcription markers in ESC-based systems. Some cultures are grown on feeder cells, which might affect the pattern of RNAs detected as well as the differentiation process and the effect of chemicals on the overall culture system. Similarly, undefined components of the culture medium, antibiotics or the adhesion matrix might have complicating effects. Most importantly, the quality of the cells is a major factor for the experimental outcome. The most serious deficiency of a test system would be infection or genetic alteration. However, also mycoplasma-free, genetically intact cells may be altered epigenetically, and this may be a major source of experimental variation (Fig. 1). Only frequent and stringent controls and efforts to avoid uncontrolled factors as listed above can lead to robust experimental test systems.

3.1.4 Genotype

In the 21st century we can begin to ask whether there is an interaction of genetic and environmental factors (gene x environment effect) for DNT and whether our test systems could also yield such information and identify groups specifically at risk. For example, hESC from different ethnicities, genders and genotypes can now be compared. New opportunities have arisen from the general availability of the technique to generate human induced pluripotent stem cells (hiPSC), which behave like hESC but can be generated from presumably all somatic cell types including skin samples of individuals. Soon, libraries will be available of human iPSC with defined genetic defects occurring in human diseases and with allelic variations as identified in many human individuals. The use of such cells for more genotype-related information in safety sciences appears very attractive. The murine counterpart is the availability of over 20,000 gene trapped mESC lines (defined reporter insertions at intron-exon boundaries that may be further genetically engineered e.g. to generate transgenic reporter or selection lines for many endogenous promoters) (Singla et al., 2010) and of thousands of transgenic and knock-out mice with the corresponding mESC derived therefrom. Such mESC may be generated by targeting of the second allele of heterozygous knock-out mESC (Madan et al., 2009) or from the mice in two different ways. Traditionally, mESC would be derived from blastocysts of homozygous matings. This process has been greatly simplified lately by small molecule chemicals that support mESC generation (Ying et al., 2008; Li and Ding, 2009). An emerging technology promises the generation of pluripotent stem cells from cells of mice by different techniques of reprogramming (Lewitzky and Yamanaka, 2007; Kim et al., 2009a; Stadtfeld et al., 2010; Carey et al., 2010). Transgenic approaches, that also allow expression of human proteins in mice, have already been applied to the examination of the role of certain genes in diseases and pathologies. Especially the availability of mESC with reporter constructs has been broadly applied to high-throughput screens, e.g. for compounds affecting DNT (Suter and Krause, 2008; Suter et al., 2009; Conti et al., 2005). Similar reporter constructs have been introduced and used in hESC or iPSC, but there is still ample room for further development and improvement.

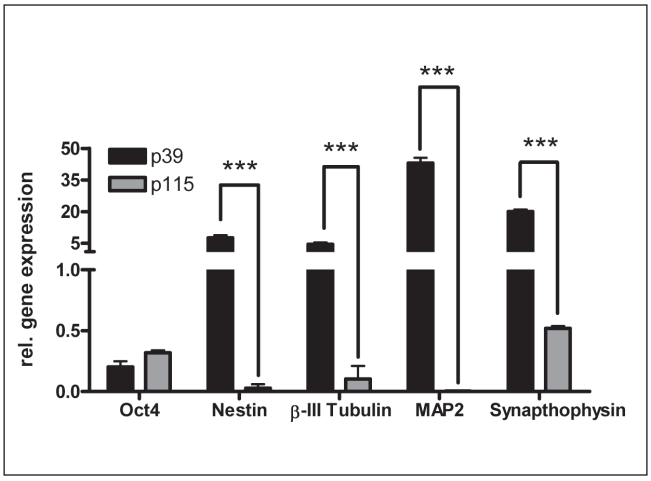

Fig. 1: Different neuronal differentiation potential of mESCs from the same strain.
CGR8 mESC were kept under routine culture conditions (details available from Leist lab). High (passage 115, p115) and low (passage 39, p39) passage cells were triggered to differentiate towards the neuronal lineage in parallel. After 20 days of differentiation, total mRNA was extracted and analysed by quantitative real time PCR for marker genes of mESC (Oct4), NSC (Nestin) or neuronal (βIII Tubulin, MAP-2, Synaptophysin). Gene expression levels were first normalised to the housekeeping gene GAPDH and then to the expression in undifferentiated mESCs (day 0), which was arbitrarily set to 1. Data represent means ± SD from triplicates. *** $p < 0.001$

3.1.5 Pluripotency status and capacity to form any neural cell

The use and culture of ESC is a demanding technology requiring high standards of good cell culture practice. The lack of standardised protocols used for cell differentiation appears to be a main source of low reproducibility. Additionally, at present no single marker can indicate conclusively that a cell has left the developmental status of mESC or hESC and that this cell may therefore not be suitable for DNT testing any longer. Only groups of markers can be used (Tab. 1). Similar questions apply when iPSC are generated but need to be evaluated for their "real" pluripotency. This practical problem is illustrated by data shown in Figure 1. The cells from different passages (mESC, CGR8 strain) behaved similarly when they were maintained in culture (similar growth rate and morphology). Only when the differentiation potential was tested did dramatic differences become obvious. Similar findings have been reported for hESC that expressed similar levels of a small set of markers (Nanog, Oct4, Tdgf1) but had dramatically different differentiation potentials (Osafune et al., 2008).

It has been shown beyond doubt that intact mESC have the full potential of a pluripotent stem cell, i.e. to generate every cellular phenotype (including every neural cell) in the organism. If DNT assays were to be developed on the basis of hESC, one objection may be that formation of complete brains has not been demonstrated. Both for scientific and ethical reasons this ultimate proof of pluripotency is unlikely ever to be provided. However, many relevant neural cell types can be formed from hESC. For instance, cells derived from hESC have been used for transplantation into brains of immunodeficient mice and integrated functionally (Laflamme et al., 2007; Elkabetz et al., 2008; Koch et al., 2009; Sharp et al., 2009). Also, 3-dimensional "brain-like" engineered neural tissue (ENT) has been generated *in vitro* from hESC (Preynat-Sauve, 2009). Thus it appears that hESC should be also suitable as a test system to cover the full range, or at least most aspects, of DNT once simple and robust protocols and a full characterisation of the functionality of resultant cultures are available.

3.1.6 DNT specific processes and endpoints

Neurodevelopment is a highly complex biological process that involves proliferation, migration, apoptosis, differentiation, synaptogenesis, neurite and network formation, as well as gliogenesis and myelinisation. All these processes need not only to be functional, but also require correct timing and complicated balances within a microenvironment often referred to as a "niche". Therefore, one single type of endpoint is unlikely to be sufficient for a comprehensive description of the overall outcome. Experimental endpoints that have been tested comprise electrophysiology, neurotransmitter release, immunostaining and other methods of protein quantification including several proteomics techniques, methods of RNA quantification, functional cellular assays and evaluations of cellular morphology. In general, endpoints that have been shown to be suitable for other cellular test systems should also be useful for mESC or hESC. However, there can be practical limitations. These are mainly due to the heterogeneity of the cultures, which precludes certain methods of quantification. This heterogeneity may be desired, e.g. for generation of "organ simulating tissues". In most cases it is accidental or stochastic, as currently-used protocols lead to the generation of different cell populations that are not homogeneously distributed but may rather grow in patches or islands within a dish. Moreover, some cells grow preferentially on top of or under other cells. In this situation it is particularly important to select endpoints that guarantee robustness (reproducible results, also when experimental conditions vary slightly), are biologically plausible and allow optimal predictivity. It is beyond the scope of this review to evaluate the usefulness of all different endpoints for DNT testing, and the experimental evidence for this. Instead, general principles of assay set-up will be discussed below in more detail for embryonic stem cell-based developmental neurotoxicity testing (ESDNT) testing.

3.2 *In vitro* DNT testing and validation: ESDNT V1.0 vs. ESDNT V2.0

Every *in vitro* toxicity test system consists of three elements: the biological system, the endpoint/test procedure and the prediction model. This is exemplified by the already validated and well-established **e**mbryonic **s**tem cell **t**est (EST) used as a general predictor of teratogenicity (Marx-Stoelting et al., 2009; Seiler et al., 2006; Genschow et al., 2004; Laschinski et al., 1991). The biological test system uses in this case mESC and murine fibroblasts under different growth and differentiation conditions, one of them being a relatively non-specific differentiation of mESC to cardiomyocytes. The test procedure is defined by INVITTOX PROTOCOL no. 113 (DB-ALM data base; http://ecvam-dbalm.jrc.ec.europa.eu/). The presence of foci of beating cells is the endpoint for cardiac differentiation. The prediction model involves mathematical comparisons between different endpoints (e.g. IC_{50} of the cytotoxicity to fibroblasts and altered efficiency of cardiac differentiation), classification of the potential results and translation of these classes into potential human toxicity classes. As evident from this example, each of the three main elements can be developed and optimised relatively independently from the others. A number of developmental neurotoxicants are also identified in this assay, presumably due to their broad teratogenic potential (Chapin and Stedman, 2009; Buesen et al., 2009).

For the validation of each test system, three major domains need to be considered (Hartung et al., 2004; Hoffmann and Hartung, 2006):

3.2.1 Reproducibility

This includes parameters like robustness of the test system, comparability of data obtained in different laboratories or by different operators, on different days or in parallel replicates. It is related to technical features of the assay.

3.2.2 Predictivity

The correlation of the *in vitro* results with the known human data or a corresponding "gold" standard (often *in vivo* animal data). Predictivity can be fine tuned by changes in the biological system, the test procedure or the prediction model. However, it remains in the end a mathematical-correlative exercise, which neither requires, nor indicates, relevance. Correlations may also be generated easily by simple mathematical tricks (Fig. 7 in Leist et al., 2008c). The definition of predictivity on the basis of correlations has some implicit consequences. As the set of compounds used for the correlations is necessarily small, compared to all possible compounds that may be used in the test system, it may not be representative to the same degree for all classes of compounds. Therefore, the prediction model has a certain applicability domain, e.g. it applies to a certain group of compounds used for the validation process (e.g. genotoxic carcinogens for the Ames test). It may fail completely when different compounds (e.g. epigenetic carcinogens in the above example) are used.

3.3.3 Biological relevance

For the above reason, this third domain is highly desired in a test system. It has been given less priority than the two other domains in the development of the first generation of alternative methods. With the rise of the tox21c idea, this should become the dominant domain in the near future. Biological relevance should be the basis of predictive systems biology. This has a major impact on the design of new test systems for DNT.

The EST would be considered a first generation test system optimised for predictivity based on correlation. With respect

to neurally-active teratogens (DNT field) it may be called an ESDNT V1.0 (embryonic stem cell based developmental neurotoxicity test, version 1.0). It operates predominantly as a black box system, similar to reproductive toxicology studies in animals. Understanding of the mechanisms is not required to derive the results and the regulatory consequences in both positive or negative cases. Moreover, and it is difficult to obtain information from this system on why positive compounds are positive and why negative compounds are negative. However, as this information is not required for regulatory testing of chemicals, a good correlation was sufficient for successful validation.

The EST may be adapted in different ways for DNT testing. However, in all cases a fundamental difference between cardioteratogenicity and neuroteratogenity needs to be considered: the heart consists of a limited number of cell types in a relatively homogeneous tissue arrangement, and most developmental effects on the heart have some form of histological or morphological correlate. The nervous system consists of many different cell populations, and DNT, as well as many CNS diseases, can have predominantly behavioural and functional consequences (e.g. on regulation of mood, intelligence, attention, concentration, motor activity) without obvious morphological correlates. This needs to be taken into account when test systems are be-

Tab. 2: Neural stem cell markers*

name	accession number	full name	comment
A230098A12Rik	NM_175485	Prtg protogenin homolog	transient neuroepithel. progenitor
Ascl1	NM_008553	Mash 1	
Atoh1	NM_007500	Math1	
Bmi1	NM_007552	Polycomb complex protein BMI-1	important for proliferation
Calcr	NM_007588[a)]	Calcitonin receptor	
Cdh2	NM_007664	N-cadherin	
Chd1	NM_007690	Chromodomain-helicase-DNA-binding prot. 1	
Chrdl1	NM_001114385[b)]	Chrodin like protein 1	
Crabp2	NM_007759	Cellular retinoic acid-binding protein 2	
CtnnB1	NM_007614	Catenin beta-1	
Cxcr4	NM_009911	C-X-C chemokine receptor type 4	adult and foetal NSC, signalling
Cyp24A1	NM_009996	Vitamin D-hydroxylase	
Dbx1	NM_001005232	Developing brain homeobox protein 1	also adult NSC
Dbx2	NM_207533	developing brain homeobox 2	
Dll3	NM_007866	Delta-like protein 3	foetal NSC; Notch ligand
EfnB2	NM_010111	Ephrin-B2	assoc. with nestin
Fabp7	NM_021272	Fatty acid-binding protein, brain	especially RG
Fgf5	NM_010203	Fibroblast growth factor 5	neurectoderm
Fgfr2	NM_201601[c)]	Fibroblast growth factor receptor 2	
FoxB2	NM_008023	Forkhead box protein B2	very early
FoxD3	NM_010425	Forkhead box protein D3	
Frzb1	NM_011356	frizzled-related protein	
Fzd1	NM_021457	Frizzled-1	Shh signalling
Fzd3	NM_021458	Frizzled-3	Shh signalling
Gata2	NM_008090	Endothelial transcription factor GATA-2	
Gpr23	NM_175271	lysophosphatidic acid receptor 4	
Gsh2	NM_133256	GS homeobox 2	
Hes5	NM_010419	Hairy and enhancer of split 5	Notch-target
Hes6	NM_019479	Hairy and enhancer of split 6	Notch-target
Id2	NM_010496	Inhibitor of DNA binding 2	BMP/TGF pathway
Ireb2	NM_022655	Iron-responsive element-binding protein 2	
Lhx1	NM_008498	LIM/homeobox protein Lhx1	
Lhx9	NM_001025565[d)]	IM/homeobox protein Lhx9	
Lrp	NM_008512	low density lipoprotein receptor-related protein	
Mbnl	NM_020007	muscleblind-like 1	
Meis1	NM_010789	Myeloid ecotropic viral integration site 1	
Metrn	NM_133719	Meteorin	also astrocytes
Msi1H	NM_008629	Musashi 1	RNA-binding
Msx1	NM_010835	Msh homeobox 1-like protein	inhibits neuronal differentiation

ing developed. For instance, the difference in the ratio between different neuronal populations needs to be detectable in the absence of an overall loss of cells. As different brain regions develop during different time windows, they display different sensitivities to neurotoxicants at different times. For instance, the DNT compound methylazoxymethanol (MAM) has different effects on the brain when given on different days of embryonic development (Penschuck et al., 2006 and references therein). Thus DNT test systems must also provide the option to apply potential toxicants in different phases of development.

Simple endpoints (for instance the number of all neurons or of functional neurons – similar to those used in the EST) are likely to be insufficient for DNT test systems. More refined endpoints that describe neuronal subpopulations and differentiation states are required. The use of RNA-based markers is suggested here as one possible approach to be explored.

Moreover, to make the test systems independent of narrow applicability domains and to design them for broad testing right from the start, the tox21c strategy suggests a toxicity pathway and mechanism-based approach (NRC, 2007). Such assays would examine quantitative cause-effect relationships with reference to relevant toxicity pathways, and the prediction model would integrate the rich information from multiple endpoints. Such future assay systems may then be labelled ESDNT V2.0.

name	accession number	full name	comment
Nedd9	NM_001111324[e)]	Enhancer of filamentation 1	
Nes	NM_016701	Nestin	Gold standard, broad profile
NeuroD4	NM_007501	Neurogenic differentiation factor 4	
Nfe2L2	NM_010902	Nuclear factor erythroid 2-related factor 2	
Nhlh2	NM_178777	Helix-loop-helix protein 2	
Notch1	NM_008714	Notch 1	
Nr2F1	NM_010151	COUP transcription factor 1	
Nr6A1	NM_001159548[f)]	Nuclear receptor subfamily 6 group A memb 1	
Ntrk3	NM_008746[g)]	NT-3 growth factor receptor	
Numb	NM_001136075[h)]	Protein numb homolog	
Otop1	NM_172709	Otopetrin-1	
Otx2	NM_144841	Orthodenticle homolog 2	
Pax3	NM_008781[i)]	Paired box protein Pax-3	also in some cells at later stages
Pax6	NM_013627	Paired box protein Pax-6	also at later stages
Prkcz	NM_008860[j)]	Protein kinase C zeta	
Prom1	NM_008935	Prominin-1, CD133	also other (haematopoietic) SC
Pxmp3	NM_008994[k)]	Peroxisome assembly factor 1	
Ror2	NM_013846	Tyrosine-protein kinase transmembrane R.	membrane receptor
Rtn1	NM_153457[l)]	Reticulon-1	
Runx1	NM_001111022[m)]	Runt-related transcription factor 1	
Rxra	NM_011305	Retinoic acid receptor RXR-alpha	
Ryr3	NM_177652	ryanodine receptor 3	
Sema5b	NM_013661	Semaphorin-5B	
Sfrp2	NM_009144	Secreted frizzled-related protein 2	
Sox1	NM_009233	Transcription factor SOX-1	
Sox11	NM_009234	Transcription factor SOX-11	in foetal NSC
Sox2	NM_011443	Transcription factor SOX-2	also in ESC
Tal2	NM_009317	T-cell acute lymphocytic leukemia protein 2	
Tcf4	NM_013685[n)]	Transcription factor 4	
Tnnc2	NM_009394	Troponin C	
Wnt5a	NM_009524	Protein Wnt-5a	
Wnt8b	NM_011720	Protein Wnt-8b	
Zic1	NM_009573	Zinc finger protein 1	

RG: radial glia; NSC: neural stem cells; OG: oligodendrocytes; SC: stem cells

Additional accession numbers:
a) NM_001042725, b) NM_031258, c) NM_010207, d) NM_001042577, NM_010714, e) NM_017464,
f) NM_010264, NM_001159549, g) NM_182809, h) NM_010949, i) NM_001159520, j) NM_001039079, k) NM_008994,
l) NM_001007596, m) NM_009821, NM_001111021, NM_001111023, n) NM_001083967

* Abranches et al., 2009; Maisel et al., 2007; Kelly et al., 2009; Vogel et al., 2009; Gaspard et al., 2008; Liu et al., 2004; Barberi et al., 2003; Ghosh et al., 2008

Here an initial basis is provided for the characterisation of the cells used in such assays.

4 The definition of stem cell genes

4.1 Transcription-based markers

For the definition of cell types and of transitions from one cell type to another, different sets of markers may be applied. These range from definition of the genome (primary sequence and epigenetic status) to definition of the proteome (protein based or antigen-based), and include the metabolome, functional characteristics (e.g. electrophysiological responses) and characterisation of the transcriptome (mRNAs and miRNAs). These approaches have different sensitivities, dynamic ranges, specificities, sample requirements, technical requirements and throughput.

The most frequently used approaches are antigen based methods and transcriptional profiling. The former have been dealt with elsewhere, and extensive studies in the stem cell field have been performed e.g. by BD Biosciences (www.bdbiosciences.com). Briefly, they are particularly useful for single cell characterisation and for sorting cells, only limited by antibody availability (works best for surface antigens). Quantitative evaluations by this approach usually involve flow cytometric analysis and work particularly well in non-adherent cultures or with cells that can be detached by enzymatic treatment without affecting the epitope. Use on adherent cells requires advanced imaging technologies and is often harder to quantify and to control. On a semi-quantitative or qualitative level, antigen staining offers an easy option to characterise mixed cell populations and to determine co-localisation of different markers within a given cell.

RNA-based measurements have been suggested to be particularly useful to characterise the differentiation of ESC (Noaksson et al., 2005) and to detect neurotoxicity and DNT (Hogberg et al., 2009; Bal-Price et al., 2009; Stummann et al., 2009). Transcriptional profiling has been used in many fields, for instance to indicate cellular activation states (Henn et al., 2009; Lund et al., 2006; Falsig et al., 2006). The method is frequently used successfully for quantitative studies in homogeneous populations of cells. More or less every gene transcript can be examined (few exceptions due to highly repetitive or highly GC-rich sequences). The expression pattern can be interpreted as a "signature" of the status of the tested cell population. The "signature" can be examined in terms of known cell specific markers, gene ontology (GO) classification systems and known gene interaction networks. For instance, different types and differentiation stages of neurons and glial cells differ in their RNA profiles, and these profiles differ from that of ESC (Tab. 1) or neural stem cells (NSC – Tab. 2). Therefore, definition of reference profiles for different culture states should permit the detection of subtle effects of developmental neurotoxicants and give information on the affected pathways. Deviations from the "default transcription signature" may permit the detection of subtle effects of developmental neurotoxicants, and give information as to the pathways affected. They may also occur as a consequence of cell cycle progression or cellular activation state. Such signatures and their alterations can also be obtained from a non-homogeneous mixed cell population and can give information on its relative composition. The transcriptional profiling approach has particular advantages for quantitative studies in inhomogeneous populations of adherent cells or for complex mixtures of cells, if appropriate cell specific markers and reference genes are available (see below – point (3)). The big disadvantage of the technology is that co-localisation studies are not readily possible, and therefore the specific cell subpopulation that undergoes changes in response to the toxin can be difficult to identify.

The use of transcription based endpoints (e.g. Northern blot, gene microarrays and PCR) also requires some technical considerations, as briefly summarised in Tab. 3. Microarray platforms may indicate relative expression differences with varying sensitivities and accuracies for different genes. Without detailed background data, information on a single gene may not be reliable. As an alternative, sets of interesting cell- or state-specific genes can be selected for detailed quantification of relative gene expression changes by quantitative real-time PCR methods. If profiling is performed by PCR on a selected set of genes, the technology is available in most laboratories at reasonable cost and throughput, and optimised primers for amplification can be derived from online databases (RTPrimerDB, http://medgen.ugent.be/rtprimerdb/).

As this review focuses on the compilation of gene lists that should be useful as background description of cellular states in DNT assays, three major technical issues of gene selection and classification will be discussed:

4.1.1 Gene annotation

First, the literature, including also relatively recent publications, is filled with strongly varying abbreviations for one given gene. This is due to the discovery and cloning process, which often occurred in parallel in different places, initial discovery in different species, protein and antigen names that differ from the

Tab. 3: Issues concerning identification and selection of transcription-based markers

Definition of applicability domain
Selection of criteria for appropriate markers (assay dependent)
Method for identification/qualification of markers
Selection of negative (exclusion) and positive markers
Assembly of set of markers (no single marker is adequate)
(Semi-)Quantitative relationship of markers (ratios; thresholds; yes/no)
Definition of differentiation status
Composition of culture over time
Selection of control population(s) for cell type specific endpoints
Biological validation of endpoint-markers with (positive and negative) controls
Timing of chemical exposure (duration and differentiation status)
Use of reference databases for cross-validation of data
Statistical and standardisation issues within and between experiments
Known species differences

gene name, and changes of names upon consolidation of the fully sequenced mouse and human genomes. We have chosen to include the currently-used official gene symbol that can be retrieved from PubMed (http://www.ncbi.nlm.nih.gov/pubmed) in addition to various other names in common use. In addition, the transcript accession number (as an unambiguous identifier) is listed. Notably, these accession numbers do not refer to the genes as such, but define cDNAs. They may e.g. characterise particular transcripts of genes with multiple splicing variants. Thus, one gene can have more than one accession number. This is highly important for expression analysis and corresponding database searches, as a given gene can form different transcripts in different cell types or at different differentiation stages. Therefore, problematic situations might arise where analysis of gene regulation by different methods (different PCR primers, different hybridisation oligos, etc.) yields different results. In such situations, different transcripts might have been analysed. To cover this situation, accession numbers for different splice and annotation variants of the same gene are also included in the tables. The NCBI RefSeq database provides annotated individual transcripts and protein sequences (derived from its predecessor, Genbank) with accession numbers that are distinguished by a two-letter prefix (http://www.ncbi.nlm.nih.gov/RefSeq/key.html). Curated transcripts for mRNA, noncoding RNA and protein sequences are distinguished by the prefixes NM_, NR_, and NP_, respectively. Other prefixes indicate original Genbank annotations (two-letter prefixes without a following underscore) or Refseq sequences that are undergoing annotation or curation (Typically XM_, XR_, XP_) among others. Ensembl, an alternative informative annotation and curation effort by the European Bioinformatics Institute (EBI) also curates sequences and splice variants derived thereof (www.ensembl.org). Typically, it is helpful to design gene expression strategies against the curated sequences, although it is important to be aware of (and design around) the potential for underlying variation in that transcript. The collective variation in gene expression can be viewed with the aid of online genome browsers as provided by the University of Santa Cruz (http://genome.ucsc.edu/) or the EBI (http://www.ensembl.org/index.html).

4.1.2 GO categories

When large-scale transcriptional profiling is performed, identified genes are often associated with gene ontologies (GO). The Gene Ontology Project is an initiative to classify genes and gene products according to known molecular functions with a defined and finite vocabulary (http://www.geneontology.org). GO classifications associate gene products with appropriate categories in the three principal areas “cellular component”, “biological process”, and “molecular function”. They are organised by a hierarchical relationship between these groups. When the transcriptional profile of a cell population changes, the altered transcripts often cluster to certain GOs, and these GOs can give useful information on the types of changes that are occurring (structural, signalling, differentiation). Thus, it may be useful to pick the genes of a hypothetical “mESC GO” to define the starting population of DNT experiments and the changes of genes characteristic for this population. Unfortunately such a GO does not exist, as GOs do not define cell types, but rather related functions of genes. Therefore mESC genes as endpoints of DNT tests need to be defined and agreed upon as in Table 1.

4.1.3 Standardisation and statistical issues

They can strongly influence the identification of marker genes. This applies in different ways to individual studies as well as to meta-analyses. In the former, normalisation, standardisation and cut-off procedures are mostly hidden in materials and methods in a way that makes them hard to control or to reproduce by peers. Alterations of expression levels are often calculated relative to housekeeping genes, but the stability and variance of these reference points is only very rarely indicated. However, these data and procedures have a large impact on specificity and sensitivity of the overall analysis. Housekeeping genes may be selected based on various criteria. Most importantly, the gene needs to be expressed in equal amounts relative to the total amount of cellular mRNA. In many cell types, this condition is fulfilled for *Gapdh, 18S ribosomal RNA* (18S rRNA), and *β2 microglobulin* (b2m). Other markers that are also used frequently comprise *Hprt, 28S ribosomal RNA* (28S rRNA), *Actb* or *Acta1*. More rarely found options are *Ribosomal protein L32* (RPL32) or *Phosphoglycerate kinase 1* (PGK1). However, these housekeeping mRNAs do not always behave according to the criteria set above (e.g. Der et al., 1998). This problem is particularly pronounced in differentiation experiments, as described here. In this case, the final cell type in the dish can be very different (overall phenotype, size, cell cycle status, metabolic activity, etc.) from the starting cell, and therefore express housekeeping genes at different levels. Similar problems may occur upon exposure to toxicants. Another type of problem lies in the heterogeneity of cells in DNT test systems. The cultures may contain different subpopulations that express house-keeping genes at different levels. Upon differentiation, the relative amounts of these subpopulations may change dramatically, leading to enormous practical challenges concerning the standardisation of gene expression levels. To circumvent this, samples are often referenced to a group of housekeeping genes instead of a single gene only. In other cases, it may be useful to employ subpopulation-specific reference points, such as *B3 tubulin or Fox-3* (NeuN) for neurons, and e.g. *Doublecortin* or *Neurogenin* to refer shifts in patterning markers more specifically to neuroblast-like cells within the overall population. Concerning meta-analysis (e.g. Assou et al., 2007; International Stem Cell Initiative, 2007; Bhattacharya et al., 2005; Bhattacharya et al., 2009), additional problems need to be considered. The statistical criteria and quality of the studies included in the meta-analysis might vary strongly, and the initial conditions and rules set within these analyses might be hard to trace. Therefore, it is dangerous to rely blindly on the summary of the outcome. This applies also to the table compilations presented here. If they are put to experimental scrutiny and trigger a constructive discussion and an improved second version, then a major goal of this review will already have been reached. Possibly subsets will have to be selected, according to the specific culture conditions and cell lines used and the questions asked.

4.2 What are stem cell genes?

At first sight, it may seem easy to extract stem cell genes from the existing literature. Dozens of papers have dealt with such genes and large numbers of microarray studies have been performed to identify such genes, but also doubt has been voiced on whether stem cells are really a defined entity at all, or whether they rather represent one of many possible transient states (Efroni et al., 2009; Zipori, 2004). The expression pattern associated with such states may vary between different stem cell lines. Such effects may be linked to higher dynamics of the genome than commonly expected. For instance, non-protein coding line elements, which make up a large proportion of the human genome, have been shown to be active as transposons in ESC and, even more commonly, in NSC. Such activity might affect the activity of classical genes directly, e.g. by insertion, or indirectly, by modification of pervasive transcription (Coufal et al., 2009; Muotri et al., 2007; Garcia-Perez et al., 2007; Muotri and Gage, 2006; Muotri et al., 2005).

For assay development we have to take a closer look at the important distinction between "stemness genes" and "stem cell marker genes". For the specific purpose of this review, the focus of the discussion will be on ESC (and NSC) markers.

Unfortunately, the term "stem cell marker gene" is less clear upon closer inspection than it appears. An easy definition would for instance be "a gene that is only expressed in mESC, and in no other cell type". Unfortunately, no such gene exists. The reverse definition may also be applied. "Negative stem cell markers" are genes that are by no means expressed in mESC. This is a definition that is useful in practice for quality control and for defining the onset of differentiation, but it is not sufficient for defining mESC (Assou et al., 2007; Bhattarachyan et al., 2009). The definition of negative markers is also not without ambiguity, as mESC cultures may often be contaminated with more differentiated cells. Upon transcriptome analysis it may then appear that apparently pure stem cells "express" certain genes usually not associated with mESC, such as *B3 tubulin, Keratins-8* and *-18* or *Alpha cardiac actin* (Ginnis et al., 2004; Bhattacharya et al., 2005). To establish an ESC database free of contaminations, cells may be sorted prior to analysis or selected on the basis of the activity of a sharply-regulated stemness gene like Utf-1 (Tan et al., 2007).

A pragmatic approach to the identification of ESC genes is to characterise transcriptome changes when ESC differentiate and to define those genes that are differentially highly expressed in ESC as stem cell genes. This approach has been taken many times in many variations (reviewed in Bhattacharya et al., 2009 and Efroni et al., 2009). The result was that these approaches consistently identified a small group of "usual suspect genes" for ESC, such as *Lefty2, Oct3/4, Nanog, Utf-1 and Tdgf1*. However, astonishingly large differences were observed between the studies. It was surprising that some studies found that mESC genes overlap with hESC genes only to a low degree, i.e. between 15 and 35% (Bhattacharya et al., 2005; Ivanova et al., 2002; Ramalho-Santos et al., 2002). This may indicate some intrinsic weaknesses of these studies (see e.g. paragraph on standardisation and statistics issue). An alternative explanation may be that the derivatisation of the lines affects their later phenotype (Navara et al., 2007). In addition, much of the variation may be due to real species differences. In fact, the biology of mESC and hESC shows distinct differences with regard to signals required to maintain pluripotency (Wei et al., 2005; Eckfeldt et al., 2005; Wang et al., 2009). In this situation, it is tempting to conclude quickly that hESC are more relevant for human physiology. However, strong evidence indicates that mESC may resemble cells of the human inner cell mass of the blastocyst more closely than hESC (Li and Ding, 2009). Moreover, it is not known whether some differences of ESC in culture have any effects on readouts for DNT. This can only be determined experimentally, and should be done so.

The overall approach of differential transcription profiling to identify ESC markers has some conceptual shortcomings: First, the factor of differential expression that is used as cut-off is often relatively low (e.g. 2-3 fold). This means that it would be very hard to identify an ESC contamination of around 30% within an otherwise fully differentiated cell population. This low cut-off also reduces the level of specificity such studies can achieve. Second, the "differentiated population" used for comparison was frequently obtained from embryoid bodies (EBs), i.e. 3-dimensional spheroids formed from ESCs when they are left to differentiate "wildly" (in a non-guided way, only triggered by withdrawal of pluripotency factors). This population contains cells from all three germ layers, and may not be relevant for the identification of differentially-expressed genes between ESC and differentiating neurons. Thirdly, this approach is bound to identify many "false positives", as two populations with different proliferation characteristics are being compared. Thus, genes involved in DNA synthesis, chromatin structuring and cell cycle regulation would be selected as putative stem cell genes. A variant of this approach was taken by the International Stem Cell Initiative (ISCI) to define hESC markers. Genes were grouped according to the similarity of their behaviour to that of Nanog when over 50 hESC lines were differentiated to EBs. The top 6 group comprises *Nanog, Tdgf1, Gabrb3, Dnmt3b, Gdf3, Pou5f1/Oct4* and the top 20 group additionally contains *Fgf4, Gal, Leftb, Ifitm1, Nodal, Tert, Utf1, Foxd3, Ebaf, Lin28, Grb7, Podxl, Cd9* and *Brix* (The International Stem Cell Initiative, 2007).

A third approach to identify stem cell genes is based on the concept that genes qualify for inclusion when they are required for the function and maintenance of ESC. These genes would be biologically defined as "stemness genes". This definition would also form the basis for the opening of a GO category under the field of "biological function". The most prominent examples for such genes are *Pou5f1/Oct4* and *Nanog* (Mitsui et al., 2003) or the *Klf* (Krüppel-like factor) genes. However, *Oct4* is also found in germ stem cells or cardiac differentiation (Stefanovic and Puceat, 2007), *Nanog* plays a role in neuronal differentiation (Molero et al., 2009) and *Klf-4* is also an oncogen (Rowland et al., 2005). The Wnt, FGF and BMP/TGF-ß pathways – and associated genes – are clearly involved in the maintenance of stemness, but they also play a role in dozens of other processes. The same type of ambiguity is found when one examines the genes that can be used for reprogramming. In addition to *Oct4*, *Nanog* and *Klf-4* above, for instance *Sox2, Lin28* and *Myc* are used. *Sox2* and *Myc* play roles not only in reprogramming but also in stem cell maintenance. However, they are not specific for ESCs,

as e.g. *Sox2* is highly expressed (and functional) in NSCs, and *Myc* is upregulated in many tumours and rapidly dividing cells.

In conclusion, simple rules for the selection of ESC marker genes cannot be applied. More advanced algorithms based on multiple markers are required as described below.

4.3 Definition of mESC markers

Based on the above, markers were filtered from the literature according to the following criteria:

(a) The gene needs to be expressed in mESC (differences between mESC and hESC need to be taken into account).

(b) The gene needs to be expressed in mESC considerably higher than in most other cell types. Frequently, ESC were compared to embryoid bodies (EBs). In other approaches mESC were compared to mNSC and other stem cell types (haematopoietic) to identify unique marker genes (Ivanova et al., 2002; Ramalho-Santos et al., 2002). An interesting approach in that direction was also taken by groups at the NIH (Bhattacharya et al., 2004; Bhattacharya et al., 2005; Bhattacharya et al., 2009; Ginis et al., 2004), when ESC were compared to RNA pools from normal differentiated tissue. This approach was taken one step further in a large meta-analysis, in which hESC expression profiles were compared to data retrieved from databases on over 100 tissue analyses (Assou et al., 2007). For the compilation of Table 1, especially co-expression at similar levels in NSC was used as an exclusion criterion. Notably, as mESC are defined by a group of genes, the criterion of absence of expression in other cells needs not be applied stringently, providing that it refers to different cell types for different marker genes. If a sufficiently large group of mESC marker genes is selected, it is likely that expression in other cells is cancelled out (averaged), while each of the genes should be expressed in mESC.

(c) The marker gene should not be expressed in neural stem cells and neuroectodermal cells and thus be different from the ones listed in Table 2. This condition is a specific limitation of condition 2 and applies particularly for mESC markers used in DNT experiments. For instance, *Galanin* is a frequently-identified mESC gene, but also plays a role in NSC and certain mature neurons. Genes with such behaviour may not be downregulated upon mESC differentiation towards the neuronal lineage and are therefore useless as mESC markers for this particular purpose. A vast amount of gene expression data is available to identify relevant genes. Here, both individual papers (e.g. Abranches et al., 2009) and databases were used for identification and exclusion of candidates. For instance, the EU fifth framework research programme (FP5)-consortium FunGenES provides extensive transcriptome profiling information on the differentiation of mESC to neurons, coupled to web-based analysis software (FunGenES consortium → http://www.fungenes.org/) (Schulz et al., 2009). Similar approaches are taken for instance by the StemBase of the Ontario Genomics Innovation Center (StemBase → http://www.stembase.ca/?path=/) (Perez-Iratxeta et al., 2005; Porter et al., 2007).

(d) Genes with a known functional role for the maintenance of mESC (e.g. loss of stemness upon their knockdown or knockout (Misui et al., 2003)) are included as markers if they do not have multiple roles also in other cell types. The reasoning is similar as for the markers identified by expression level. Stemness genes are not included when their expression is relatively low compared to neural tissue expression.

(e) Only mRNAs coding for proteins have been considered for this analysis. Information on micro RNAs (miRNA) is still relatively limited. However, it appears that expression of miRNA can be relatively cell type-specific. Thus, miRNAs with relatively high expression levels in mESC compared to other cell types may be identified (Lakshmipathy et al., 2007a,b). Similarly, miRNAs important for defined steps in neuronal development have been identified (Yoo et al., 2009). Future profiles may therefore also include miRNAs. A further step may be a more detailed analyses of the promoters themselves and their epigenetic state by chip-on ChIP experiments (microarray analysis of chromatin immunoprecipitates), bisulfite sequencing (identification of methylcytosine as altered base in the DNA structure) or one of the many related new technologies. For instance, it has been suggested that the ESC genome may be characterised and defined by relatively open chromatin architecture (Zipori, 2004; Eckfeldt et al., 2005). This has been corroborated on the molecular level by genome-wide mapping of the chromatin state of ESC and other cells and indeed has functional consequences (Mikkelsen et al., 2007). The resultant pervasive transcription is particularly prominent in ESC, and a major difference between ESC state and more lineage committed differentiation stages may be the extent of this genome wide transcriptional activity (Efroni et al., 2009), that involves many non-protein coding RNAs (Berretta and Morillon, 2009; Dinger et al., 2009; Jacquier, 2009; Mikkelsen et al., 2007). To transform this knowledge into robust test endpoints and defining markers, the identification of ESC-specific non-coding RNA would appear useful. Indeed, recently over a thousand conserved large intervening non-coding RNAs (lincRNAs) have been identified (Guttman et al., 2009). About 100 were regulated by Oct4 and Nanog and functionally implicated in a stemness network, and at least one was only expressed in ESC. Thus, lincRNAs are candidates for future lists of differentiation and cell activation-defining lists of markers.

(f) Last, but definitely not least, negative markers should be used in transcription-based cell characterisations. The mESC table contains only positive markers, as naturally all genes listed in Table 2 (or other tables presented here) represent the corresponding negative markers. Typical markers for endodermal differentiation (e.g. intestine, glands, liver) would be *VegfR2, Sox17, Ttr, ApoA1, Lim1, Cytokeratin19, FoxA2, Alphafetoprotein* or *Gata-4* (also mesendoderm and cardiac mesoderm); for mesoderm (e.g. muscles, bones, heart, blood): *Hand1, Brachyury, Smooth muscle actin, Cd31, Cd34, Cd325* or *Eomes* (also trophoblast), and e.g. *Ncam1* or certain keratins (*Krt 18*) indicate ectoderm. Other useful and sensitive markers for initial differentiation away from ESC may be *Fibronectin-1, Naalad2, Profilin-1* and *Slc40a1*.

4.4 Neurodevelopmental biology and definition of neural stem cell markers

Differentiation of mESC towards neurons triggers coordinated waves of gene transcription that can be identified by unbiased cluster analyses (Abranches et al., 2009; Schulz et al., 2009). Accordingly, the cells move from the multipotent stem cell state

over an early neuroectoderm state to a state in which they can form rosettes that still have the potential to develop to central and peripheral neurons. This state is closely linked to the production of neural precursor cells or NSC. Such NSCs (human or murine) may be enriched and clonally expanded under appropriate culture conditions (Ying and Smith, 2003; Conti et al., 2005; Barberi et al., 2003; Koch et al., 2009; Elkabetz et al., 2008; Okabe et al., 1996). NSC markers may be derived from gene expression profiling of clonally-expanded NSC-like cells. This has for instance been done for human rosette-type cells vs. hESC (Elkabetz et al., 2008), but multiple comparisons against different populations (including more mature neurons) would be required to define the genuine NSC genes. NSCs, while sharing common properties of undifferentiated progenitors, may exhibit distinct regional capacities for neural differentiation to specific lineages or neurotransmitter phenotypes (Klein and Fishell, 2004). Similarly, ESC-derived NSC-like cells can acquire region-specific phenotypes depending on the differentiation protocol (Bouhon et al., 2006; Gaspard et al., 2009). Therefore, not all cells fulfilling basic criteria for NSC can still be differentiated to all CNS cell types. Differences also exist between ESC-derived NSC, and brain-derived NSC (both can only be obtained by extensive *in vitro* culturing, potentially leading to artefacts), for instance in the readiness to generate astroglial cells, or between spinal cord NSC and cortical NSC in the expression of many patterning marks and genes with broadly varying biological function (Kelly et al., 2009). Thus, it is not a straightforward and unambiguous approach to define NSC markers by characterising one given NSC population that can be maintained in culture.

Defined protocols for mESC neuronal differentiation typically involve initial neural specification and expansion under NSC growth conditions, followed by withdrawal of growth factors

Tab. 4: Markers for fine mapping of DNT effects in developing neural cells

category	name	accession number	full name	comment
rostral-caudal	FoxG1	NM_008241[a)]	forkhead box G1 (Bf1)	very rostral
	Emx1	NM_010131	empty spiracles homolog 1	very rostral
	Emx2	NM_010132	empty spiracles homolog 2	forebrain
	Dlx1	NM_010053	distal-less homeobox 1	forebrain
	Nkx2.1	NM_009385	Titf1, NK2 homeobox 1	forebrain
	Gsx2	NM_133256	GS homeobox 2	forebrain
	En1	NM_010133	engrailed 1	midbrain
	Otx1	NM_011023	orthodenticle homolog 1	dorsal fore- and midbrain
	Otx2	NM_144841	orthodenticle homolog 2	dorsal fore- and midbrain
	Atoh1	NM_007500	Math1, atonal homolog 1	hindbrain
	Irx3	NM_008393	Iroquois related homeobox 1	caudal
	HoxA2	NM_010451	Homeobox A2	rostral hindbrain
	HoxB1	NM_008266	Homeobox B1	rostral hindbrain
	HoxB4	NM_010459	Homeobox B4	caudal hindbrain
	HoxB6	NM_008269	Homeobox B6	spinal cord
dorsal-ventral	Shh	NM_009170	sonic hedgehog	ventral
	Gli3	NM_008130	GLI-Kruppel family member	dorsal forebrain
	Olig2	NM_016967	OC transcription factor 2	hindbrain
	Isl1	NM_021459	ISL1 transcription factor	forebrain
	Nkx2.2	NM_010919[b)]	NK2 transcription factor	ventral
	Neurog2	NM_009718	neurogenin 2	dorsal forebrain
	Nr2f1	NM_010151	nuclear receptor subfamily 2	ventral forebrain
	Ascl1	NM_008553	Mash1, achaete-scute complex homolog 1	ventral forebrain
	Msx1	NM_010835	homeobox msh-like 1	ventral midbrain
	Dll1	NM_007865	delta-like 1	ventral midbrain
neuronal subtypes	GAD2	NM_008078	glutamic acid decarboxylase 2	GABAergic neurons
	Gat3	NM_144512	solute carrier family 6a13	GABAergic neurons
	Calb1	NM_009788	calbindin 1	GABA subtypes
	Calb2	NM_007586	calbindin 2, calretinin	GABA subtypes
	TH	NM_009377	tyrosine hydroxylase	dopaminergic neurons
	VGlut2	NM_080853	solute carrier family 17a6	glutamatergic neurons
	Adra2b	NM_009633	adrenergic receptor	adrenergic neurons
	Tph1	NM_009414	tryptophan hydroxylase 1	serotonergic neurons
	Chat	NM_009891	choline acetyltransferase	motor neurons
	Mnx1	NM_019944	HB9, motor neu. And panc. homeobox 1	motor neurons

and neuronal differentiation. The differentiation process is usually not 100% synchronised, and cellular differentiation stages form a continuum. Therefore, the wave of NSC gene expression may overlap with the antecedent mESC gene expression and with the following wave of NSC-derived neuronal/glial gene expression. Consequently, it is difficult to strictly define NSC patterns of gene expression solely within the context of an *in vitro* differentiation system. For this reason, changes in gene expression are interpreted with reference to those observed during neural specification and lineage progression *in vivo* (Rubenstein and Puelles, 1994; Rubenstein et al., 1998).

A basic characteristic of the nervous system is the high diversity of different cell types, which is necessary for appropriate function. Neuronal differentiation proceeds in a region-specific manner, depending on the position of neuroepithelial progenitors along the rostrocaudal or dorsoventral axes (Fig. 2). This regional patterning is thought to be achieved by cell-extrinsic, contrasting gradients of morphogens and growth factors, including Bone Morphogenetic Proteins (BMPs), Sonic Hedgehog (Shh), Retinoic acid, Fibroblast Growth Factors (FGFs), etc. These chemical gradients establish a positional axis that confers region-specific patterns of gene expression and directs lineage-specific differentiation. This information has been used to compile the list of markers for *in vitro* differentiation (Tab. 2). The translation of knowledge from developmental gene expression to *in vitro* gene expression is not without caveats. For instance, the gradients formed *in vivo* are complex and not stable over time. For instance, NSC formation in the neural tube structure begins rostrally, and zones of NSC formation and patterning are moving in a rostro-caudal (from head to tail) way along the neural tube (Wilson and Maden, 2005). *In vivo* neurulation is also a desynchronised process. Homogenates used for transcriptional

category	name	accession number	full name	comment
NCC	Sox10	XM_128139	SRY-box containing gene 10	transcription factor
neuroblast	Dcx	NM_010025[c)]	Doublecortin	cytoplasmic protein
	NeuroD4	NM_007501	neurogenic differentiation 4	transcriptional activator
	Tubb3	NM_023279	tubulin beta 3	cytoskeleton protein
	Elavl4	NM_010488[d)]	emb. lethal abnormal vision-like 4	RNA binding protein
	Epha7	NM_010141[e)]	Eph receptor A7	growing axons
non ectodermal germ layers	Sox17	NM_011441	SRY-box containing gene 17	mesoderm
	Acta2	NM_007392[f)]	SMA, actin, alpha 2, smooth muscle	mesoderm
	T	NM_009309	Brachyury	mesoderm
	Gata4	NM_008092	GATA binding protein 4	endoderm
	Afp	NM_007423	alpha fetoprotein	endoderm
ECM components	Col4a1	NM_009931	collagen type IV alpha 1	early marker in diff.
	Ncan	NM_007789[g)]	Neurocan	early marker in diff.
	Tnc	NM_011607	tenascin C	late marker in diff.
	Col1a1	NM_007742	collagen type I, alpha 1	late marker in diff.
neuronal marker	Syp	NM_009305	Synaptophysin	synaptic vesicle assoc.
	Grin1	NM_008169	NMDA1	ionotropic glutamate R.
	Nrg1	NM_178591	neuregulin 1	schiz. assoc.
	Nrxn 1	NM_020252	neurexin I	autism, schiz. assoc.
	Stx1a	NM_016801	syntaxin 1A	synapse assoc.
	Snca	NM_009221[h)]	Synuclein alpha	parkinson assoc.
	Mapt	NM_010838[i)]	microtubule-associated protein tau	alzheimer assoc.

OC: oligodendrocyte, panc: pancreas, neu: neuron, NCC: neural crest cell, emb: embryonic, R: receptor, schiz: schizophrenia, assoc: associated

Additional accession numbers: a) NM_001160112, b) NM_001077632, c) NM_001110222, NM_001110224, NM_001110223, d) NM_001038698, e) NM_001122889, f) NM_183274, g) XM_913832, h) NM_001042451, i) NM_001038609

profiling will contain both NSCs and differentiating neurons. Therefore, NSC and their progeny can be hard to disentangle at the level of transcription without reference to cellular, spatial distinctions in gene expression profiles (e.g. as determined by high resolution *in situ* hybridisation).

Ideally, mESC-derived NSC gene expression should broadly recapitulate developmental patterns of gene expression observed during neuroepithelium specification, or within proliferative progenitor zones at later stages. Such information has also been used here (amongst others) for filtering of suggested marker genes (Fig. 2). For our compilation, we used the strong expression and/or significant role of a gene in differentiated neurons and/or glia as exclusion for its definition as an NSC marker, even though the gene may be expressed in NSCs. This important filtering step is admittedly biased, and new information will require adaptations.

A different approach would be to look at functional importance. While some of the most frequently used mESC markers also have a functional role in stemness, many of the typical NSC and radial glia markers are e.g. cytoskeletal elements (*Nestin, Gfap, Vimentin*) without known function in NSC maintenance. Others, with known and important functions, such as the transcription factors *Sox2* or *Zic1*, or signal transduction molecules like *Jak2, Hes5* and *Fgfr3* also have roles in other cells. Thus, at present, it appears necessary to combine all different approaches to select candidate NSC genes and to further filter those by manual cherry-picking based on literature studies. Such attempts have been made repeatedly, and the table (Tab. 2) presented here is strongly based on the publications of Abranches et al., 2009; Maisel et al., 2007; Kelly et al., 2009; Vogel et al., 2009; Gaspard et al., 2008; Liu et al., 2004; Barberi et al., 2003; Ghosh et al., 2008.

4.5 Definition of differentiation markers for different neuronal stages

After the generation of NSC, neuronal differentiation proceeds. The currently accepted model of neural developmental proposes that extracellular signalling molecules act on NSC and their progeny and determine what type of neurons or glia they will become. This would be accompanied by migration of the neuroblasts and by generation of new signalling gradients due to factors secreted from neural cells themselves. Cells at a differentiation stage after the NSC stage that are not yet mature neurons are frequently referred to as neuroblasts. However, the definition of these cells has some caveats. During organ development, the neuroblast is essentially a post-mitotic neuron that is distinguished from a maturing or mature neuron by its specialisation for migration rather than for functional integration. Postnatally, neuroblasts are a distinct population of cells, which are capable of proliferation and are neuronally committed.

Therefore, we use the expression NSC in this review to signify proliferative cells with self-renewal capacity, the ability to form neuronal and glial cells, and a dependence on EGF and bFGF for optimal proliferation (mESC depend on LIF). The neuronally committed progeny of NSC includes maturing neurons at different stages, which we denote as "neuroblasts" when referring to early stages and as neurons when referring to late stages.

Similar processes occur also in *in vitro* culture, which explains that e.g. the density of cells has a major impact on the end result of the differentiation. Chemicals can act in this phase on the cells or their signalling molecules, and the exposure may result in a shift of the balance between neuronal subtypes (Gaspard et al., 2008) or between glia and neurons (Fritsche et al., 2005; Steinhart et al., 2007). Such early events have been speculated to have a late impact e.g. on development of neurodegenerative disease (Landrigan et al., 2005), and it has been demonstrated experimentally that e.g. exposure to polychlorin-

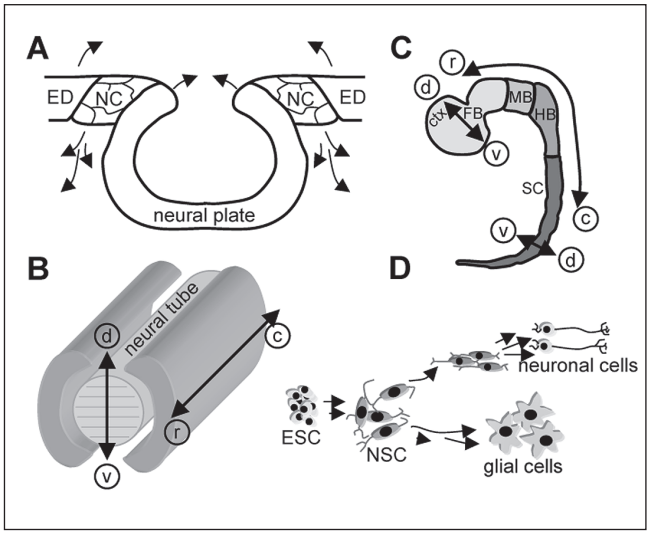

Fig. 2: Basic concepts of neurodevelopment
Very early during embryonic development (about day 7.5) the neural plate forms as an area of early neuroectodermal tissue, whereas flanking regions form ectoderm (ED).

A. Within the next 24 h this plate invaginates and closes to form the neural tube, which is the precursor stage of the central nervous system. Cells at the lateral margins of the neural tube form the neural crest cells (NC) that migrate to various locations and form parts of the peripheral nervous system among other cell types.

B. The neural tube (light gray) is flanked by non-neural tissue (dark grey) and extends from the head region towards the prospective tail region. At this stage, clear patterns of neurons along different axes are established, which lead to different neuronal subpopulations in the adult. The major axes are from back (dorsal = d) to belly-side (ventral = v) and from head (rostral = r) to tail-side (caudal = c).

C. lateral view of a day 10.5 embryo (E10.5): the caudal end (c) represents the spinal cord (SC), the rostral end (r) develops into the brain, where forebrain (FB), midbrain (MB) and hindbrain (HB) can be distinguished. The dorso-ventral axes (d-v) remains present both in the spinal cord (motor neurons in the ventral part) and in the brain (e.g. the dorsal forebrain differentiates to cortical structures (Ctx).

D. Embryonic stem cells (ESC) can differentiate to neural stem cells (NSC) with characteristics resembling those of proliferating cells found in the early development of the nervous system. The mESC-derived NSC-like cells, like their in vivo counterparts, retain the capacity to acquire region-specific identities and differentiate into neurons and/or glia via intermediate lineage-restricted progenitor cell stages *in vitro*.

ated biphenyls (PCBs) *in utero* can affect the outcome of stroke in later life without major effects on brain development (Dziennis et al., 2008). Thus, we have to assume that DNT does not necessarily affect the number of neurons or other major cell types, but the specific patterning of the nervous system and relationships between neuronal populations. For such endpoints, transcriptional profiling at different phases of development appears to be a useful approach to detect deviations from the normal pattern. An example of markers to define such patterning is displayed in Table 4. A future refinement may be the selection of differentially spliced genes that form highly cell-type or development-specific transcripts. For instance the well known neuronal marker NeuN affects neuronal-specific splicing (Kim et al., 2009b), and the mitochondrial fusion-fission-regulating gene *Drp1* expresses a specific splice variant only in brain (Uo et al., 2009). New microarray platforms that allow reliable detection of exon splicing may enable detailed analysis of post-mitotic neuronal differentiation.

4.6 Astrocyte markers

A discussion of all markers relevant for the different developmental phases is beyond the scope of this review. As one example for the complexity, we chose a relatively simple neural population: astrocytes. Although these cells make up more than half of the brain mass, they have been relatively neglected as potential targets of toxicity or DNT. It is generally assumed that astrocytes are identified by the intermediary filament protein GFAP. However, recent research has shown that antibodies to this protein also label radial glia (NSC-related cells) (Seri et al., 2001; Ganat et al., 2006; Götz and Steindler, 2003; Buffo et al., 2008), and that about 50% of astrocytes in the brain may not express significant amounts of GFAP (Lovatt et al., 2007; Cahoy et al., 2008). Moreover, knockout of *Gfap* has no major effects on astrocyte development or brain function (Pekny et al., 1995; Gomi et al., 1995). Thus, a broader panel of astrocyte markers, as compiled here (Tab. 5), is urgently needed, similar to the markers for mESC and mNSC presented in Tables 1 and 2.

Tab. 5: Marker genes for astrocytes

name	accession number	full name	comment	reference
ActA2	NM_007392	Alpha-actin-2	also in smooth muscle	[1]
Aldh1L1	NM_027406	Aldehyde dehydrogenase family 1 member L1	also in GFAP-neg AC	[2]
AldoC	NM_009657	Fructose-bisphosphate aldolase C		[3]
ApoE	NM_009696	Apolipoprotein E	also synthesised by MG	[4]
Aqp4	NM_009700	Aquaporin-4	also RG, endfeet at vessels	[5], [6]
Bysl	NM_016859	Bystin	in reactive AC	[7]
Car2	NM_009801	Carbonic anhydrase 2	also in OC	
Cbs	NM_178224[a)]	Cystathionine beta-synthase		[3]
Csad	NM_144942	Cysteine sulfinic acid decarboxylase	taurine biosynthesis	
Gfap	NM_001131020[b)]	Glial fibrillary acidic protein	labels subset of AC	[8], [9]
GjA1	NM_010288	Connexin 43	AC specific in the brain	[6], [10]
GjB6	NM_001010937[c)]	Connexin 30		[10]
Glul	NM_008131	Glutamine Synthetase	also in GFAP-neg AC	[11], [12]
Kcnj10	NM_001039484	ATP-sensitive inward rectifier potassium channel 10, Kir4.1	absent in immature AC	[3]
MaoB	NM_172778	Monoamine oxidase type B	mitochondrial	
NFIA	NM_010905[d)]	Nuclear factor 1 A-type	also in OC, NSC	[13]
NFIB	NM_001113209[e)]	Nuclear factor 1 B-type	also in OC, NSC	[13]
NFIX	NM_001081982[f)]	Nuclear factor 1 X-type	also in OC	[13]
Pla2g7	NM_013737	PAF acetylhydrolase	useful RNA marker	[2]
PygB	NM_153781	Glycogen phosphorylase, brain form	specific for AC in brain	[14]
S100b	NM_009115	S100beta	also in early AC, NSC	[15]
Slc1A2	NM_001077515[g)]	GLT-1, excitatory amino acid transporter 2	early AC, also NSC, RG	[16]
Slc1A3	NM_148938	Glast-1, excitatory amino acid transporter 1	mature AC	[16]
SparcL1	NM_010097	SC1, SPARC-like protein 1		[17]
Vim	NM_011701	Vimentin	also RG, NSC, early AC	[18], [19]

AC: astrocytes; RG: radial glia; NSC: neural stem cells; OG: oligodendrocytes; MG: microglia

Additional accession numbers:
a) NM_144855, b) NM_010277, c) NM_008128, d) NM_001122952, NM_001122953,
e) NM_008687, NM_001113210, f) NM_010906, NM_001081981, g) NM_011393, NM_001077514

1: Lecain et al., 1991; 2: Cahoy et al., 2008; 3: Hatada et al., 2008; 4: Gee et al., 2005; 5: Nakahama et al., 1999; 6: Fatemi et al., 2008; 7: Sheng et al., 2004; 8: Rodnight et al., 1997; 9: Ghandour et al.,, 1979; 10: Lovatt et al., 2007; 11: Steffek et al., 2008; 12: Wu et al., 2005; 13: Wilczynska et al., 2009; 14: Pfeiffer et al., 1992; 15: Burette et al., 1998; 16: Chaudhry et al., 1995; 17: McKinnon et al., 1996; 18: Dahl et al., 1981; 19: Zamora et al., 1988

Most notably, the underlying principle for the definition of astrocyte markers differs from that of stem cell markers. All of the stem cell markers are expected to be expressed in all stem cells. In contrast to this, not all "astrocyte markers" are expressed in all astrocytes. They rather define subpopulations of astrocytes and different developmental stages of such subpopulations. Only this comprehensive picture based on multiple markers will yield meaningful information on the fate of the diverse group of astrocytes as a whole and on effects of chemical and other influences on their development.

4.7 Toxicity pathways

In this review we have focussed on markers useful for the description of subtle phenotypic effects caused by toxicants – independent of their mode of action. An interesting additional aspect of transcription-based endpoints may be the possibility to identify cellular toxicity pathways and fingerprints involved in the effect of the chemicals. Especially fingerprinting is already established for other organ toxicities, in particular hepatotoxicity (Ruepp et al., 2005; Steiner et al., 2004; Blomme et al., 2009). These two different approaches may be applied independently or be combined. An example may best demonstrate the underlying principle: For instance, a chemical may be identified as a potential developmental neurotoxicant based on shifts in the patterning markers presented in table 5. It may e.g. increase dorsal markers and decrease ventral markers relative to house keeping genes. On closer (mechanistic) examination, one may notice, that in particular sonic hedgehog (Shh) target genes were down-regulated upon exposure to the chemical. The mechanism of toxicity may thus involve inhibition of Shh signalling. Alternatively, chemicals may be screened specifically for disturbances of key signalling pathways by reporter assays or transcriptome analysis coupled with systems biology approaches. One compound may be identified to block the Shh signalling pathway, and upon subsequent examination of DNT effects, it would lead to a dorsalisation of the developing neurons. Cyclopamine is a substance that behaves as described above. Other toxicity pathways, which may be identified in a similar manner, involve e.g. retinoic acid synthesis, notch processing or Wnt, TGF-beta or Ah-receptor signalling. The above examples show the independence of mechanistic and phenotypic approaches and the huge potential of using and combining both. In the context of ESC-based neurodevelopmental test systems, it is important to note that the phenotypic approach necessarily requires a complex and difficult experimental test system (differentiating ESC). In contrast to this, the mechanistic approach may also be applied to (and work much better in) much simpler systems involving the respective pathways. Differentiating ESC are in fact, due to their complexity, not very suitable as a mechanistic screen system. This review has predominantly focussed on the markers that may be useful for DNT/teratogenicity screening approaches in the nearer future.

5 Conclusions

The transcription-based markers discussed in this review represent an effort to characterise subtle disturbances in the waves of gene inductions leading from mESC to differentiated neural cells. With more experience, and for specific applications, possibly small subgroups of markers can be selected to obtain relevant information. A step further would be the use of genetically-modified mESC with reporter constructs. In this case, easily quantifiable enzymes, like luciferase or secreted alkaline phosphatase (Suter et al., 2009; Volbracht et al., 2009), driven by cell- and stage-specific promoters would be used as endpoints of gene induction, also in very complex cell mixtures. As with all transcription-based assays, the endpoints suggested here do not necessarily correlate with protein or function, and this issue will require further characterisation and validation (Schrattenholz and Soskić, 2008). In the end, the proof of the pudding is in the eating. In extreme cases, certain well-established markers cannot be detected at all on the RNA-level for technical reasons (e.g. highly repetitive sequences) or because they are antigenically defined. These comprise the early astrocyte precursor marker A2B5, the mESC marker SSEA-1 or the NSC marker polysialylated-NCAM (PSA-NCAM). Such markers are linked to specific glycosylations or keratin sulfates, and in extreme cases glycosylation itself (GalNAc-Epitopes on multiple proteins) can be an excellent marker for mESC (Nash et al., 2007). Such antigenic markers are ideally combined with RNA markers and, in the future, RNA marker sophistication will increase by the inclusion of miRNAs, lincRNAs or other non-coding RNAs, and of tissue specific splice variants. Altogether, this approach seems to be powerful enough to define exactly the differentiation capacity of ESC, also to germ layers other than neuroectoderm, and it should therefore be a suitable substitute for old-fashioned assays testing teratoma formation *in vivo* to establish pluripotency of a cell population. This review focussed mainly on mESC differentiation, but the underlying principles also apply to hESC. For the human counterparts, some excellent compilations of stem cell markers exist (Assou et al., 2007; International Stem Cell Initiative, 2007; Bhattacharya et al., 2005, 2009), and it needs to be noted in this context that clear species differences may exist (Ginis et al., 2004; Sato et al., 2003). For instance, the above mentioned marker SSEA-1 does not work for hESC, while those are characterised by SSEA-3/4, which do not work for mESC. Genes like *threonine dehydrogenase* (Tdh) (Wang et al., 2009), *FoxD3* or the genes coding for the receptor of the mESC growth factor LIF (which is dispensable for hESC) are regulated in a species-specific manner. With these well known differences taken into account, mESC still remain a very robust system for studying neural development and are possibly able to provide human DNT relevant information on compounds more sensitively than the currently used animal models.

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Acknowledgements

This work was supported by the Doerenkamp-Zbinden Foundation, the FP7 ESNATS project, the DFG international research training school IRTG1331, and the Konstanz Research School Chemical Biology of the German Excellence Initiative. We are grateful to many who gave useful advice and help on many occasions and especially to Brigitte Schanze for excellent secretarial support.

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