Neurodevelopmental toxicity detection by transcriptomics in an embryonic stem cell differentiation assay

CHAPTER 9

Summary & General Discussion

Summary of main findings

In this thesis the development of the neural embryonic stem cell test (ESTn) as a high throughput system for detection of compound induced neurodevelopmental toxicity is described: from developing and characterizing the model to implementation of transcriptomics techniques in the testing strategy and finally the formation of a biomarker gene set for neurodevelopmental toxicity in this test system. In addition, the time-related and compound induced gene expression changes across ESTn and other in vitro and in vivo models are compared. A schematic summary of the main findings is provided in Figure 1.
Development and molecular characterization of the ESTn is described in chapter 2. A 20-day neural differentiation protocol was designed based on two existing protocols published by Okabe et al. and Bibel et al. [52, 103]. The main steps in the protocol are embryoid body (EB) formation using the hanging drop method, exposure of the EB to the morphogen retinoic acid (RA) at a physiological concentration in a suspension culture, step-wise reduction of the serum percentage from 20% to 0% in the medium and addition of an extra-cellular matrix and growth factors stimulating neural differentiation. At the end-stage of the differentiation culture, EB were for 75-100% surrounded by neural outgrowth (Figure 2).

In order to increase the high-throughput of the test system, two additional shorter variants of the protocol (13 and 17 days), with a reduced 'serum-free' phase were designed and the three protocols were characterized morphologically and at the molecular level for development related protein and mRNA markers using immunocytochemistry, gene expression and flow-cytometry. Early in the protocols, which are identical the first seven days, markers for all three primary germ layers are expressed and in addition, an increase in ectodermal and specific neuron type differentiation was observed, whereas pluripotency markers were decreased over time. This demonstrates neural differentiation was induced early in the protocols. Furthermore, at the end-stage of the three protocols, expression level of specific neuron type markers was comparable between the three protocol variants, except for GFAP, a marker for astrocytes, for which gene expression was lower but still significantly present in the shortest protocol. Due to the shared morphological and molecular characteristics of the three protocols, further experiments to test compound induced effects on neural differentiation were performed in the shortest protocol.
As a proof-of-principle, perturbation of neural differentiation by the model neurodevelopmental toxicant methylmercury (MeHg) was assessed at multiple

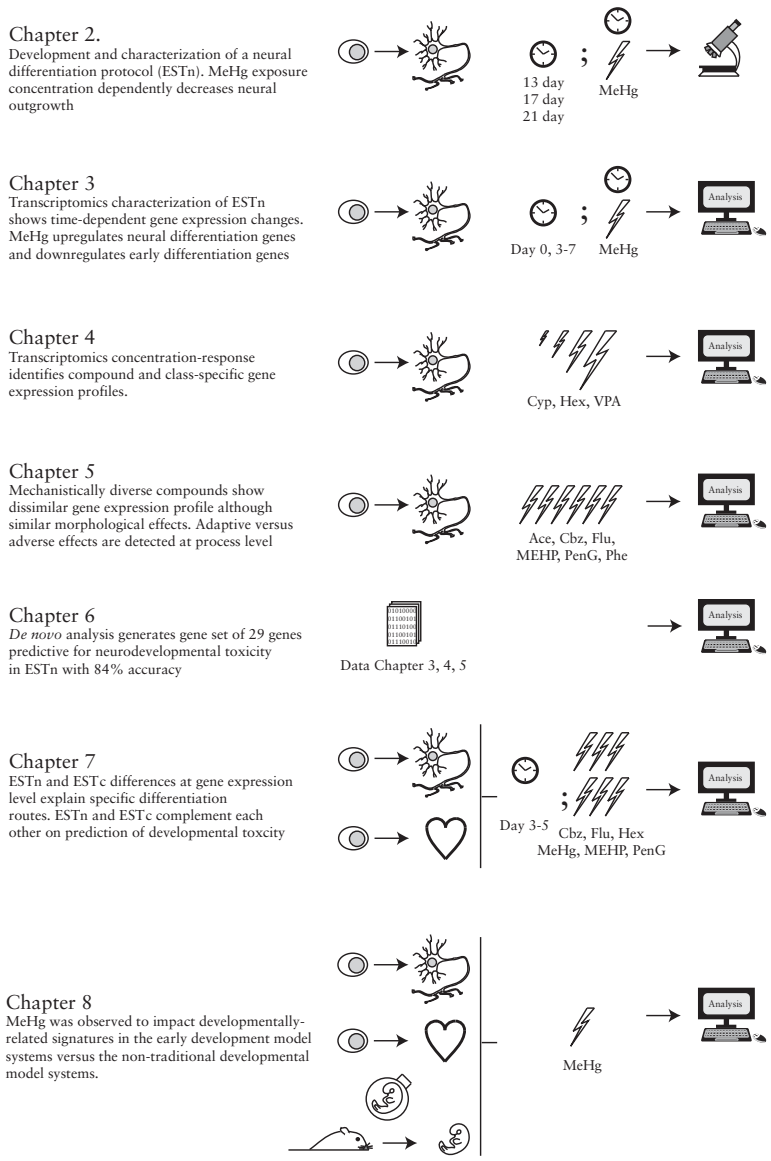

Figure 1 A Schematic summary of the main findings

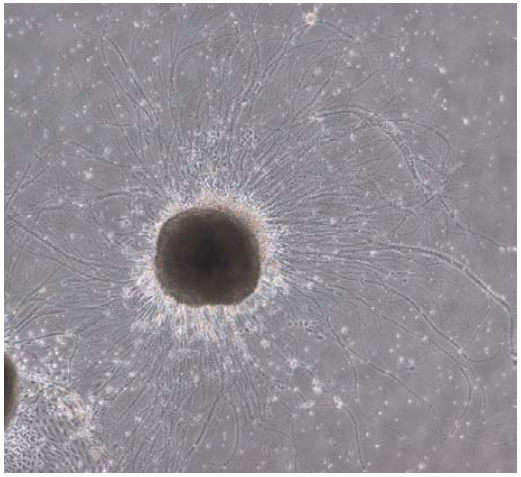

Figure 2 Example of an embryoid body (EB) completely surrounded by neural outgrowth.

concentrations. Neurite outgrowth was assessed by morphologically scoring the percentage of the EB surrounded by neural outgrowth and by measuring the amount cells positive for the pluripotency marker SSEA1, neural precursor marker nestin and neuronal marker βIII-tubulin. Due to the possible binding of MeHg to proteins in the serum, three different MeHg exposure designs were performed: exposure only during the serum containing period (days 3-5), only during the serum-free period (days 6-13) or a complete exposure (days 3-13). MeHg exposure effects on morphological neural outgrowth at the serum-only and complete exposures showed a similar concentration response, whereas the serum-free exposure was more sensitive. An explanation may be that at start of the serum-free exposure, the cell population in culture is different compared to the population with exposure from day 3 onwards. Furthermore, a complete absence of neural outgrowth observed exclusively after late exposure (serum-free and complete exposure) to the highest concentration tested, may be related to cytotoxicity of the compound at this concentration, which may be more pronounced under serum-free conditions. Due to the similar concentration-response observed after serum-containing and complete exposure and possible effects of lack of protein binding in the serum-free phase of the method, exposure for following experiments was performed from day 3-5. A scheme of the final version of the ESTn is depicted in chapter 2, figure 1.

In chapter 3 the ESTn is further characterized by monitoring temporal gene expression changes related to differentiation. Cultures were sampled at days 0, 3, 4, 5, 6 and 7 of the ESTn protocol and whole genome mRNA expression was assessed. Temporal regulated genes could be subdivided into eight separate clusters, each describing a distinct gene expression pattern over time. Multiple analysis techniques (including hierarchical clustering, DAVID and Tox-profiler) showed that early up-regulated gene clusters were involved in processes related to transcription and pluripotency and that late up-regulated gene clusters were related to processes involved in neural development. Furthermore, using principal component analysis (PCA) a differentiation track describing differentiation over time could be constructed with all genes regulated over time, as was shown before for cardiac differentiation in the cardiac stem cell test (ESTc) [187]. The differentiation track showed that for each time-point, samples clustered together, but that variation between samples increased over time, indicating cells in culture differentiated to become more heterogeneous in gene expression profile.

MeHg induced effects on gene expression over time were studied after 24, 48, 72 and 96h exposure, starting from day 3 of culture onwards. MeHg was observed to up-regulate neural development related processes and down-regulate general development related processes and to a lesser extent, endoderm and mesoderm related processes. In addition, the PCA-based differentiation track method was used to study temporal MeHg effects on gene expression after 24, 48 and 72h exposure. In this approach, a differentiation track is set up using the genes regulated by the time-matched control and the two control days surrounding the day of interest (e.g. for 24h exposure, controls days 3, 4 and 5 are used to setup the track). Then the exposed samples are compared to its day-matched control and significant deviation from the track is calculated. MeHg induced samples were most significantly deviating from the differentiation track after 24h exposure. Based on these results, for following transcriptomics experiments a 24h compound exposure with controls on day 3, 4 and 5 was performed.

Compound induced concentration-dependent effects on gene expression during embryonic stem cell (ESC) differentiation were studied in chapter 4. Three known (neuro)developmental toxic compounds were selected as test compounds; the triazoles cyproconazole and hexaconazole and the anticonvulsant valproic acid (VPA). Compound-mediated effects on gene expression were identified after 24h exposure in relation to morphological changes on day 11 of culture. All three compounds showed a concentration-dependent effect in gene expression and biological processes, providing information on mechanisms and concentration-response characteristics.

General and specific effects on development could be discriminated between compounds. For example, concentration-dependent gene expression for the biological process embryonic morphogenesis was enriched for all compounds, indicating an effect on general development. However, cyproconazole and VPA (both neurodevelopmental toxicants) but not hexaconazole (developmental toxicant) significantly enriched the neuron development process. In addition, compound class specific mechanisms could be detected, including sterol biosynthetic process enriched by the triazoles. Implementation of transcriptomics in this study was shown to precede morphological changes and provided a more sensitive measure to assess possible developmental toxicity.

In order to increase knowledge on the applicability domain of the test system, effects on gene expression of six additional compounds with a different mechanism of action was assessed at equipotent and non-toxic concentrations in **chapter 5**. Five compounds tested were known (neuro)developmental toxicants (acetaldehyde, carbamazepine, flusilazole, monoethylhexyl phthalate (MEHP), and phenytoin) and penicillin G was tested as a negative control. Compounds induced transcriptomic profiles were assessed at two concentrations for gene expression profiling; a toxic concentration inhibiting differentiation by approximately 50% or a concentration at which 80% of the cells was still viable and a non-toxic concentration thirty times lower. Both compound-specific and common gene expression changes were observed between subsets of tested compounds, in terms of significance, magnitude of regulation and functionality. However, overlap between the compounds was very low, indicating the specific mechanisms behind each of the compound's developmental toxicity. In addition, gene expression and GOBP enrichment showed concentration dependence, allowing discrimination of non-toxic versus toxic concentrations on the basis of transcriptomics. This information may be used to define adaptive versus toxic responses at the transcriptome level.

In **chapter 6**, transcriptomics data from chapters 3, 4 and 5 is used to identify a biomarker gene set to predict neurodevelopmental toxicity in the ESTn. Gene expression responses were distinguished using the differentiation track algorithm with data from ten compounds and nineteen exposures. Using classification feature selection in combination with a leave-one-out cross-validation, a set of 29 genes was obtained consisting of genes mainly involved in neural development. This set allowed for 84% prediction accuracy and may contribute to further improve ESTn transcriptomics studies aimed at compound risk assessment.

The ESTn was developed as a companion test system to the ESTc, therefore differences and similarities in transcriptomics responses between the two

systems are discussed in **chapter 7**. Gene expression profiles of both ESTn and ESTc over time early in both methods (days 3, 4 and 5) and changes induced by seven compounds, including six *in vivo* developmental toxicants and one negative control, are compared. Although temporal gene expression responses were generally comparable between models, a relatively small group of genes could be identified behaving differently in each model. These genes were mainly related to cardiac development and early developmental signaling pathways in ESTc and neural development and retinoic acid signaling in ESTn. Compound induced gene expression changes were generally different in either model, predicting mechanism behind compound developmental toxicity better in one model or in the other (e.g. MeHg in ESTn and FLU in ESTc). However, VPA induced gene expression changes were comparable in both ESTn and ESTc, but varied at the process level. A comparison between the two models shows that a tiered approach of compound screening in ESTc and ESTn may improve prediction and understanding of the compound mechanism of action. A transcriptomic based *in vivo* versus *in vitro* comparison is performed in **chapter 8** in order to obtain insight on differences in prediction and mechanism of action in different models. The impact of MeHg on a diverse set of *in vitro* and *in vivo* models was assessed. Models predicting developmental toxicity were the *in vitro* rat whole embryo culture (WEC), ESTc and ESTn and two mouse-embryo developmental toxicity studies. Models predicting general toxicity were an embryonic fibroblast system, adult rat liver brain studies. Evaluation of common and unique aspects at the functional and gene level showed stronger similarities in MeHg response between the development related studies compared to the general toxicity studies. This comparison shows *in vitro* / *in vivo* similarities supporting the relevance of *in vitro* developmental models for predicting *in vivo* developmental toxicity.

General discussion

Development of a neural differentiation cell culture

ESC differentiation models are currently very promising as alternative and animal-free models for developmental toxicity [306]. Due to the pluripotent nature of ESC, in theory the effect of compounds on any differentiation route of the three germ layers can be studied, which is not possible in primary cell lines, immortalized cell lines, or cells already in a more advanced stage of differentiation, such as neural progenitor cells (NPC) [19, 130-132]. In the ESTc,

the ESC default differentiation pathway is induced, mainly differentiating cells towards mesodermal and endodermal lineages, resulting in approximately 40% cardiomyocytes at end of culture. Due to the relatively weak prediction of the ESTc for neurodevelopmental toxicants such as MeHg, the need for addition of a neural differentiation based EST was recognized [17, 36, 37].

As was described in chapter 2, we developed the ESTn as a companion model to the ESTc, leaving as many as possible characteristics of the original ESTc differentiation model intact. This approach introduced advantages, but also some minor disadvantages of the ESTn protocol compared to other possible methods. Similarly as the ESTc, standardized embryoid body (EB) formation using the hanging drop method is implemented in the ESTn, decreasing variation in EB size compared to spontaneous EB formation [21]. Three-dimensional EB formation furthermore allowed differentiation to mimic the in vivo developmental process in a more complete manner compared to monolayer differentiation, due to the presence of all three germ layers [307]. However, in the EB no physical structures such as formation of a neural tube or blood vessels is observed. The three-dimensional induced multi-lineage character of the assay may allow improved detection of developmental toxicity in the assay. On the other hand, this three-dimensional complexity can be a disadvantage when a relatively simple morphological readout for compound effects on differentiation is preferred. EB structure is maintained throughout the complete protocol, from EB formation to the morphological readout, supporting stable culture conditions. In previously described ESC neural differentiation methods for neurodevelopmental toxicity, ESC monolayer culture was used [106], or EB were dissociated to single cells during the protocol [41], ensuing a less stable protocol. Further similarities between the ESTn and ESTc, the suspension phase after the hanging drop phase and plating of the EB on day 5, are maintained to keep comparability between protocols.

In order to induce neural differentiation, three distinct steps are implemented in the protocol, which were earlier described in neural differentiation protocols by Okabe et al. and Bibel et al. [52, 103]. The first being addition of the morphogen all-trans retinoic acid (RA) to the culture at the suspension phase at a physiological concentration during neural development in vivo [308]). The first developed ESC neural differentiation models already used RA as a morphogen to induce differentiation [52]. RA, an active metabolite of vitamin A, is known to play an important role in normal embryogenesis. Subtle gradients of RA direct morphogenesis throughout the embryo, including limb development and neural development [271-273]. In addition, during early

organogenesis in vivo, RA levels are observed to be highest in the neural tube [272]. The second critical step in the ESTn is gradual serum deprivation of the medium as an induction and selection for neural cell types. Serum deprivation is currently widely used to induce ESC neural differentiation and was first described in an ECC cell line [56, 309]. In ECC, serum deprivation was described to induce neural differentiation and in addition induce apoptosis in other cell types which depend on serum for growth [309, 310]. However, specific mechanisms behind the effects of serum deprivation on neural differentiation remain unclear. The last critical step in the ESTn is facilitating neural growth by offering a an extracellular matrix (laminin and poly-L-ornithine) and addition of growth factors to the medium [311]. These growth factors include progesterone, sodium selenite, transferrin and bFGF and are used in serum free primary neuron cultures to create a viable environment for neural cell types [308]. Together RA supplementation, serum deprivation, the extracellular matrix and addition of growth factors create the environment for ESC to differentiate into neural cell types in ESTn.

Molecular characterization of ESC neural differentiation

Characterization at the single gene level

Characterization of differentiation over time in ESC differentiation cultures has been performed using distinguished gene expression markers for differentiation of all three germ layers, as was also performed in ESTn in chapter 2 [19]. Using this approach, differentiation can be studied in broad terms with markers for pluripotency (e.g. Pou5f1 and SSEA1), markers for progenitor cells of the three germ layers (e.g. T, nestin, Nkx2-5) and markers for mature cell types (e.g. Myh6, Tubb3, GFAP), which has shown characteristics reminiscent of germ layer development and specific lineage differentiation in ESTn and other models [19, 312]. Although this approach can provide information on general temporal regulation of predetermined differentiation routes, obtained information remains limited due to the pre-selection of genes.

Characterization at the whole genome level

In recent years, transcriptomics analysis have provided a more complete view of ESC differentiation dynamics. A number of studies published described a range of differentiation routes, including cardiac, neural and pancreatic differentiation [80, 169, 313-315]. In these studies it was confirmed that gene expression profiles of specialized cellular differentiation protocols were concurrent with functional processes observed for specific regions of the embryo during development. However, in these studies transcriptome profiling

was performed on very distinct morphological stages during ESC differentiation with relatively much time between time-points studied, by which especially during early ESC differentiation, subtle short changes in gene expression were not identified. More recently, early temporal gene expression changes over a relatively small time-span (one day or less) were studied in a number of ESC differentiation protocols, including the ESTn and ESTc, as described in chapter 3 and chapter 7 [177, 187, 235]. In these studies, it was shown that the continuous developmental changes occurring in various mouse and human ESC differentiation models could be visualized using PCA to describe a 'differentiation track' [177, 187, 235, 316]. This approach makes it possible to visualize and quantify differentiation status and developmental potency status of cells cultures. In the ESTn, the differentiation track over time showed that all time-points clustered together and separate time-points were ordered chronologically. However, over time, variation between samples increased, probably due to increased heterogeneity of the cell culture. Studies on ESC differentiation have been instrumental in describing differentiation processes, showing a reduction of pluripotency and an induction of neural differentiation-related gene sets over time [177, 187, 235]. In addition, these studies showed that early ESC differentiation in diverse differentiation protocols is characterized by a cascade of time-dependent early-, mid- and late-regulated gene expression profiles and processes. For instance, in ESTn, ESTc and other ESC differentiation models, genes and processes involved in pluripotency (including Pou5f1, Nodal and Eomes) and proliferation (including Ccnd1 and Ccne1) were up-regulated early and down-regulated over time, genes up-regulated for a short duration during the midterm of the protocol related to early differentiation (T, Cyp26a1, Frzb, Lhx1) and genes and process up-regulation of (end-stage) differentiation as a late response (including Tubb3, Pax6, Gata6 among others). These 'coordinated waves' of differentiation have also been observed in vivo during the gastrula stage in multiple species, including the mouse [317].

Comparing temporal gene expression profiles in ESTn and ESTc

In order to further characterize early ESC differentiation, in chapter 7 early temporal gene expression signatures (days 3, 4 and 5) for neural differentiation in ESTn and cardiomyocyte differentiation in ESTc were compared. This analysis showed for a large part (71% of genes regulated in either model) early gene expression profiles in the two models were regulated similarly, illustrating that temporal differentiation related genes are conserved in both models. In addition, genes regulated early specifically in ESTn (12%) could be related to connected processes involved in patterning (mainly homeobox related), RA

metabolism and neural development, whereas genes regulated specifically in ESTc (17%) were related highly connected to Wnt-, Tgfβ- and BMP signalling and cardiac development, showing early gene expression responses are highly related to the morphological phenotype later in the protocols.

Temporal gene expression profiles in ESC differentiation in context of in vivo organogenesis

A useful in vitro model should to a certain extent be comparable to the in vivo situation [318]. Mouse ESC are collected in vivo from the inner cell mass of the blastocyst, approximately 4 days after fertilization [19]. Therefore, the first week of ESC differentiation is expected to be correlated to day 4 - 10, during gastrulation of the developing embryo [19]. Mitiku and Baker performed a very detailed genomic profiling of gastrulation and organogenesis in the mouse embryo from day 6.5 through 9.0 post conception, which was compared to our data in the discussion in chapter 3 [171]. Seven gene clusters were identified, describing specific processes during development, including two clusters involved in neurogenesis and cardio-vasculature development, which were up-regulated from day 8.0 onwards, in a comparable manner to late responses observed in ESTn and ESTc [171, 177, 187]. Interestingly, another cluster involved in blood vessel development and neurogenesis consisted of genes with a peak up-regulation at day 8, which was again down-regulated at day 9.0, similar to the observation in ESTn and ESTc. Comparisons of gene clusters with late expression profiles in ESTn with the in vivo data were performed and corresponding genes, and GO terms were observed. Although evidence is limited, this similar gene signature provides an additional indication that processes involved in ESTn and ESTc are comparable to the in vivo situation.

ESTn as a test system for assessment of neurodevelopmental toxicity

Developing an endpoint for neurodevelopmental toxicity in ESTn

For detection and prediction of adverse effects on neural differentiation in the ESTn, a readout method was needed. In earlier described models for neurotoxicity and neurodevelopmental toxicity, a number of readouts have been proposed [319], including cell morphology by assessing neural outgrowth, axonal and dendritic morphology, cytotoxicity/viability and migration in monolayer or neurosphere cultures [149, 150], functional parameters determined by electric potential or calcium homeostasis [147, 320] or assessing neural differentiation using mRNA transcripts of a small selected number of

genes [41, 321]. In order to perform quick readout screens, a relatively fast readout system was required for ESTn. Due to the complexity of the neural outgrowth at the end of culture, fast measurement of functional parameters was not possible. Therefore, initially a combination of a morphological and molecular approach was chosen, assessing neural outgrowth from EB microscopically and compound effects on protein markers using flow cytometry, as was described in chapter 2. Morphology of neural outgrowth was scored as the percentage of neural corona surrounding each EB, irrespective of the distance of outgrowth from the EB. A scoring was performed for 100, 75, 50, 25, <10 and 0% neural outgrowth surrounding the EB (Figure 3). For scoring compound effects on neurite outgrowth, a cutoff was chosen at ≥75% neural outgrowth around the EB, based on historical control data. Thus, when more than 75% of the corona around an EB contained microscopically observable neural outgrowth, the EB was scored positive, whereas below 75% it was scored negative. Using a concentration-response curve, the concentration by which a 50% inhibition of differentiation (ID50) (EB with >75% neural outgrowth) was observed, could be identified. This ID50 method has also been used to define developmental toxicity in the EST [17].

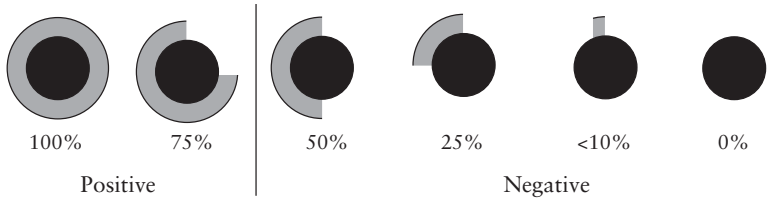

Figure 3 Schematic overview of Morphological Scoring criteria for determining the percentage of neural outgrowth around the EB.

Next to morphology, initially flow cytometry was used to count the number of cells positive for the pluripotency marker SSEA1, neural precursor marker nestin and neuron marker βIII-tubulin. Flow cytometry has been successfully implemented in the validated EST using the cardiomyocyte marker MYH as readout parameter [322]. As a proof-of-principle, effects of the model neurodevelopmental toxicant MeHg were assessed using the morphology and flowcytometry readouts. A strong concentration-response was observed in the morphological readout, but only limited prediction was observed by the flow cytometry method. Therefore, for further experiments, morphological scoring

of neurite outgrowth was used as a parameter to define compound effects on neural differentiation and outgrowth.

Exposure strategy: Selection of exposure duration and concentration ranges

Due to the complexity of the ESTn, including changes of serum percentage in culture medium and the differentiating character of the culture cell types, effects of compound exposure duration and concentration ranges should be taken into consideration, in order to obtain an optimum exposure strategy for assessing compound perturbation on neural differentiation in ESTn (chapter 2). The ESTn was designed to detect compound induced effects specifically on neural development. In an optimal setting, a compound has an effect on neural differentiation, but not on cell viability or cytotoxicity [17]. Therefore, a viability assay (the Resazurin assay) on ESC was performed to determine the cytotoxic effects of a compound, and to determine a concentration-range to be tested in ESTn [323]. Furthermore, previous studies performed in our laboratory showed that proliferation and differentiation of ESC in the ESTc are highly intertwined processes [137]. It was recommended that, to largely limit exposure to the proliferation phase of the assay, it would be advantageous that EB be exposed to compounds from day 3 in the assay onwards [137]. Up to day 3, the ESTc and ESTn are identical. Therefore, compound exposure was performed from day 3 onward in all studies. Another variable influence on exposure in culture was serum deprivation of the culture medium [324]. Effects of MeHg exposure only during the serum-containing period (days 3-5), only during the serum-free period (days 6-13) or a complete exposure (days 3-13) was assessed. Due to the similar concentration-response observed after serum-containing and complete exposure and possible differences in exposure due to compound binding to protein between the serum-containing and serum-free phase of the method, exposure for following experiments was performed from day 3-5. In addition, temporal transcriptomics profiling of ESTn differentiation described in chapter 3 showed that greatest changes in gene expression are observed early in the ESTn (day 3-5) compared to later (days 6 and 7), indicating greatest compound effects can be expected during this stage of culture [177].

Limitations of scoring of neural outgrowth as a readout for neurodevelopmental toxicity

Although the morphological readout in ESTn was successful in predicting neurodevelopmental toxicity for a number of compounds, including MeHg, valproic acid, carbamazepine and phenytoin (chapters 3, 4 and 5), there are limitations to this readout. The scoring only takes into account the

morphological outgrowth, however, this method does not take into account effects on the variety of cell types in culture, such as shifts in ectodermal versus other germ layer type cells or even differences in neuron types or neurons versus astrocytes [319]. In addition, as stated before, neuron functionality cannot be taken into account using this model. Furthermore, morphological scoring provides insight on the ultimate effect of a compound on neurite outgrowth, while the adverse compound effect might have occurred much earlier in the differentiation process, as was observed in ESTc [155]. Although the morphological readout in ESTn and other readouts in previous test systems show promise in being used for the prediction of neurodevelopmental toxicity, the complexity of the developing nervous system warrants a more comprehensive assessment at the molecular level of exposure effects by neurodevelopmental toxicants [60].

Implementation of transcriptomic techniques in ESTn for prediction of neurodevelopmental toxicity

The usefulness of transcriptomics as a mechanistic approach for prediction of (neuro)developmental toxicity has been shown in vivo and in vitro [79, 80, 82, 151]. The use of molecular screens for toxicity testing is furthermore predicted to play an important role in near-future risk assessment [217]. The aim of transcriptomics implementation in the ESTn was to increase test throughput by measuring compound toxicity at an earlier stage, gain additional mechanistic insight in compound specific toxicity and to find biomarkers for (neuro) developmental toxicity [177, 263]. Performing a time-response transcriptomics study to determine most robust gene expression changes over time has been proven useful before in mouse developmental toxicity studies [152]. In order to determine the most informative exposure duration, MeHg exposure effects at the morphological ID50 concentration after 24, 48, 72 and 96h exposure were assessed in chapter 3. Using multiple analysis tools, from the single gene level to process enrichment, MeHg was observed to increase neurectodermal related genes and decrease mesodermal, endodermal and early differentiation genes over all time-points. Similar observations made in the WEC and in vivo in mice confirmed that transcriptomics in the ESTn can provide additional information on compound mechanism of action [82, 152]. In vivo mechanism of action was later also verified in ESTn for multiple other compounds in chapter 4 and chapter 5, including VPA, cyproconazole and flusilazole [203, 263].

In order to make a statistical prediction for compound induced neurodevelopmental toxicity in ESTn, the differentiation track approach was used in chapter 3 determining best exposure timing, chapter 5 to explain differences in

mechanism of action between compounds and for biomarker discovery in chapter 6. The differentiation track approach was first described by van Dartel et al. [187]. Using this technique, differentiation over time can be visualized by PCA and compound induced deviation from the track can be statistically defined [187]. Earlier we observed an increase in variation of late time-point samples for differentiation over time, indicating an increased heterogeneity between samples of one time-point [177]. Furthermore, greatest dynamics in gene expression changes were observed at day 4 (which is the same time-point as a 24h exposure) on the differentiation track compared to the remaining time-points, as was also observed in ESTc [177, 187]. Compound induced effects on the differentiation track were further studied on a 24, 48 and 72 hour exposure differentiation track. The 24 hour MeHg exposure provided the most significant deviation from the differentiation track, whereas 48 and 72 hour exposure samples did not deviate significantly from the track, due to increased time- and compound-induced sample variance. Furthermore, a shorter exposure of 6 and 12 hours with monobutyl phthalate in the ESTc did not induce any significant gene expression changes [40]. Therefore, based on the least variance, greatest observed dynamics in temporal gene expression and most significant compound deviation from the track, a 24 hour exposure was chosen for following experiments. Use of the differentiation track in chapter 5 was furthermore instrumental to visualize compound induced changes on differentiation [263].

In chapter 6, the differentiation track was further used to obtain a biomarker set for prediction of neurodevelopmental toxicity in the ESTn. Using a de novo analysis, including a leave-one-exposure-out approach, a gene set consisting of 29 genes was developed using data from chapters 3, 4 and 5. This gene set described both ESC differentiation and predicted neurodevelopmental toxicity. Evaluating the performance of this gene set in combination with the differentiation track algorithm showed that developmental toxicity of 10 compounds and 19 toxic and non-toxic exposures could be correctly predicted with an accuracy of 84%. These genes were mainly involved in processes related to neural development and general development. A similar approach was successfully performed for the ESTc for 15 compounds, resulting in a set of 52 genes with an accuracy of 83% [84]. In contrast to the prediction accuracy of 78% under the original ECVAM validated EST, the results for both ESTn and ESTc are promising [17]. However, additional compounds have to be evaluated, including negative controls and pharmacological active compounds which are not developmental toxic, to further confirm prediction of these differentiation track-based novel identified gene sets.

Implementation of transcriptomic concentration-response in ESTn to identify adaptive and adverse responses

The value of taking into account concentration-response in combination with transcriptomics to identify compound toxic properties has been shown in various fields of toxicological research [151, 229, 230, 305, 325]. In the field of reproductive toxicology, Naciff and Daston performed a range of studies in the male and female reproductive tract, showing individual endocrine disruptor induced gene expression changes followed a dose-response relationship [73, 325-327]. Comparable findings were observed in developmental toxicity studies by Robinson *et al.*, where exposure to the metals MeHg, cadmium and arsenic showed a dose-dependent effect on development related gene expression and process enrichment during gestational development in the mouse [152, 279]. Furthermore, a study in the ESTc described flusilazole concentration-dependent mechanisms of action at the transcriptome level [230]. Effects on processes related to development were observed at lower concentrations than those related to sterol metabolism, followed by enrichment of cell cycle gene expression at even higher cytotoxic concentrations. Similar findings were observed in **chapter 4** where exposure to VPA, cyproconazole and hexaconazole in the ESTn showed a concentration-dependent effect on individual gene expression, fold change and process enrichment. Using a concentration-response approach, mechanistic effects on development related processes could be identified at concentrations below observed effects on morphology, providing a more sensitive measure of concentration-dependent effects. Furthermore, compound specific process enrichment could be identified, enabling discrimination between the neurodevelopmental- (e.g. VPA and cyproconazole) and more general developmental toxicants (e.g. hexaconazole). In addition, compound class specific responses on sterol metabolism could be identified, discerning the triazoles from VPA.

Although using transcriptomics enables to detect compound induced effects on gene expression with greater sensitivity, a definition is required to discriminate adaptive from adverse gene expression changes. This definition should identify the level of regulation of a functional process representing an adverse response, in order to discern compound adverse effects exclusively with gene expression. This distinction has gained importance in view of interpreting gene-expression findings for risk assessment [226]. Until adverse and adaptive responses are identified, application of toxicogenomics for risk assessment remains dependent on traditional toxicology parameters [328]. Based on available human data, it has been proposed that reproductive and developmental toxicants have a threshold of adversity [329]. Current in vivo developmental toxicity testing paradigms are also build upon this hypothesis [10, 11]. In order to discern

adverse from adaptive effects on gene expression, Daston and Naciff proposed an approach for validation of in vitro alternatives, in which not the compound per se but an "exposure", defined as a compound concentration or dose, is classified as a developmental toxicant, enabling the use of a compound exposure as both a 'positive' and a 'negative' toxicant [101]. In line with this approach ESTn gene expression responses between a non-developmental toxic and a developmental toxic exposure concentration were compared in chapter 5. The study design was aimed at distinguishing between adaptive and adverse responses at the gene expression level. The developmental toxicants carbamazepine and flusilazole were observed to enriched pathways involved in transcription and compound metabolism at the low concentrations, whereas development related processes were enriched only at the high concentration. Additionally, the low concentration carbamazepine enriched histone methylation related processes which represent a known pharmacological mechanism of action of carbamazepine [227]. Taken together current evidence in literature suggests that up to a certain level of induction, general physiological pathways such as related to transcription and metabolism may indicate adaptive responses, whereas the induction of developmental pathways may be more specifically indicating adverse responses [151, 203, 229, 230, 305, 325]. In addition, the magnitude of induction should be considered. More detailed studies and development of additional concentration-response modeling tools will be needed to define the thresholds between adaptive and adverse responses in *in vitro* assays, in the interest of their interpretation and in view of hazard and risk assessment [217].

ESTn in risk assessment

Risk assessment determines the risks of human exposure to a compound or pharmaceutical [330]. Regulatory risk assessment is currently based on animal toxicity studies [9, 11]. Intelligent testing strategies are used to assess toxicological profiles, using decision schemes to determine the requirements of animal studies needed for hazard assessment [331]. Intelligent testing strategies use a stepwise approach (battery or tiered) with tests of increasing complexity to gather information on compound toxicity, weighing the information to determine further testing after each test performed, with the aim of limiting the number of animals needed. To further reduce the number of animals and decrease the time consuming process of toxicity testing, enhancing the use of *in vitro* developmental toxicity testing methods within intelligent testing strategies is desirable. In an intelligent testing strategy, such as the OECD conceptual framework [11], initial prioritization based upon all existing

information is followed by non-testing information such as read-across and (Quantitative) Structure-Activity Relationships ((Q)SAR). Simple *in vitro* screening assays, such as receptor binding assays, will provide more mechanistic data and as a next step, more complex (multi)cellular *in vitro* models such as the ESTn, can be studied. In this phase, multiple test systems each describing a part of development could be combined to provide a more complete indication of possible untoward effects on embryogenesis, functioning as a prioritizing tool for determining whether *in vivo* developmental toxicity studies are necessary. An initiative combining multiple *in vitro* models in an effort to determine compound induced toxicity in humans is the US Environmental Protection Agency (EPA) ToxCast program [332]. In this project 650 *in vitro* assays, including a variant on the ESTc, are combined to obtain a chemical fingerprint of currently more than 2000 chemicals. Using computational technology, it is thought a prediction model can be constructed for developmental toxicity based on high- throughput systems [266]. The optimal place of ESTn within an intelligent testing strategy is dependent on a clear definition of its applicability domain, in terms of the biological processes represented in the assay. The term applicability domain was defined by European Centre of Validation of Alternative Methods (ECVAM) as: *Definition of the chemical classes and/or ranges of test method endpoints for which the model makes reliable predictions* [35]. According to this definition, the predictive value of the method for different compound classes should assist in determining its applicability domain. Furthermore, the biological mechanisms incorporated within the system need to be characterized, to define the scope and limitations of the assay.

Defining the applicability domain

Developmental toxicants are structurally diverse and induce a broad range of developmental defects [333]. Furthermore, it has been shown that compounds inducing comparable birth defects, can do this through multiple mechanisms of action. For instance, in the WEC it was observed that four compounds inducing spina bifida *in vivo* showed limited overlap in toxicogenomic responses [82]. In **chapter 5** a similar finding is observed in the ESTn, where five compounds inducing neural tube defects *in vivo* showed no overlap in gene expression responses. To deal with this issue, a compound category approach may be useful. This strategy allows the assessment of specific chemical classes and their biological mechanisms for which the ESTn can make accurate prediction. A category approach could lead to a substantial reduction in animal use for developmental toxicity testing. When a chemical class has been correctly

predicted *in vitro*, chemicals belonging to the same class but with unknown embryotoxic properties could reliably be tested in the ESTn. In addition, using a read-across approach a prediction can be made as to the embryotoxic potency of that compound. Implementing a compound category approach and relative-potency ranking within a compound class have proven to be reliable methods for determining relative toxic properties in various models for developmental toxicity [15, 78, 81, 277, 334-336]. In the ESTc, de Jong *et al*. were able to rank relative potency of a series of homologous compounds, glycol ether alkoxy acid metabolites, valproic acid analogs and triazoles, matching the potency ranking to *in vivo* ranking [15, 334, 335]. In addition, using transcriptomics, categorization of phthalates and triazoles was performed in the ESTc, using gene sets of class specific differentiation modulation [81, 337]. Furthermore, within classes, known *in vivo* developmental toxicants and non-developmental toxic compounds could be distinguished using gene expression profiling [81]. In line with these studies, in **chapter 4** a transcriptomic comparison between gene expression profiles induced by two triazoles, the neurodevelopmental toxicant cyproconazole and developmental toxicant hexaconazole, was performed in ESTn. For both compounds, effects on general embryonic development terms could be identified, however, only for cyproconazole effects on neuron development were observed. Although further research is needed to determine the applicability domain of the ESTn, these results provide promising insight. To further increase knowledge on the applicability domain, a proposed method is identification of perturbations in pathways of toxicity in a model [217]. In the National Research Council's proposed 21st century toxicity testing strategy, these pathways of toxicity are identified as the biological signaling pathways important in compound perturbation [217]. In their vision, a systems approach can be used to describe the fundamental biologic events involved in toxicity pathways and to provide evolving biologic modeling tools that describe cellular circuits and their perturbations by compounds [338]. When the specific pathways of toxicity are identified for the ESTn, the applicability domain of the test system will be better understood.

Extrapolating transcriptional profiling in the ESTn to other test systems and species

Transcriptomic ESTn and ESTc comparisons

The ESTn was developed as a companion test system to the ESTc, and in both models transcriptomics studies have been performed to determine developmental toxicity. In **chapter 7** differences and similarities in transcriptomics responses between the two systems are discussed. Gene expression profiles of both ESTn and ESTc over time early on days 3, 4 and 5 are compared. Although temporal gene expression responses were generally comparable between models, a relatively small group of genes could be identified behaving differently in each model. These genes were mainly related to cardiac development in ESTc and neural development in ESTn. Gene expression changes induced by seven compounds, including six *in vivo* developmental toxicants and one negative control were assessed further. Compound induced gene expression changes were generally different in either model, predicting better in one model or in the other. For instance, the neurodevelopmental toxicant MeHg, which was misclassified in the validated EST [17], specifically induced gene expression changes related to neural development in ESTn, and only general development in ESTc. Furthermore, the tested concentration in ESTn was 200 fold lower compared to ESTc, indicating a higher sensitivity of neural differentiating cells to MeHg. On the other hand, specific effects of flusilazole on cardiac differentiation were observed in ESTc, whereas at a comparable concentration, no specific effects on neural differentiation were observed in ESTn. Furthermore, VPA induced gene expression changes were comparable in both ESTn and ESTc, but varied at the process level. Testing of developmental toxicants in both ESTc and ESTn in a tiered approach can provide additional information for *in vitro* developmental toxicity prediction screens, especially when a compound is suspected to be a neurodevelopmental toxicant, as shown with MeHg.

Interspecies- and in vitro / in vivo extrapolations using transcriptomics

Transcriptomics assessments provide a sensitive, robust and common endpoint that can be compared across in vivo and in vitro models, which usually use a variety of model-specific morphological endpoints to determine developmental toxicity. Additionally, cross-comparisons across in vitro and in vivo models may facilitate the identification of relevant biomarkers associated with compound exposure and developmental toxicity, and increase our understanding in determining how systems correlate in terms of response to compounds.

For extrapolation between species in vivo, there is recent transcriptomics evidence that the phylotypic stage plays a critical role in development [339]. Irie and Kuratani performed a quantitative comparative transcriptome analysis of four model vertebrate embryo models (mouse, chick, zebrafish and frog) and showed that the phylotypic stage is the most conserved phase in development across vertebrates (e.g. GD8.5 in mouse and 24hpf in zebrafish) [339]. During this stage, forming of the blastula, gastrulation and neurulation occur. In addition, at earlier (cleavage and morula) and later stages (somitogenesis and

organogenesis) transcriptomic profiles between vertebrates is less conserved. Extrapolation of compound induced developmental toxicity between species at the phylotypic stage may be more relevant than at earlier or later stages. Current transcriptomic tests performed in vivo, and in the WEC, zebrafish, ESTn and ESTc are performed during this stage, increasing the probability of finding effects which can be extrapolated to the human situation [77, 82, 84, 177, 215].

Although limited, initial comparative studies across models have shown the ability to match gene alterations linked with developmental toxicity in vivo with biomarkers in vitro. In a study by Kultima et al. [340], targets of valproic acid associated with effects on neural tube development in vivo were identified to be commonly altered in P19 neuroblastoma cells due to valproic acid. Genes commonly altered between the two models represented known neural tube defect candidate genes and pathways critical for early embryogenesis (e.g. retinoic acid). In a parallel transcriptomic comparison study between unexposed cultured whole embryos (WEC) and embryos in vivo, high similarities in gene expression changes over time were identified between the two model systems, including significance of gene expression changes, direction in regulation (increase or decrease) and corresponding functionality [341], suggesting the WEC to be a good representation of this particular window of in vivo development at the molecular level. Similarly, in response to all-trans-retinoic acid exposure, high similarity in gene expression changes were observed between WEC and embryos in vivo despite both common and unique developmental toxicity endpoints [77].

In **chapter 8**, a larger comparison study between *in vivo* model systems and *in vitro* systems, including the ESTn and ESTc, MeHg-induced transcriptomic response was compared to identify commonalities and dissimilarities in response [342]. Greater similarity were observed in terms of response between mouse embryos exposed in utero (2 studies), differentiating ESC in the ESTc and ESTn and WEC as compared to adult mouse liver, juvenile mouse brain and mouse embryonic fibroblast MeHg-studies. MeHg was observed to impact developmentally-related signatures in the early development model systems (*in vivo* or *in vitro*) versus the non-traditional developmental model systems. Future comparisons between ESTn and *in vivo* (as well as other alternative systems) using the emerging rich toxicogenomic databases should increase the mechanistic understanding of the relevancy of compound induced response in the ESTn, and therefore, the applicability domain of the ESTn for developmental toxicity testing.

Conclusion

In this thesis the development of the ESTn and implementation of transcriptomics into the ESTn testing strategy have been described, ultimately resulting in a biomarker gene set for prediction of neurodevelopmental toxicity. Compound effects on gene expression have been studied both over time and concentrations, comparing mechanism of action of a range of neurodevelopmental toxicants. Furthermore, comparisons between ESTn, ESTc and other developmental toxicity models show the ESTn can add mechanistic insight and increased prediction to the developmental toxicity testing strategy. In order increase the prediction of the ESTn, the applicability domain of the ESTn should be further defined by defining the pathways of toxicity important in ESTn neural differentiation and compound perturbation.

Nederlandsche Samenvatting

Inleiding

De wetenschap van de ontwikkelingstoxicogie houdt zich bezig met het onderzoeken van de giftige effecten van stoffen op de ontwikkeling van de ongeboren vrucht. Om geboorteafwijkingen door giftigheid van stoffen te voorkomen, verplicht huidige internationale wetgeving fabrikanten om stoffen en medicijnen te testen op ontwikkelingstoxiciteit. De bepaling van toxiciteit van een stof wordt uitgevoerd op proefdieren. Van alle proefdieren die tegenwoordig gebruikt worden voor toxicologische testen, wordt ongeveer 65% gebruikt voor fertiliteit- en ontwikkelingstoxicologie. Dit komt doordat in deze studies het effect van stoffen over meerdere generaties dieren wordt bestudeerd. Omdat het gebruik van proefdieren ethisch ongewenst is, is het belangrijk om nieuwe alternatieve testsystemen te ontwikkelen voor het voorspellen van ontwikkelingstoxiciteit, waarbij minder of zelfs géén proefdieren gebruikt hoeven te worden. Daarnaast zijn de huidige testen te arbeidsintensief om voorspellingen te kunnen doen over de giftigheid van een groot aantal stoffen in een korte tijd.
Een veelbelovend alternatief model voor het voorspellen van ontwikkelingstoxiciteit is de embryonale stamcel test (EST). Deze test maakt gebruik van de pluripotente eigenschap van embryonale stamcellen (ESC), waardoor deze cellen kunnen differentiëren tot elk celtype in het lichaam. In deze test worden stamcellen gedifferentieerd tot hartspiercellen (cariomyocyten), welke onder de microscoop waarneembaar zijn doordat deze cellen contraheren. Het verstoren van deze differentiatie ten gevolge van blootstelling aan ontwikkelingstoxische stoffen wordt gezien als een maat voor ontwikkelingstoxiciteit. Dit wordt gemeten door met behulp van de microscoop 'handmatig' het aantal celculturen met kloppende gebieden te tellen. Uit validatie van de EST bleek dat de test een redelijk goede voorspelling gaf voor ontwikkelingstoxiciteit, maar niet voor stoffen welke een nadelig effect hebben op de ontwikkeling van het zenuwstelsel. Om de voorspelbaarheid van de EST te verbeteren, werden daarom een aantal voorstellen gedaan. Een van deze verbeteringen was het ontwikkelen van een neurale variant van de EST, om de giftigheid van neuraal ontwikkelingstoxische stoffen toch goed te kunnen voorspellen. Een ander voorstel was om de subjectieve handmatige scoringsmethode van de EST te vervangen door een objectievere maat. Een methode om dit te doen is met behulp van transcriptomics, een relatief nieuwe techniek waarmee de expressie van tienduizenden genen in één experiment gemeten kan worden. Wanneer met deze techniek het verschil in genexpressie tussen blootgestelde en niet blootgestelde cellen gemeten wordt, kan een profiel ontwikkeld worden welke stof geïnduceerde effecten op ontwikkeling in een testsysteem beschrijft. Daarnaast kan deze methode opheldering geven over de vele moleculaire mechanismen die ten grondslag liggen aan ontwikkelingstoxiciteit.

Samenvatting

In dit proefschrift wordt de ontwikkeling van de neurale embryonale stamcel test (ESTn) beschreven. De ESTn is ontwikkeld als een snel, of 'high-throughput', test systeem voor het detecteren van neuraal-ontwikkelings toxische stoffen. Alle stappen, van ontwikkeling en het karakteriseren van het model, naar implementatie van transcriptomics technieken in het test systeem tot het ontwikkelen van een biomarker genen set voor neuraal-ontwikkelings toxiciteit worden besproken in dit proefschrift. Daarnaast worden de genexpressie profielen, welke de differentiatie over de tijd en effecten van stoffen beschrijven in de ESTn, vergeleken genexpressie profielen in andere *in vitro* en *in vivo* modellen.

De ontwikkeling en het karakteriseren van de ESTn is beschreven in **hoofdstuk 2**. Een 20 dagen lang neuraal differentiatie protocol was ontworpen, gebaseerd op twee eerder beschreven protocollen door Okabe *et al*. en Bibel *et al*. De belangrijkste stappen om stamcellen te laten differentiëren tot neurale cellen in deze methode zijn de volgende: Ten eerste de vorming van embryoid bodies (EB) door middel van de 'hanging drop' methode. Een EB is een agglomeratie van cellen die de karaktereigenschappen heeft van een zeer vroeg stadium embryo. Ten tweede worden deze EB bloot gesteld aan retinolzuur (RA), een actieve metaboliet van vitamine A. Deze metaboliet heeft tijdens de ontwikkeling onder andere een belangrijke functie in de vroege vorming van het zenuwstelsel. Ten derde wordt het serum percentage in het medium in twee stappen verlaagd van 20% naar 0% en worden groeifactoren toegevoegd aan het medium, beiden om ESC differentiatie richting neurale cellen te stimuleren. Aan het einde van de kweek worden de EB compleet omringd door neurale uitgroei, die bestaat uit neuronen en glia cellen (Hoofdstuk 9, Figuur 2).

Omdat dit protocol voor neurale differentiatie redelijk lang is voor een snelle screening van stoffen, ontwikkelden we twee kortere varianten, een 13 dagen en een 17 dagen lange methode. Dit deden we door de fase waarin het medium geen serum bevat te verkorten. De drie protocollen werden vergeleken wat betreft morfologie van de neurale uitgroei en op moleculair niveau, waarbij we keken naar RNA en eiwit markers welke belangrijk zijn tijdens de vroege ontwikkeling. Dit gebeurde met behulp van immunocytochemie, genexpressie bepalingen en flow-cytometrie. Tijdens de eerste zeven dagen zijn de drie protocollen identiek aan elkaar. Tijdens deze fase zagen we dat markers voor de drie primaire kiemlagen (ectoderm, mesoderm en endoderm) aanwezig waren in de kweek. Daarnaast werd gezien dat neurale en ectodermale markers

over de tijd verhoogd waren en markers voor pluripotentie over de tijd verlaagd werden. Hiermee werd aangetoond dat neurale differentiatie al vanaf vroeg in het protocol geïnduceerd werd. Vergelijking van de eindstadia van de drie protocollen laat zien dat de mate van genexpressie voor verschillende neuronen typen vergelijkbaar is. Uit dezelfde analyse blijkt dat het genexpressie niveau voor een marker voor astrocyten lager, maar nog steeds significant verhoogd is in het korte protocol ten opzichte van de andere twee langere protocollen. Aangezien de drie protocollen wat betreft morfologie en moleculaire expressie vergelijkbaar zijn, werd voor de volgende experimenten gebruik gemaakt van het kortste protocol, wat we vanaf nu de ESTn zullen noemen.

Om te bepalen of het mogelijk was om specifieke neurale ontwikkelingstoxiciteit te kunnen voorspellen in de ESTn, stelden we de cellen bloot aan methylkwik (MeHg) op verschillende concentraties. We bepaalden het effect van MeHg op de neurale differentiatie door bij verschillende EB de mate van neurale uitgroei rond de EB te bepalen. Daarnaast gebruikten we de flow-cytometrie methode om het percentage cellen te tellen dat positief was voor de volgende drie markers: SSEA1, een marker voor pluripotentie; nestin, een marker voor neurale voorlopercellen; en βIII-tubuline, een marker voor neuronen. Doordat MeHg aan bestanddelen van het serum in het medium kan binden, kan de effectieve concentratie en daarmee de blootstelling aan de cellen dalen. Aangezien in de ESTn het serum percentage in het medium verlaagd wordt van 20% naar 0%, voerden we drie verschillende blootstellingen uit: blootstelling tijdens de serum periode (dag 3-5), tijdens de serum vrije periode (dag 6-13) of tijdens de gehele periode (dag 3-13). Effecten van MeHg op de neurale uitgroei waren vergelijkbaar voor blootstellingen tijdens de serum periode en gehele periode, terwijl de serum vrije periode gevoeliger was. Daarnaast was bij de hoogst geteste concentratie nog steeds neurale uitroei waargenomen bij de serum periode blootstelling, terwijl bij de twee andere blootstellingen geen neurale uitgroei meer was. Dit komt waarschijnlijk doordat tijdens de serum vrije periode meer MeHg vrij beschikbaar is in het medium waardoor de effectieve concentratie toeneemt. Door de vergelijkbare concentratierespons tijdens de serum periode en complete blootstelling werd voor de toekomstige experimenten gebruik gemaakt van de blootstelling van dag 3 tot dag 5.

In **hoofdstuk 3** wordt de ESTn verder gekarakteriseerd door genexpressie over de tijd te bestuderen met behulp van transcriptomics. We namen monsters van celkweken op dag 0 van de kweek (pluripotente ESC) en op de dagen 3, 4, 5, 6 en 7 tijdens de neurale differentiatie. Genen die gereguleerd waren over de tijd konden, op basis van hun genexpressie profiel, onderverdeeld worden in acht

clusters, elk met een specifiek genexpressie patroon over de tijd. Met verscheidene analyse technieken kon aangetoond worden dat genen met een vroeg verhoogde genexpressie gerelateerd waren aan pluripotentie en transcriptie en dat laattijdig gereguleerde genen te maken hadden met neurale ontwikkeling. Daarnaast kon differentiatie over de tijd beschreven worden met behulp van de 'differentiatie track', waarbij door 'principal component analysis' het patroon van genexpressie van alle genen over de tijd weergegeven kan worden per monster. De 'differentiatie track' liet zien dat voor elk getest tijdspunt de monsters samen clusterden en dat deze groepjes chronologisch over de track verdeeld lagen. Daarnaast werd de variatie tussen monsters binnen een tijdsmeting groter bij monsters genomen op een later tijdspunt. Dit geeft aan dat cellen in de kweek differentieerden en ten opzichte van elkaar steeds meer verschillend werden.
Op eenzelfde manier werd ook het effect van MeHg bestudeerd met behulp van transcriptomics. Cellen werden blootgesteld aan MeHg vanaf dag 3, waarna monsters werden genomen na 24, 48, 72 en 96 uur blootstelling. Deze data lieten zien dat op elk tijdspunt MeHg de genexpressie verhoogde van neuraal gerelateerde genen en genexpressie verlaagde van genen gerelateerd aan algemene en vroege ontwikkeling. De 'differentiation track' techniek werd gebruikt om veranderingen van MeHg op de genexpressie aan te tonen na 24, 48 en 72 uur blootstelling. MeHg monsters verschilden het meest van de 'differentiation track' na 24 uur blootstelling. Op 48 en 72 uur blootstelling was de variatie binnen de tijd en blootgestelde monsters te groot om een significant verschil op te kunnen pikken. Op basis van deze gegevens werden monsters voor de volgende transcriptomics experimenten blootgesteld voor 24 uur en, namen we controles mee van dag 3, 4 en 5 om de 'differentiatie track' op te kunnen zetten.

Een belangrijk gegeven in de toxicologie is dat giftigheid toeneemt met de concentratie van de blootstelling en dat er dus een concentratie hoort te zijn waarbij een stof niet giftig is. In **hoofdstuk 4** gebruiken we transcriptomics om het effect van de blootstelling van een stof te bestuderen bij verschillende concentraties. Hiervoor gebruikten we drie stoffen waarvan bekend is dat ze ontwikkelingstoxisch zijn: de twee triazolen cyproconazole en hexaconazole en valproaat. Triazolen worden normaal gebruikt als antischimmel middelen en valproaat is een medicijn dat gebruikt wordt tegen epilepsie. Effecten van deze stoffen werden gemeten op genexpressie niveau na 24 uur blootstelling en vergeleken met de uitkomst van de morfologische score op dag 11. Alle drie de stoffen lieten een concentratie gemedieerd effect zien op genexpressie en biologische processen, wat informatie verschafte over mechanismen en concentratierespons karakteristieken. Algemene en specifieke effecten van de verschillende stoffen konden zichtbaar gemaakt worden. Het biologische proces 'embryonic morphogenesis' werd bijvoorbeeld door elk van de stoffen beïnvloed, wat aangeeft dat deze stoffen een effect op 'standaard' ontwikkeling hebben. Verder werden voor cyproconazole en valproaat specifieke effecten op neurale ontwikkeling gevonden, terwijl deze niet gevonden werden voor hexaconazole. Dit komt overeen met de literatuur, aangezien cyproconazole en valproaat neuraal ontwikkelingstoxisch zijn en hexaconazole dat niet is. Verder konden klasse specifieke effecten worden gevonden voor de triazolen, aangezien zij het sterol biosynthese proces beïnvloedden, een belangrijk mechanisme in de antischimmel werking van deze stoffen. Het gebruik van transcriptomics in deze studie toonde verder aan dat effecten op genexpressie eerder waarneembaar waren dan effecten op morfologie, waardoor dit een meer gevoeligere methode kan zijn om ontwikkelingstoxiciteit te meten.

In de volgende transcriptomics studie in **hoofdstuk 5** werden effecten op genexpressie van zes stoffen met verschillende werkingsmechanismen bestudeerd, om het toepassingsdomein van het testsysteem te bepalen. Vijf van deze stoffen zijn bekende ontwikkelingstoxische stoffen en één stof was meegenomen als een negatieve controle. De stoffen werden getest op een toxische concentratie waarop het effect op morfologie vergelijkbaar was tussen de stoffen én een concentratie die 30 keer lager, en dus niet toxisch, was. Stof specifieke en algemene genexpressie veranderingen werden waargenomen tussen de verschillende stoffen. Er werden echter geen processen gevonden die door alle ontwikkelingstoxische stoffen aangedaan waren. Dit toont aan dat effecten op neurale differentiatie via meerdere mechanismen geïnduceerd kunnen worden. Ook konden genexpressie profielen en proces verrijking gebruikt worden om de verschillen in genexpressie in een niet toxische en ontwikkelingstoxische concentratie te vergelijken. Deze informatie kan gebruikt worden om verschillen in genexpressie veranderingen ten opzichte van adaptieve of ongunstige effecten te verklaren.

In **hoofdstuk 6** zijn de transcriptomics data van hoofdstukken 3, 4 en 5 gecombineerd om een biomarker genen set te verkrijgen om neurale ontwikkelingstoxiciteit in de ESTn te voorspellen. Genexpressie veranderingen werden vergeleken met behulp van de 'differentiatie track' waarbij data van 10 stoffen en 19 blootstellingen werd gebruikt. Met behulp van een genen set evaluatie gekoppeld aan een 'leave-one-out cross-validatie' kon een set van 29 genen gevonden worden die vooral te maken hadden met neurale ontwikkeling. Met deze genen set kon neurale ontwikkelingstoxiciteit in de ESTn voor 84% van

de blootstellingen goed voorspeld worden. Deze set verbetert de predictie van de ESTn en kan in de toekomst eventueel gebruikt worden voor risico evaluaties.

De ESTn was ontwikkeld om gebruikt te worden in combinatie met de originele ESTc. De overeenkomsten en verschillen tussen de twee modellen op genexpressie niveau worden beschreven in **hoofdstuk 7**. Tijd gereguleerde genexpressie profielen van ESTn en ESTc op dag 3, 4 en 5 en blootstelling aan zeven stoffen werden tussen de modellen vergeleken. Hoewel genexpressie verschillen over de tijd tussen de twee protocollen voor een groot deel vergelijkbaar waren, was er een kleinere groep genen die zeer specifiek gereguleerd werd door één van beide modellen en niet in het andere model. Deze genen waren gerelateerd aan hartspierdifferentiatie in de ESTc en neurale differentiatie in ESTn. Stof geïnduceerde genexpressie veranderingen waren over het algemeen zeer verschillend tussen de modellen, waardoor de ontwikkelingstoxiciteit óf in de ESTc (bijvoorbeeld flusilazole) óf in de ESTn (bijvoorbeeld MeHg) goed werd voorspeld. Een uitzondering hierop was valproaat, waarbij genexpressie veranderingen in beide modellen vergelijkbaar waren, hoewel er op het proces niveau grote verschillen waren. Een vergelijking op genexpressie niveau tussen ESTn en ESTc laat zien dat beide modellen een eigen specifieke bijdrage kunnen leveren aan voorspelling van ontwikkelingstoxiciteit.

Een vergelijking tussen ESTn en andere *in vitro* en *in vivo* modellen op transcriptomics niveau is uitgevoerd in **hoofdstuk 8**, om inzicht te krijgen in overeenkomsten en verschillen van mechanismen in deze modellen. Het effect van MeHg op genexpressie in verschillende *in vitro* en *in vivo* modellen was bestudeerd. Modellen voor ontwikkelingstoxiciteit waren de *in vitro* rat 'whole embryo culture', ESTc en ESTn en twee *in vivo* embryotoxiciteitstesten. Daarnaast waren modellen vergeleken welke acute toxiciteit voorspellen zoals een embryonale fibroblast cellijn en het jong-volwassen brein en de nier. Evaluatie van vergelijkbare en unieke aspecten op het functionele en genexpressie niveau toonden aan dat deze een vergelijkbaar patroon lieten zien in de ontwikkelingstoxiciteit modellen, maar niet in de andere modellen. Deze vergelijking laat zien dat extrapolatie van *in vitro* naar *in vivo* modellen relevant is en dat genexpressie veranderingen over modellen heen voor een groot deel overeen komen.

Conclusie

In dit proefschrift wordt de ontwikkeling van de ESTn en implementatie van transcriptomics in de ESTn test strategie beschreven. Dit leidde uiteindelijk tot een biomarker genen set voor het voorspellen van neurale ontwikkelingstoxiciteit in de ESTn. Stof effecten op genexpressie over tijd en concentratie zijn beschreven en bestudeerd, waardoor een vergelijking tussen mechanismen van verschillende neuraal ontwikkelingstoxische stoffen gemaakt kan worden. Verder is er een vergelijking gemaakt tussen de ESTn, ESTc en andere *in vitro* en *in vivo* modellen waaruit blijkt dat de ESTn een bijdrage kan leveren aan mechanistisch inzicht en een verhoging van de predictie in ontwikkelingstoxiciteit test strategieën. Om de predictie van de ESTn verder te verhogen is het nodig om het toepassingsdomein van de ESTn verder te definiëren, waarbij het belangrijk is om de mechanismen achter toxiciteit in de ESTn in kaart te brengen.