

Video Article

Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology Data Generation

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Abstract

Efficient protocols to differentiate human pluripotent stem cells to various tissues in combination with -omics technologies opened up new horizons for *in vitro* toxicity testing of potential drugs. To provide a solid scientific basis for such assays, it will be important to gain quantitative information on the time course of development and on the underlying regulatory mechanisms by systems biology approaches. Two assays have therefore been tuned here for these requirements. In the UKK test system, human embryonic stem cells (hESC) (or other pluripotent cells) are left to spontaneously differentiate for 14 days in embryoid bodies, to allow generation of cells of all three germ layers. This system recapitulates key steps of early human embryonic development, and it can predict human-specific early embryonic toxicity/teratogenicity, if cells are exposed to chemicals during differentiation. The UKN1 test system is based on hESC differentiating to a population of neuroectodermal progenitor (NEP) cells for 6 days. This system recapitulates early neural development and predicts early developmental neurotoxicity and epigenetic changes triggered by chemicals. Both systems, in combination with transcriptome microarray studies, are suitable for identifying toxicity biomarkers. Moreover, they may be used in combination to generate input data for systems biology analysis. These test systems have advantages over the traditional toxicological studies requiring large amounts of animals. The test systems may contribute to a reduction of the costs for drug development and chemical safety evaluation. Their combination sheds light especially on compounds that may influence neurodevelopment specifically.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52333/>

Introduction

The ability of human embryonic stem cells (hESC) to differentiate into various types of cells opened up a new era of *in vitro* toxicity testing¹, disease modelling and regenerative medicine². The stem cells are endowed with the capacity to self-replicate, to keep their pluripotent state, and to differentiate into specialized cells^{3,4}. The properties of hESC (capacity to differentiate to all major cell types) are also found in other human pluripotent stem cells, such as human induced pluripotent stem cells (hiPSC) or cells generated by nuclear transfer⁵. For instance, many different hESC lines have been differentiated into neurons⁶, renal cells⁷, neural crest cells⁸, cardiomyocytes⁹⁻¹², or hepatocytes like cells^{13,14}. Moreover, hESC can spontaneously differentiate into cells of all three germ layers¹⁵⁻¹⁸ in embryoid bodies (EBs)^{19,20}. Early embryonic development is regulated by differential expression of various genes related to the different germ layers which has been captured at mRNA level by transcriptomics using microarray technology¹⁵. These efforts resulted in the establishment of organ specific toxicological models based on hESC/hiPSC and transcriptomics analysis (for review see^{21,22}). These models have advantages over the traditional use of laboratory animals for toxicological studies, as preclinical studies using laboratory animals are not always predictive of human safety. The drug induced toxicities encountered in patients are often related to metabolic or signaling processes that differ between humans and experimental animals. The species difference has prevented the reliable early detection of developmental toxicity in humans, and for instance drugs such as thalidomide^{23,24} and diethylstilbestrol^{25,26} were withdrawn from the market due to teratogenicity. Thalidomide has not shown any developmental toxicity in rats or mice. Environmental chemicals such as methyl mercury²⁷ resulted in prenatal developmental toxicity with respect to the nervous system in various species, but human manifestations have been hard to model in animals. To address the problem of species specificity issues, scientists working under different projects based on stem cells like ReProTect, ESNATS, DETECTIVE *etc.* are engaged in the development of different models for embryonic toxicity, neurotoxicity, cardiotoxicity, hepatotoxicity and nephrotoxicity using human toxicants suspected to affect humans.

Under the European consortium project 'Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS)' five test systems have been established. One test system the so called UKK (Universitätsklinikum Köln) test system partially captures early human embryonic development. In this system human embryonic H9 cells are differentiated in to three germ layers (ectoderm, endoderm and mesoderm)¹⁵ and germ layer specific signatures have been captured by transcriptomics profile using the Affymetrix microarray platform. Various developmental toxicants like thalidomide²⁸, valproic acid, methyl mercury^{16,17}, or cytosine arabinoside¹⁵ have been tested in this system, and toxicant specific gene signatures have been obtained. In a second test system, the so called the UKN1 (University of Konstanz) test system 1, H9 cells are differentiated to neuroectodermal progenitor cells (NEP) for 6 days. This is evidenced by high expression of neural gene markers such as *PAX6* and *OTX2*. During differentiation for 6 days, NEP cells have been exposed to developmental neuro-toxicants such as VPA, methyl mercury. Toxicant-specific de-regulated transcriptomics profiles have been obtained as well by using the Affymetrix microarray platform^{16,29}.

The new vision for toxicology of the 21st century envisages that test systems do not only yield phenotypic descriptions like histopathology *in vivo*, or transcriptome changes at the end of long-term toxicant incubations. It rather suggests that assays provide mechanistic information³, and that this information can be mapped to so-called adverse outcome pathways (AOP) that provide a scientific rationale for hazardous effects³⁰. To provide such information, the test systems applied have to be highly quality controlled³¹, as for instance documented by robust standard operation procedures. Moreover, time-dependent changes need to be mapped with high resolution. This requires test systems with synchronized changes³². The UKN1 and UKK test systems described here have been optimized for these requirements.

Protocol

The following protocol was performed using human Embryonic Stem Cell line (hESC) H9. This cell line was routinely cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in hESC culture media supplemented with bFGF and then cultured in stem cell media on 6 cm Petri plates coated with basement membrane matrix such as matrigel, to get rid of MEFs. The H9 cells from >80% confluent plates were used for further passage. H9 cells cultured on basement membrane matrix plates were used for EBs formation. All procedures mentioned in the following protocol have been performed using standard methods for aseptic and good cell culture practices.

Part 1. UKK test System

1. Human Embryonic Stem Cell Culturing

- Splitting and maintenance of H9 on feeder cells
 - Pipette 2 ml 0.1% gelatin into each 6 cm plate and incubate for 30 min in cell culture incubator (37 °C and 5% CO₂). Aspirate gelatin solution with sterile Pasteur pipette.
 - Add 2 ml MEF medium containing 0.1 x 10⁶ MEF cells/ml into the two gelatin coated plates and incubate them in cell culture incubator (37 °C and 5% CO₂) O/N.
 - On next day, remove the H9 cells vial from the liquid nitrogen storage tank using forceps and thaw the vial in a 37 °C water bath using long forceps.
 - Remove the vial from water bath, bath it with 70% ethanol, air dry in the biosafety cabinet for 15 to 30 sec and transfer the cells to 15 ml falcon tube.
 - Add 9 ml of H9 culture medium slowly on the inner wall and centrifuge the cells at 200 x g for 5 min.
 - Aspirate the supernatant and re-suspend the cells in 6 ml culture medium containing ROCK inhibitor (10 µM, Y27632) and gently pipette to mix. Aspirate MEF medium from the 6 cm plate and add 3 ml cell suspension in each plate. Change the medium on day 3 and then every other day. Subculture >80% confluent plate cells with split ratio 1:3.
Note: Usually in 5 to 7 days plate becomes confluent. Feeders used are obtained from CF1 mice embryo and inactivated by exposure to γ radiation.
- H9 cell culturing on basement membrane matrix coated plates
 - Thaw stem cell medium (5x) supplement at RT and add 100 ml into 400 ml basal medium in biosafety cabinet.
 - Thaw basement membrane matrix on ice. Add suggested volume of basement membrane matrix (refer to certificate of analysis for each batch) in 24 ml chilled DMEM/F-12 basal medium for twelve 6 cm plates. Mix by pipetting up and down.
 - Add 2 ml in each 6 cm plate. Keep the plate at RT for 1 hr. Remove the medium and add 2 ml of stem cell medium.
 - Take out four confluent H9 plates on MEFs. Remove the differentiated colonies with 1 ml pipette tip under stereomicroscope kept in biosafety cabinet.
 - Aspirate the medium and wash the cells with 4 ml PBS and add 2 ml stem cell medium in each plate. Cut the undifferentiated colonies with 26 G needle in to 6 to 9 pieces each.
 - Gently collect the cells in 50 ml falcon tube. Centrifuge at 200 x g for 5 min.
 - Aspirate the supernatant and re-suspend in 12 ml stem cell medium. Count the clumps by putting 20 µl on glass slide under the microscope and adjust the volume for 150 clumps per ml. Add 2 ml of suspension in each 6 cm plate.
 - Move the plates back-and-forth and side-to-side motions for uniform clump distribution and incubate the plates in cell culture incubator (37 °C and 5% CO₂).
 - Remove the differentiated colonies and give medium change every alternate day.

2. Embryoid Bodies (EBs) Formation

Perform all procedure mentioned below as per aseptic precautions and in the biosafety cabinet.

- Day 0 — Plating of H9 cells on V bottom plates

1. Prepare 5% block copolymer such as Pluronic F 127 in PBS and filter through vacuum driven filtration system using 0.22 μ m sterile filter.
 2. Coat V bottom 96 well plates with 40 μ l of 5% block copolymer per well and incubate at RT for 45 min.
 3. Remove the confluent basement membrane matrix plates with H9 cells from incubator and remove the differentiated colonies with 1 ml pipette tip under stereomicroscope in biosafety cabinet.
 4. Aspirate the medium and wash the cells with 4 ml PBS. Add 2 ml random differentiation medium (H9 culture medium without bFGF, RD medium) in each plate. Use passage tool and cut the H9 cell colonies in clumps of uniform size and shape by observing under stereomicroscope in biosafety cabinet and then gently scrape with the cell scraper.
 5. Collect the clumps in 50 ml falcon tube and centrifuge at 200 x g for 5 min. Aspirate the supernatant and re-suspend the cell in RD medium to get 1,000 clumps per ml.
 6. Aspirate the block copolymer from V bottom plates. Pour the clumps in sterile square plate and with help of multichannel pipette add 100 μ l of suspension to each well of V bottom plate.
 7. For the force aggregation of clumps, centrifuge the V bottom plates at 4 °C for 4 min at 400 x g. Incubate plates in cell culture incubator (37 °C and 5% CO₂) for four days.
2. Day 4 — Collection of EBs
1. Collect the EBs in the sterile square plate from V bottom plates using multichannel pipette and wide bore 200 μ l tips.
 2. Collect the EBs from sterile square plate in to 15 ml falcon tube with 10 ml sterile serological pipette. Allow EBs to settle for 2 min. Aspirate the supernatant and wash the EBs with 5 ml PBS.
 3. Allow EBs to settle for 2 min and aspirate the supernatant. Re-suspend EBs in 5 ml RD medium.
 4. Pipette out 10 ml RD medium in 10 cm bacteriological plates. Transfer the EBs in 10 cm bacteriological plates.
 5. Incubate bacteriological plates on horizontal shaker (reciprocation motion 50/min) kept in cell culture incubator (37 °C and 5% CO₂) for required time period. Give medium change (15 ml RD medium) every alternate day.
- Note: Gentle handling is required while culturing hESCs. The size of EBs varies on day 4. Select the uniform size EBs (\pm 20%) by observing under stereomicroscope for further experiment. Approximately 50% EBs formed with this method are of uniform in size. The transfer of EBs on shaker results in uniform shape.

3. Cytotoxicity Assay for IC₁₀ Determination

1. Transfer of EBs on optical bottom plates
 1. Thaw 0.1% gelatin in water bath at 37 °C for 15 min and coat optical bottom plates with 50 μ l of 0.1% gelatin per well using multichannel pipette. Incubate the plates at RT for 45 min. After incubation aspirate the gelatin from optical bottom plates.
 2. Take out the EBs collected on day 4 in 10 cm bacteriological plate containing RD medium.
 3. Keep optical bottom plates in slanted position in biosafety cabinet. Transfer two uniform size of EBs in 100 μ l RD medium per well in optical bottom 96 well plate by observing under stereomicroscope. Keep 12th column empty.
 4. Incubate plates in cell culture incubator (37 °C and 5% CO₂) for 24 hr.
2. Drug exposure from day 5 to day 14
 1. Weigh the test compound and make highest concentration in known solvent.
 2. Perform half-logarithmic dilution of the test compound serially till 8 dilutions in the solvent containing falcon tubes numbered with A to H. Keep tube no. I as vehicle control, tube no. J as negative control (RD medium) and tube no. K as positive (70% ethanol) control.
 3. Thaw the RD medium in water bath at 37 °C for 15 min. Take out 5 ml RD medium each in 11 sterile falcon tubes labelled from 1 to 11.
 4. Transfer 5 μ l of solution from tube A to tube K in to tube 1 to 11 respectively and vortex the tubes. Take out the optical bottom plate from the incubator and carefully remove the media with use of multichannel pipette.
 5. Add 200 μ l of media from tube number 1 to 11 into the respective columns of the optical bottom plate. Give medium/drug change every alternate day.

Note: For half-logarithmic dilutions take 6.48 μ l solvent in 7 tubes labeled from 2 to 8. From highest concentration tube no.1 transfer 3 μ l to tube no.2, vortex and serially transfer 3 μ l to next tube. Keep tube no.9 for vehicle control and tube no.10 for negative control. Tube no.11 is 70% ethanol.
3. Day 14 : Resazurin exposure and fluorescence measurement
 1. Thaw RD medium in water bath at 37 °C for 15 min. Perform all procedure mention below in absence of light in the biosafety cabinet.
 2. Take 10 ml RD medium in 15 ml tube (A) and add recommended volume of resazurin reagent and mix by pipetting. Take out the optical bottom plate from the incubator and carefully remove all medium with multichannel pipette.
 3. Add 100 μ l of medium from tube A in each well. Incubate the plate in cell culture incubator (37 °C and 5% CO₂) for 90 min.
 4. Measure the fluorescence using spectrophotometer (560_{Ex}/590_{Em}).
4. IC₁₀ value determination
 1. Import the values in graph pad prism after subtracting the blank values. Set x-axis as a dose and y-axis as a fluorescence units.
 2. Normalize the values to obtain percentage on y axis and transform the values (x-axis as log scale). Calculate IC₅₀ value by using sigmoidal-dose response (variable slope) parameter. Calculate log IC₁₀ values by using following equation:

$$F = 10 \log EC_{50} = \log ECF - (1/\text{HillSlope}) * \log(F/(100-F))$$

$$Y = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + 10^{((\log EC_{50} - X) * \text{HillSlope})})$$
 3. Determine the IC₁₀ values to be taken for further studies.

4. Biomarker Study Based on Microarrays

- Day 0 to day 5:
 - Embryoid body formation and transfer to 10 cm bacteriological plates — follow the steps mentioned in point 2 for embryoid body formation.
 Note: Use three biological replicates for each study. Divide each biological replicate in to two parts — Drug treatment at IC₁₀ concentration and vehicle control. Prepare drug concentration 10,000 fold above the IC₁₀ concentration in vehicle and from this add 10 µl to 100 ml RD medium with H9 cell clumps in 50 ml Eppendorf tube mix well and seed it on V bottom plates. Follow the same procedure for vehicle control group.
- For drug exposure on Day 5 to 14, collect the EB's and transfer them in 10 cm bacteriological plates on day 4 as per the steps mentioned in point 2. Transfer the plates on horizontal shaker (reciprocation motion 50/min) in cell culture incubator (37 °C and 5% CO₂) for 14 days. Give medium change every alternate day.
- For sample collection, on day 14, collect the EBs from 10 cm plates in to 15 ml falcon tube with sterile serological pipette. Allow EBs to settle for 2 min. Aspirate the supernatant and wash the EBs with 5 ml PBS. Allow EBs to settle for 2 min and aspirate the supernatant. Re-suspend EBs in 1 ml RNAlater solution or TRIzol reagent, vortex and store the sample at -80 °C till further processing.
 Note: Perform all procedure in biosafety cabinet as per good laboratory practices. Rotate the plates in circular motion around the center to bring all EBs in center, aspirate the medium from surrounding with the help of sterile glass pasture pipette, add 15 ml RD medium and then add 15 µl of drug / vehicle for respective group.

5. RNA Isolation and Integrity Testing

- RNA Isolation:
 Most of the steps mentioned below are to be performed for RNA purification using RNeasy Mini Kit as per the instruction manual. Always use nuclease free tubes, pipette tips and water. While working with TRIzol carry out all procedure in chemical safety hood and wear protective glasses as well as chemical protective gloves.
 - Thaw the samples on ice. If samples are stored in RNAlater solution, centrifuge the tubes at 12,000 x g for 5 min at 4 °C. Discard the supernatant and add 1 ml TRIzol reagent.
 - Triturate the samples using 24 G needle and 1 ml syringe. Approximately 15 times trituration is sufficient for disruption of EBs, cell wall and plasma membranes.
 - Add 200 µl of chloroform in each sample. Vortex to mix the contents uniformly. Centrifuge at 12,000 x g for 15 min at 4 °C. Remove the RNeasy mini spin columns, 1.5 ml tubes and label them properly.
 - Collect the supernatant in 1.5 ml tubes (While collecting supernatant do not disturb the middle or bottom layer). Add equal volume of chilled 70% ethanol. Mix the contents by gentle shaking.
 - Apply 700 µl from the tubes to respective mini spin columns and centrifuge them at 12,000 x g for 20 sec at RT. Perform all further steps at RT.
 - Discard the filtrate and apply remaining solution to the respective columns and centrifuge them at 12,000 x g for 20 sec. Discard the filtrate.
 - Apply 350 µl of RW1 buffer to the column and centrifuge them at 12,000 x g for 20 sec. Discard the filtrate and apply 10 µl of DNase and 70 µl RDD buffer to the column.
 - Incubate at RT for 15 min. Apply 350 µl of RW1 buffer to the column and centrifuge them at 12,000 x g for 20 sec. Discard the filtrate. Apply 500 µl of RPE wash buffer to the column and centrifuge them at 12,000 x g for 20 sec. Discard the filtrate. Again Apply 500 µl of RPE wash buffer to the column and centrifuge them at 12,000 x g for 2 min. Discard the filtrate.
 - Shift the columns to new 2 ml collection tubes and centrifuge them at 12,000 x g for 1 min. Transfer the columns to labelled 1.5 ml collection tube and apply 22 µl of nuclease free water. Centrifuge the tubes at 12,000 x g for 1 min.
 - Remove the collection tube and put them on ice. Quantify RNA using automated electrophoresis system.
- RNA concentration, purity and integrity testing.
 For RNA purity and integrity testing use automated electrophoresis system and respective kit³³.

6. Microarray Studies

- Perform transcriptional profiling using commercial available Human array chips. For RNA target preparation, fragmentation, hybridization³⁴ and array chip staining, washing³⁵ use commercial available kits.
- Perform array chip scanning and quality control check by using standard fluidics station, array scanners and standard operating softwares³⁶. For gene expression analysis import the files generated from scanners to the standard commercial available software³⁷, perform background correction, summarization and normalization with Robust Multi-array Analysis (RMA).
- For obtaining list of differentially expressed genes (DEG's) perform one way ANOVA analysis. From this list filter out the genes based on the fold change (± 2) and FDR-controlled p-value (< 0.05). Obtain the Principal Component Analysis (PCA), Heat Map, etc. using this software.

Part 2. UKN 1 Test System

1. Maintenance of hESC

- Seeding of MEFs
 - For the differentiation use the NSCB#8534 (H9) cell line. Culture cells on mouse embryonic fibroblasts (MEFs) as feeder cells. Coat T25 flask with 4 ml of 0.1% gelatin and incubate for 30 min at 37 °C.

2. Thaw MEFs in 37 °C water bath and transfer the cells into pre-warmed DMEM/10%FBS.
3. Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells to obtain 1×10^7 cells/ml. Plate MEFs $4 \times 10^4/\text{cm}^2$ in T25 flasks on gelatin. Optionally, use the MEFs for the next two days. Quality of MEF batches are a very critical issue for hESC maintenance. Therefore it is advisable to elucidate the best company and preparation method for the H9 cells. We use PMEF P3.
2. Splitting and maintenance of H9
 1. Add 1 ml pre-warmed dispase per T25 flask H9 and incubate 9 min at 37 °C.
 2. Add 2 ml wash medium to dispase treated cells and pipet 5 times up and down with 5 ml pipet and transfer cell solution to a falcon tube.
 3. Wash the flask with 9 ml wash medium and add cells to the others. Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells in 10 ml hESC medium.
 4. Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells in 4 ml hESC medium. Add 0.5 ml cell suspension and 4.5 ml hESC medium and plate in a new (PBS washed) T25 flask with MEFs. Change entire hESC medium (5 ml) of the flask every day.

2. Differentiation of hESC towards Neuroectodermal Progenitor Cells (NEP)

1. Prepare hESC medium and KCM medium. Coat one 10 cm dish with gelatine (0.1% in PBS) per T25 flask and incubate for 30 min at 37 °C. Remove medium from hESC and add enough accutase to cover the whole bottom of the flask (1 ml per T25 flask) and incubate 25 to 30 min at 37 °C.
2. Prepare basement membrane matrix coated plates during accutase incubation. Add cold DMEM/F12 to frozen basement membrane matrix pellet and resolve it 1:20. Filter basement membrane matrix solution through a 40 µm cell strainer. Add filtered solution to plate, the whole bottom has to be covered (1 ml per 6-well is required) and incubate for 2 hr at RT.
3. After incubation period remove the basement membrane matrix supernatant and seed cells on the coated wells. After accutase step (2.1) stop reaction by addition of 1.5 ml HES medium. Scrape cells from the flask, add 8 ml hESC medium and produce a single cell solution by pipetting with 10 ml pipet thoroughly. Filter cells through a 40 µm cell strainer.
4. Spin cells 3 min with 500 x g, remove supernatant and re-suspend cells in 10 ml of hESC. Spin cells again 3 min with 500 x g, remove supernatant and re-suspend cells in hESC containing ROCK inhibitor Y-27632 at a final concentration of 10 µM.
5. Remove supernatant of gelatin coated dish. Plate cell suspension on gelatin coated dish to remove the MEFs and leave in the incubator for exactly 1 hr.
NOTE: During this step the MEFs will settle onto the gelatine coated plate, whereas the hESC cannot attach to gelatin. Therefore this is crucial to obtain a feeder-free differentiation. It is a critical step as too long incubation results in hESC clumps and too short incubation in inefficient removal of MEFs. After 45 min of incubation the plate should be investigated for already settled MEFs and hESC clumps.
6. When the MEFs have attached, gently wash non-adherent cells (hESC) off after incubation with the medium already in the plate. If several T25 were used to get more cells, single cells now can be combined. Wash plate once with hESC medium.
7. Spin cells 3 min with 500 x g, remove supernatant and re-suspend cells in approximately 4 ml KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2.
8. Count cells in a hemocytometer using Trypan blue. Plate 18×10^3 cells/cm² on basement membrane matrix coated plates in KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2 (for 6 well use 1.5 ml per well). It is crucial to plate the cells in the right density to differentiate them successfully into NEPs.
9. After 24 hr, change medium to fresh KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2. After further 24 hr, change medium to fresh KCM containing 10 ng/ml FGF2.
10. 72 hr after seeding the cells, differentiation starts by medium change towards KSR medium. This time point is referred to as day of differentiation 0 (DoD0). The addition of test substances is possible now.
11. On DoD1 and DoD2 the medium is changed exactly as on DoD0. Next medium change is at DoD4 containing 25% N2-S and 75% KSR. At DoD6 the differentiation is stopped and cells are harvested for analysis.

3. Chromatin Immunoprecipitation (ChIP) of hESC and NEP

1. Preparation of nuclei
 1. Add 500 µl accutase to each 6 well which should be analyzed and incubate for 25 to 30 min. Count cells in a Neubauer chamber using Trypan blue.
 2. Resuspend cells in 1% formaldehyde in DMEM/F12 for crosslink. Add Tris pH 7.5 to a final concentration of 125 mM after 10 min to stop the crosslink.
 3. Spin cells 3 min with 500 x g at 4 °C, remove supernatant and re-suspend cells in cold PBS.
 4. Spin cells 3 min with 500 x g at 4 °C, remove supernatant and re-suspend cells in 1 ml L1- buffer / 1×10^6 cells.
 5. Incubate for 5 min on ice. Spin 5 min with 800 x g at 4 °C, remove supernatant and re-suspend nuclei in 1 ml L2- buffer / 2×10^6 cells.
2. Sonication and quality control
 1. Sonicate so that DNA fragments of 300–700 bp length are generated. Spin 1 min with 10,000 x g at 4 °C. Transfer supernatant to a new tube. The fragments need to have the correct size, otherwise the immunoprecipitation will be inefficient as well as the followed qPCR.
 2. Remove 50 µl and mix with 50 µl L2 buffer to check efficiency of sonication by running an agarose gel.
 3. Reverse crosslink by incubation at 65 °C for 4 hr and 500 rpm. Load samples 1:5 with Orange G loading dye on a 1.5% agarose gel and run 45 min at 110 V in 1x TBE buffer. Control fragment size (should be between 300–700 bp).
3. Chromatin Immunoprecipitation
 1. Dilute samples 1:5 in dilution buffer and aliquot 1 ml per IP in siliconized tubes.
 2. Remove 5% (volume) from diluted chromatin sample (step 3.3.1) and store at 4 °C as "input".
 3. Incubate samples with antibodies of your choice and with unspecific IgG O/N at 4 °C on a rotator.

4. Add 50 μ l Protein-A/G Sepharose beads to each sample after immunoprecipitation. Incubate samples 3 hr at 4 °C on a rotator. Spin 1 min with 1,500 x g at 4 °C and remove supernatant.
5. Wash with 1 ml washing beads. Spin 1 min with 1,500 x g at 4 °C and remove supernatant. Repeat step g to h. Wash with 1 ml final washing buffer. During the washing steps you should not lose any of the beads, because this alters the amount of eluate directly.
6. Centrifuge 1 min with 1,500 x g at 4 °C and remove supernatant. Add 125 μ l elution buffer and incubate 15 min with 65 °C at 1,000 rpm on a shaker.
7. Spin 1 min with 1,500 x g and transfer supernatant to a new tube Repeat step k and l. Add 200 μ l elution buffer to input (3.3.2). Add Proteinase K and RNase to each sample and incubate 30 min with 37 °C at 500 rpm on a shaker and afterwards 4 hr with 65 °C at 500 rpm on a shaker.

NOTE: For DNA extraction use commercial available ChIP DNA Clean and Concentrator Kit³⁸.

Representative Results

Methyl mercury exposure in UKK test system

The cytotoxicity assay was performed with H9 EBs to obtain an IC₁₀ value (reduction of viability by 10%) for the cytotoxicity of methyl mercury (**Figure 1**). We also performed a microarray based (affymetrix platform) biomarker study. The H9 EBs have been exposed to methyl mercury (0.25 and 1 μ M) for 14 days. On day 14, samples have been collected using TRIzol and RNA was isolated. Transcriptional profiling was performed using Human Genome U133 plus 2.0 array chips. The data have been analyzed with Partek Genomic SuiteTM 6.6. First data overview was obtained by Principle Component Analysis (**Figure 2A**), generation of Venn diagrams (**Figure 2B**) and construction of heat maps (**Figure 2C**). The principle component analysis represents the overall distribution of gene expression and it clearly visualized segregation of MeHg 1 μ M from the vehicle control and MeHg 0.25 μ M groups (PC # 25.2) (**Figure 2A**). A list of differentially-expressed genes (DEG) was obtained after statistical treatment (one-way ANOVA) and filtering of the data using a fold change cut-off of ± 2 and a multiplicity-corrected (Benjamini-Hochberg method) p-value < 0.05 (**Table 1**). The 1 μ M MeHg treatment resulted in 276 DEGs and 0.25 μ M in 31 DEGs (**Figure 2B**). The heat map showed that MeHg 1 μ M treatment mainly reduced gene expression (**Figure 2C**). Information on overrepresented gene ontology terms was obtained by using the DAVID bioinformatics tool. **Table 2** represents the significantly overrepresented GO gene categories that contained more than 5 genes. The down-regulated transcription factors related to the nervous system development were identified. *SEPP1*, *DDIT4*, *AK4*, *FRZB* (brain development), *PITX* (neural nucleus development) and *ERBB3*, *UGT8*, *APOB*, *APOA1* (nervous system development) were down-regulated in a dose dependent manner for methyl mercury treatment (**Table 3**).

UKN 1 test system

This differentiation protocol uses dual SMAD inhibitor⁶ to generate a pure population of NEP within six days of differentiation. The resultant cells are characterized by an up-regulation of the neural precursor genes *PAX6* and *OTX2*. The stem cell markers *OCT4* and *Nanog* are down regulated during the differentiation towards NEP (**Figure 3A**). Due to the highly synchronous and homogenous differentiation, it is also possible to get information on the histone modifications during this early stage of development. We adapted the protocol for chromatin immunoprecipitation (ChIP) using the cells either at the beginning of differentiation or after 6 days of differentiation. A switch of methylation sites on the promoter regions of *PAX6* and *OTX2* was evident from these studies (**Figure 3B**). The investigated methylation sites histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 27 trimethylation (H3K27me3) were highly dynamic during the differentiation. Also on protein level a down regulation of *Oct4* could be observed (**Figure 4**). The up-regulation of *Pax6* and the neural stem cell marker *Nestin* was observed by immunofluorescence microscopy on protein level (**Figure 4**). The cell population showed a homogeneous and pure differentiation after six days of differentiation. Therefore the cultures can be easily used for analysis of RNA and protein. The system provides also the possibility to test substances and the effect they have on early neural development^{16,29}.

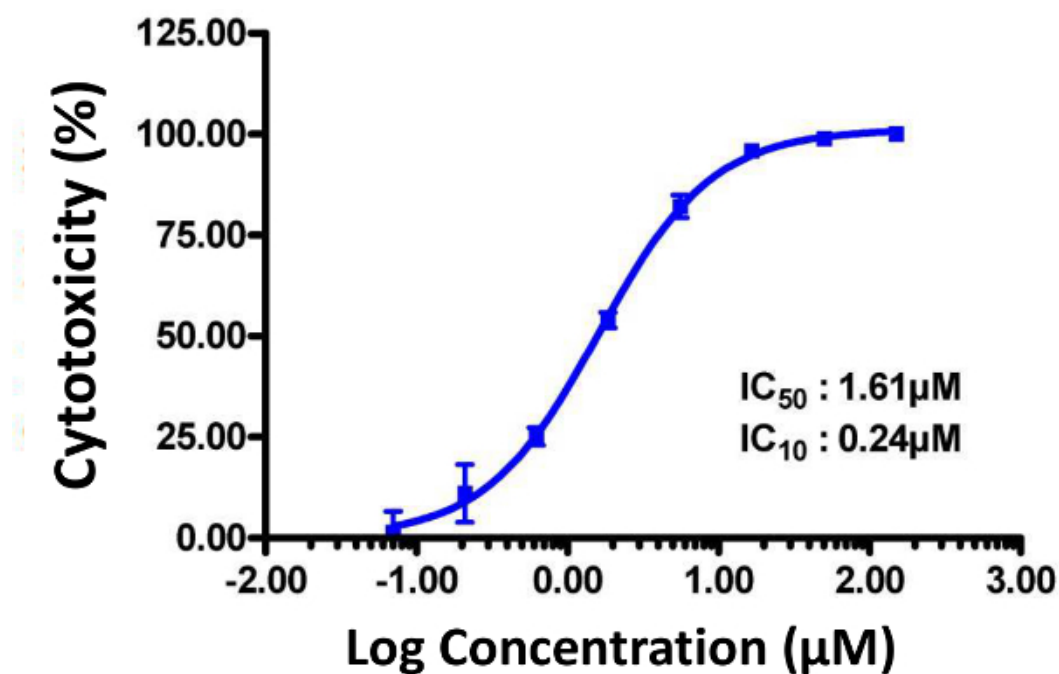


Figure 1

Figure 1. Cytotoxicity Assay (H9 differentiation) for MeHg. The assay has been performed as per the protocol to define the IC_{10} value for methyl mercury.

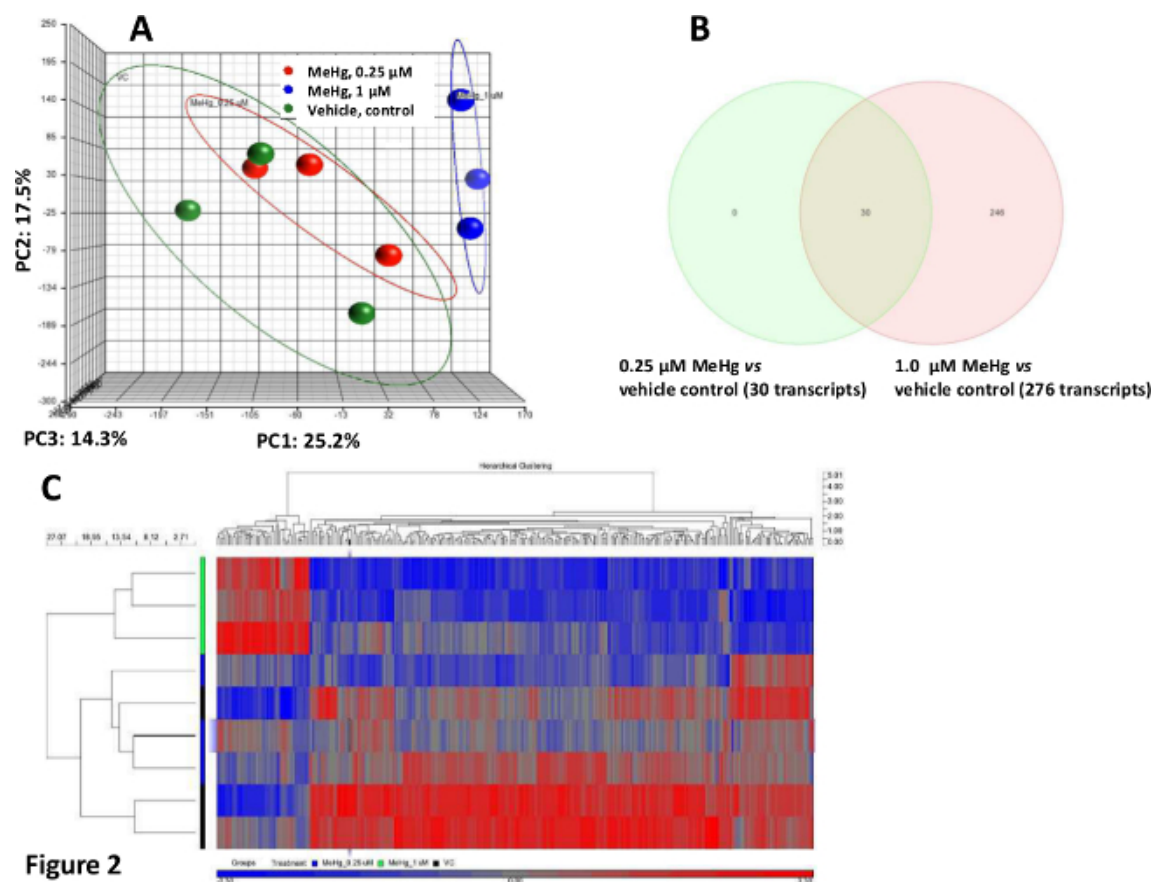


Figure 2

Figure 2. Representative analysis of the differential expressed genes induced by 0.25 and 1 μ M MeHg after application of the UKK test system. The hESCs were treated with 0.25 and 1 μ M MeHg according to the UKK test system. Analysis of the differential expressed transcripts in 14-day differentiated EBs has been performed using the Partek Genomic Suite™ 6.6 software. **(A)** Principal component analysis (3-Dimensional) of the microarray data. **(B)** Venn diagram obtained from microarray analysis of gene expression. The diagram shows the number of genes modulated by the MeHg treatment (fold change $> \pm 2$, p value < 0.05). **(C)** Hierarchical clustering of the gene expression data (fold change $> \pm 2$, p value < 0.05). The highly expressed genes in vehicle control group are repressed by 1 μ M MeHg treatment. The 1 μ M MeHg treatment resulted in 233 transcripts with lower expression and 43 probes with higher expression as compare to vehicle control group. [Please click here to view a larger version of this figure.](#)

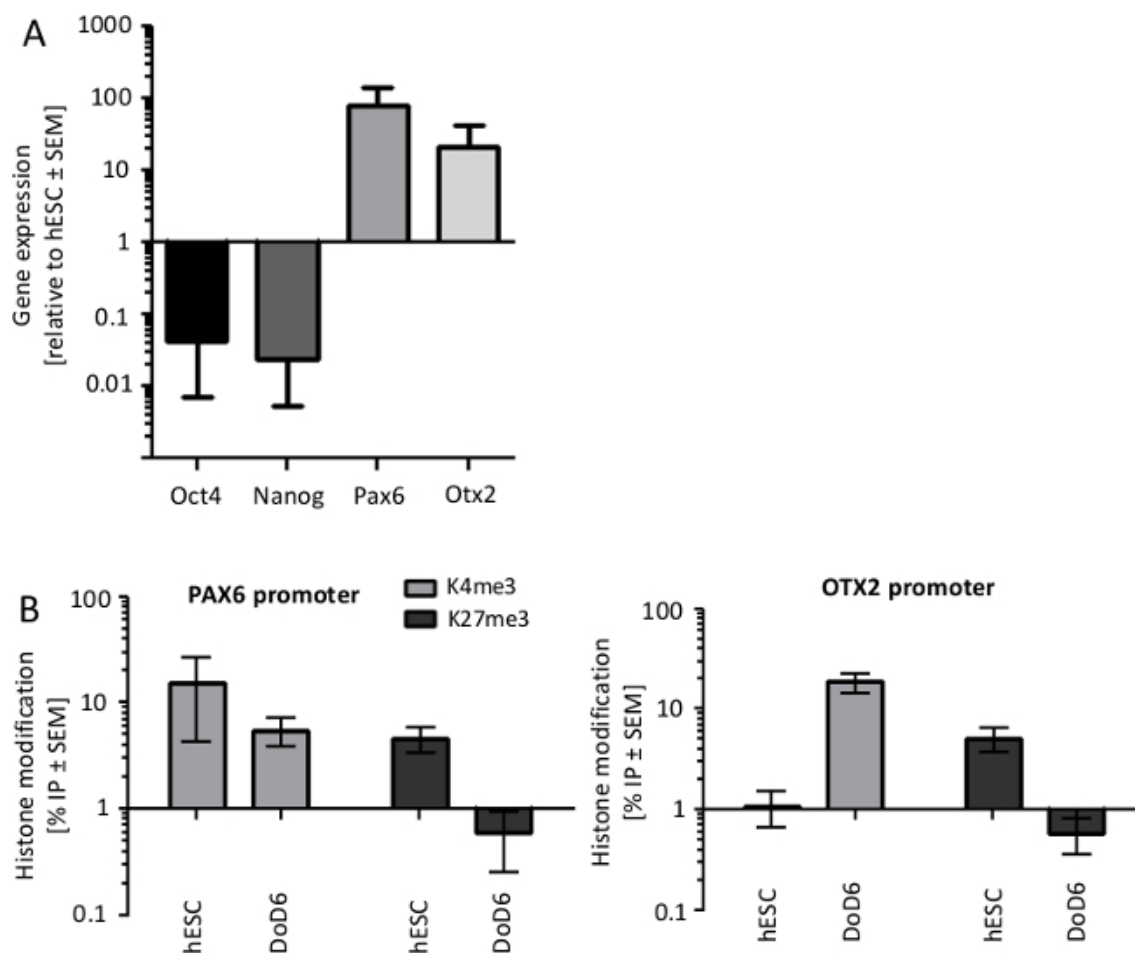
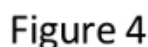


Figure 3

Figure 3. Gene expression and histone methylation pattern during differentiation from hESC towards NEP. For all experiments, hESC were differentiated to neuroectodermal precursor cells (NEP). (A) Samples were taken at day 6 of differentiation, and transcript levels of marker genes of neural differentiation were determined by RT-qPCR. Data (gene expression relative to hESC) are means \pm SEM of 5 experiments. (B) Samples for chromatin immunoprecipitation (ChIP) were prepared at day 6 of differentiation. ChIP was performed with antibodies specific for H3K4me3 or H3K27me3 or control IgG. The enrichment factors of promoter sequences are given as % input for H3K4me3 (grey) and H3K27me3 (black). Data are means \pm SEM of 3 independent cell preparations. [Please click here to view a larger version of this figure.](#)



Column #	Probeset ID	Entrez Gene	Gen	Gene Symb	Gene Title	RefSeq	1 seq	1 UniGene	1 p-value	(Gr	p-value)	(Tr	p-value)	(Mr	Ratio)	(MeH	Fold-Chang	Fold-Chang	Fold-Chang	p-value)	(Mr	Ratio)	(MeH	Fold-Chang	Fold-Chang	Fold-Chang	(Groups	SS(Groups)	F(Treatment	SS(Treatment	SS(Error)	
15391	205935_2	2294	FOXF1		forkhead b	NM_002445	HS.155591		0.002325	7.49E-05	0.000127	0.588692	-1.69868	MeHg_2.0 ¹	3.13E-05	0.469762	-1.28784	MeHg_1.0 ¹	39.4807	0.323635	229.153	1.87843	0.618595									
15388	205932_3	4487	MSX1		msk homeobox	NM_001044	HS.424414		0.002373	0.138E-05	0.02765	0.638977	-1.565	MeHg_2.0 ¹	0.005403	0.484389	-2.06446	MeHg_1.0 ¹	39.0536	0.426852	15.2867	1.67082	0.216988									
36431	227167_5	283349	RASSF3		Ras associi	NM_128118	HS.643605		0.000703	0.000575	0.02476	0.625262	-1.53242	MeHg_2.0 ¹	0.00029	0.473821	-2.1105	MeHg_1.0 ¹	29.4933	0.730844	70.7686	1.75365	0.119358									
27038	217744_3	64005	PERP		PERP, TP53	NM_022112	HS.201446		0.007024	0.000157	0.10823	0.651483	-1.53496	MeHg_2.0 ¹	0.000583	0.389429	-2.56787	MeHg_1.0 ¹	21.5644	1.22096	49.1782	1.78463	0.043626									
28399	219106_3	10324	KLHL41		kelch-like	NM_006067	HS.505501		0.008174	0.057882	0.134696	0.654361	-1.52821	MeHg_2.0 ¹	0.023764	0.447046	-2.2369	MeHg_1.0 ¹	20.1209	6.45594	6.313	2.02557	0.647174									
6175	1561657_2	---	---	---	---	---	HS.659667		0.081001	0.052027	1.3218	1.3218	MeHg_2.0 ¹	0.024048	0.200509	2.00509	MeHg_1.0 ¹	20.089	1.3027	23.6059	1.56071	0.129693										
34100	224833_3	2113	ET1		v-ets erythr	NM_001143	HS.369438		0.08792	0.01056	0.0227	0.581063	-1.72098	MeHg_2.0 ¹	0.04462	0.418813	-1.3877	MeHg_1.0 ¹	19.3292	2.74045	17.021	2.41319	0.283555									
23603	214295_2	57235	KLIAA048S		uncharacteri	---	HS.607554		0.011016	0.007638	0.678896	1.30534	1.30534	MeHg_2.0 ¹	0.030202	0.203628	2.03628	MeHg_1.0 ¹	17.8852	1.4128	20.4051	1.61185	0.157885									
13153	203697_2	2487	FRZB		frizzled-rel	NM_001048	HS.128453		0.010132	0.018799	0.187731	0.776571	-1.28771	MeHg_2.0 ¹	0.079955	0.456899	-1.8867	MeHg_1.0 ¹	17.8693	2.83322	12.587	1.9957	0.317105									
22451	213139_2	6591	SNAI2		snail family	NM_003016	HS.360174		0.010327	0.044398	0.06785	0.619562	-1.61404	MeHg_2.0 ¹	0.018895	0.479719	-2.08455	MeHg_1.0 ¹	17.6808	4.09772	7.49175	1.73629	0.463521									
13488	240032_2	8412	BCAR3		breast can	NM_001218	HS.36958		0.010798	0.003734	0.109005	0.651849	-1.5341	MeHg_2.0 ¹	0.00145	0.475031	-2.10513	MeHg_1.0 ¹	17.2468	0.978208	30.7283	1.74285	0.113436									
13795	204339_3	5999	RGS4		regulator c	NM_001118	HS.386726		0.011381	0.013651	0.018386	0.567983	-1.76061	MeHg_2.0 ¹	0.005978	0.456697	-2.18963	MeHg_1.0 ¹	16.7472	2.26365	15.1179	2.04343	0.270332									
13390	203934_2	3791	KDC		kinase inse	NM_002275	HS.479756																									

Table 2. List of significantly enriched and selected GO categories (p value < 0.05, > 5 genes) with dysregulated transcripts for MeHg versus vehicle control in 14 day old EBs.

GO Term	Count	P value	Genes
Regulation of Apoptosis	18	0.0068	ARHGEF3, TBX3, ERBB3, MITF, BNIP3, CDH1, IGF2, IFI16, HGF, GCH1, AMIGO2, SERPINB9, KRT18, MSX1, ETS1, VEGFA, PERP, IGFBP3
Regulation of Cell Proliferation	17	0.0123	RBP4, LYN, TBX3, ERBB3, MITF, IGF2, KDR, RERG, MSX1, ADM.

			ETS1, VEGFA, BNC1, ADAMTS1, FABP1, IGFBP3, FIGF
Vasculature Development	12	0.0001	PLAT, APOB, HAND1, TBX3, EPAS1, FOXF1, LEPR, VEGFA, COL3A1, LOX, FIGF, KDR
Skeletal System Development	12	0.0008	RBP4, MSX1, LGALS3, TBX3, HOXB6, COL3A1, STC1, IGF2, POSTN, FRZB, IGFBP3, AHSG
Heart Development	11	0.0001	RBP4, ACTC1, MSX1, HAND1, TBX3, ADM, PKP2, ERBB3, GATA6, COL3A1, ADAMTS1
Glucose Metabolic Process	9	0.0003	PDK1, RBP4, LDHA, PGM5, PYGL, HK2, PFKF, IGF2, PGK1
Lung Development	7	0.0008	RBP4, EPAS1, GATA6, FOXF1, VEGFA, LOX, KDR
Epithelium Development	7	0.0386	F11R, FREM2, GATA6, FOXF1, VEGFA, DSP, KDR
Mesoderm Development	5	0.0088	HAND1, TBX3, FOXF1, VEGFA, SNAI2

Table 3. List of significantly down-regulated transcripts related to the developmental nervous system with MeHg treatment in 14 day old EBs.

Term	Gene Symbol	Fold Change*	
		0.25 μ M MeHg vs VC	1 μ M MeHg vs VC
Brain Development	SEPP1	-2.17	-4.13
	DDIT4	-1.20	-3.11
	AK4	-1.41	-3.08
	FRZB	-1.29	-2.19
Neuronal nucleus development	PITX2	-2.08	-4.90
Nervous system development	ERBB3	-1.86	-2.89
	UGT8	-1.67	-2.14
	APOB	-3.59	-5.72
	APOA1	2.63	-2.90
	VEGFA	-1.28	-3.06

* p value < 0.05

Table 4. Composition of culture media.

Sr. No.	Medium / Buffer Name	Composition	
		Contents	Amount
1	MEF Medium	DMEM High glucose	
		FCS	10%
		Penicillin	100 units/ml
		Streptomycin	100 μ g/ml
		L-Glutamine	2 mM
2	H9 Culture Medium	DMEM F12	
		KOSR	20%
		NEAA	1%
		Glutamax	1x
		β -mercaptoethanol	0.1 mM
		Penicillin	100 units/ml
		Streptomycin	100 μ g/ml

		bFGF	4 ng /ml
3	RD Medium	H9 culture medium without bFGF	
4	Wash Medium	DMEM/F12	
		Knockout Serum Replacment	20%
		1x GlutaMAX	1x
		MEM non-essential amino acids	
		HEPES	15 mM
		β -mercaptoethanol	90 μ M
5	KCM Medium	DMEM	
		FBS	10%
		incubated for 24 hr on MEFs	
6	Knockout Serum Replacement (KSR)	Knockout DMEM/F12	
		Knockout serum replacement	15%
		1x GlutaMAX	
		1x MEM non-essential amino acids	
		β -mercaptoethanol	15 μ m
		Noggin	35 ng/ml
		Dorsomorphin	600 nM
		SB431542	10 μ M
7	N2-S	DMEM/F-12	
		Apotransferin	100 μ g/ml
		Glucose	1.55 mg/ml
		Putrescine	10 mM
		Selenium	500 μ M
		Progesteron	20 μ M
		GlutaMAX	200 μ M
		Insulin	25 μ g/ml
8	L1 Buffer	Tris pH 8	50 mM
		EDTA	2 mM
		NP-40	0.10%
		Glycerol	10%
9	L2 Buffer	Tris pH 8	50 mM
		EDTA	10 mM
		SDS	1%
10	Elution Buffer	NaHCO ₃	100 mM
		SDS	1%
11	Wash Buffer	Tris	20 mM
		EDTA	2 mM
		SDS	0.10%
		NP-40	0.50%
		NaCl	150 mM
12	Final Wash Buffer	Tris	20 mM
		EDTA	2 mM
		SDS	0.10%
		NP-40	0.50%

		NaCl	500 mM
13	Stem Cell Medium	mTESAR™ basal medium	400 ml
		mTESAR™ supplement	100 ml

Discussion

Traditional approaches to toxicological testing involve extensive animal studies thus making testing costly and time-consuming. Moreover, due to the interspecies differences the preclinical animal safety studies are not always valid to predict toxicity effects of potential drugs relevant for humans. Although non-human primates are most predictable, still strong ethical, and socioeconomical demands are rapidly raising by modern societies for developing sensitive and robust *in vitro* test system relevant to human safety.

The unique ability of hESCs to differentiate into all somatic cell types, therefore recapitulating *in vivo* human developmental processes in the combination with sensitive toxicogenomics approaches has been proposed as an alternative to the traditional approaches for drug safety testing^{6,21}. Under the 'ESNATS' project the 'UKK' test system has been developed to predict the developmental embryonic toxicity based on transcriptomics profiling. In this system hESC have been differentiated in to the embryoid bodies for 14 days. The time kinetic transcriptomics profile obtained shows high expression of differentiation marker specific to the three germ layers ectoderm, endoderm and mesoderm on day 14 which partially recapitulate early human embryonic development. Based on these results, known teratogenic drugs have been exposed during differentiation for 14 days and differential expressed gene profile have been obtained. Impressively, gene signatures associated with the teratogenic effects of thalidomide observed in humans, could be predicted by this test system²⁸. The representative results for methyl mercury in UKK system show concentration-dependent down regulation of the transcription factors related to the nervous system development. The other developmental neuro-toxicants were also tested in this system and efforts are going on to identify the common toxico-markers across the compounds at mRNA level and validate them at the protein level. The UKK test protocol provided here gives basic guideline for conducting the experiment with human embryonic stem cell H9 to identify the transcriptomic signature for developmental toxicant.

The optimized standard operation procedure (SOP) for differentiation of pluripotent stem cells according to the UKN1 protocol allows a robust and synchronized differentiation of hESC to NEP. Already after six days of differentiation, a homogeneous cell population with high PAX6 expression levels is generated. The cells grow in adherent cultures, which allow analysis by immunostaining. Immunocytochemical analysis with high resolution and by confocal microscopy requires that cells are grown on thin glass surfaces. This is possible for these cultures if the glass is coated optimally, but it needs to be mentioned that the cells grow very dense, in more than one single layer after six days. Therefore, routine analysis of lineage-specific markers is more easily performed by RT-qPCR, ChIP or western blot. A big advantage for the biochemical analysis of the cultures is the high yield of cells which can be achieved by this differentiation protocol from a small starting population of hESC. One drawback of this protocol is the high cost of the medium supplements (e.g., noggin) required to force the homogeneous neural differentiation. Another drawback for some applications may be that some small molecules (kinase inhibitors) need to be present in the culture medium as part of the protocol. Thus, certain signal pathways cannot be examined toxicologically, as the change of the culture conditions also changes the differentiation²⁹.

The advantage of test system combination is the better understanding of DNT. Whereas UKK covers a broader range and adverse effect on early germ layer formation can be investigated, UKN1 allows to investigate more neural-specific mechanisms such as epigenetics. Although the two culture systems presented here have been shown to predict developmental neurotoxicity for few model toxicants¹⁶, there is still a need for higher throughput versions of the protocols that allow screening of a large number of potential developmental neurotoxicants. Moreover, more work is required to identify and validate common markers of toxicity at the mRNA or protein level, and to establish them as a part of preclinical drug safety evaluation.

More than 20 billion US dollars per year are invested by the pharmaceutical industries for drug discovery³⁹. As a proof of concept, we have developed *in vitro* toxicity test systems based on hESC and transcriptomics that may be suitable to predict human relevant toxicity effects of potential drug compounds in a cost-effective and less-time consuming manner.

Disclosures

The authors have nothing to disclose.

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