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1. Executive Summary

The need for a profound shift in the way toxicology testing is carried out for chemicals in the pharmaceutical and cosmetic industry is clearly acknowledged by all, in the industry and academia as well as in institutional bodies. The extrapolations —across species, from high test doses to low exposures, and from descriptive endpoints in animals to their possible human correlates— are handicapped by the lack of underlying mechanistic information. A number of expert reports and publications have called for re-orienting testing to the molecular level, highlighting the concept of "toxicity pathways" within human cells that would be triggered by a toxicant exposure at a low dose that by itself does not provoke major cell toxicity but induces changes in cell homeostasis to cope with the phenomenon.

The *SCR&Tox* consortium has been formed in order to evaluate the value of human pluripotent stem cell lines for elaborating assays of toxicity pathways that meet all challenges of the new strategy. The aim of the *SCR&Tox* program was to provide biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources could be reliably and robustly implemented at the required scale.

The first part of the *SCR&Tox* Project was dedicated to the provision of biological resources required for assaying toxicity pathways. Human embryonic stem cells and induced pluripotent stem cells have been harnessed. Their use requested mastering and orienting their major attributes –self renewal and pluripotency. Cell lines were banked at the undifferentiated stage, and new technologies, including automation, developed in order to obtain ES and iPS cell lines optimized for use in standardized assays. Protocols were then designed for differentiating pluripotent cells into derivatives for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, Muscle).

The second part of the *SCR&Tox* project provided all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways. This involved validating the applicability on pluripotent stem cells derivatives of a multi-parametric array not only of available techniques analysing molecular components but also of newly designed methods for exploring functionally cells responses to potential toxicants, with special mention to bioelectronics. Optimization of the biological resources to their use for industrial-scale assays additionally required genetic engineering.

The third part of the project explored toxicity pathways "on the bench", making use of the obtained biological and technological resources. The existence and reliability of toxicity pathways in the biological resources developed from pluripotent stem cells were demonstrated. Then, drug toxicology assays were designed and tested in academic-scale conditions. This involved the discovery of the protein components of the toxicity pathway and how the pathway is altered by test chemicals. Implementation of prototype assays was carried out and demonstrated the value of the resources developed by the consortium. Unfortunately, due to delays introduced by the inappropriate withdrawal of the key partner in the task, the last objective of that part, i.e. prevalidation of the assay and documenting test methods according to ECVAM criteria, fell beyond the life time of the network.

The fourth and last part of the *SCR&Tox* Project aimed at operating the promotion of the selected cell-based assay up to the industrial scale. The transfer of the technologies of the assay developed on the bench was performed toward use on appropriate platforms for industrial-scale implementation through establishment of all standard operating procedures (SOP) and associated instructions for biological resources. Protocols were then adapted them to the industrial platforms, with particular emphasis on miniaturization and standardization. Unfortunately, the demonstration of the robustness, specificity and sensitivity of the prototype assay for testing toxicology could not be fully performed within the life time of the SCR&Tox program.

2. Summary description of project context and objectives

The need for a profound shift in the way toxicology testing is carried out for chemicals in the pharmaceutical and cosmetic industry is clearly acknowledged by all, in the industry and academia as well as in institutional bodies. Change is inevitable because the current system is not based on fundamentally sound science, but rather on descriptive data from high dose animal tests. The extrapolations —across species, from high test doses to low exposures, and from descriptive endpoints in animals to their possible human correlates— are handicapped by the lack of underlying mechanistic information. Although this has been often instrumental in the past, it has also shown sometimes clearly unreliable. In addition, our current approach is too expensive and too slow, capable of only limited throughput.
A number of expert reports and publications have called for re-orienting testing to the molecular level, highlighting the concept of “toxicity pathways” within human cells that would be triggered by a toxicant exposure at a low dose that by itself does not provoke major cell toxicity but induces changes in cell homeostasis to cope with the phenomenon. Evaluation of toxicants calls, therefore, for new models to be created that will allow assessing toxicity pathway responses *in vitro*, that will deliver a more accurate profile of acute toxicity in humans and possibly also reveal more subtle chronic toxic contraindications. Implementation of this new strategy based upon *in vitro* tests requires the most relevant and reliable model systems, which should also be robust and scalable in order to be instrumental at an industrial scale.
The *SCR&Tox* consortium has been formed in order to evaluate the value of human pluripotent stem cell lines for elaborating assays of toxicity pathways that meet all challenges of the new strategy, from the most basic issues on mechanisms of differentiation up to the demonstration of normalized assays on industrial-scale platforms and validation. It has used fully the diversity and versatility offered by those cell lines, for analysis of multiple cell phenotypes (in 5 different organs of interest for toxicology), multiple conditions of exposure (single *vs.* repeated –low *vs.* high doses) and multiple approaches (both in terms of analyses, in particular with “functional ‘omics”, and engineering to optimize and standardise).
The aim of the *SCR&Tox* program was to provide biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources could be reliably and robustly implemented at the required scale.

The first part of the *SCR&Tox* Project was dedicated to the provision of biological resources required for assaying toxicity pathways. Human embryonic stem cells and induced pluripotent stem cells have been harnessed. Their use requested mastering and orienting their major attributes – self renewal and pluripotency – toward their specific use in testing toxicity of chemicals, namely their diversity of origins, scalability at the undifferentiated stage and pluripotency in order to create the conditions for production in needed quantity and quality.
The first objective was to provide cell lines at the undifferentiated stage, with emphasis on a diversity of donors relevant to analysis of the impact of genetic polymorphism on toxicity assays.
Diversity is a major advantage of pluripotent stem cell lines, as it allows exploring impact of genetic polymorphisms on the responses to chemical in Human. The first task of the program was to master that capacity by managing banking of the many cell lines deemed necessary for the project. Procedures for amplification and quality control of hES cell lines were well established and banking of cells at the undifferentiated stage were straightforward. This was not fully the same for iPS cell lines. First, a small set of induced pluripotent stem cell lines was available at the start of the program, in order to explore iPS capacities in parallel to hES. Then, newly developed technologies were used to obtain iPS cell lines optimized for use in standardized assays.
A second objective was to establish conditions for scaled-up production of large amounts of pluripotent cells at the undifferentiated stage. Scalability is a major advantage of pluripotent stem cell lines since they can be amplified without ever entering into a senescence process. The consortium aimed at developing culture systems that would allow growing cells in flasks in a scalable way and at implementing conditioned media or more defined culture media for feeder cell replacement.

A third objective was to identify protocols for differentiating pluripotent cells into derivatives –both at a “full” terminal stage and in applicable cases at an intermediate, amplifiable stage- for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, Muscle). Pluripotency is a main attribute of ES and iPS cell lines: cells are theoretically capable of providing any cell phenotype at any stage of differentiation, with the only qualification that they spontaneously do so in a stochastic non selective way, i.e. they differentiate into multiple cell phenotypes at the same time if left undirected. Specific protocols have been designed and implemented in order to obtain specifically the cell phenotypes of interest.
The fourth and last objective in the first part of the program was to make use of the technologies and methodologies described above in order to provide cells ready, quantitatively and qualitatively for toxicology pathway assays in dedicated formats for direct use in different assays.

The second part of the SCR&Tox project aimed at providing all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways. This involved validating the applicability on pluripotent stem cells derivatives of a multi-parametric array not only of available techniques analysing molecular components but also of newly designed methods for exploring functionally cells responses to potential toxicants. Optimization of the biological resources to their use for industrial-scale assays additionally required genetic engineering and new developments compatible with the overall objective of standardization.
The first objective of this second part was to implement classical “omics” for gene and protein expression analysis on ES and iPS cells-derivatives in order to demonstrate the value of the biological resources for exploring signalling pathways. Large-scale evaluations of the status of gene expression and protein concentrations in cells has allowed us to understand the integrated biologic activities and to catalogue changes after in vitro treatment with toxicants. Gene and protein profiling have been adapted to pluripotent stem cells derivatives in order to define capacities and limitations of those technological resources applied to the test cells.
A second objective was a search for scalable techniques applicable to pluripotent stem cells derivatives that would allow dynamic identification of genes involved in signalling pathways triggered by toxicants, to develop functional genomics based upon large-scale screens for siRNA or cDNA, functional proteomics with particular emphasis on protein-protein interaction, and bioelectronics. Functional approaches are especially useful in addition to classical ‘omics for seeking and characterizing toxicity pathways, and the consortium has set-up and characterized value and limitations of such approaches. Functional analysis used high-throughput methods that permitted automation of cell-based assays with libraries of cDNAs and siRNAs. Functional proteomics was explored for complementary data. Last, an important effort was put on bioelectronics that allows us to evaluate electrogenic changes associated to exposure to toxicants in cells that are physiologically electro-active like cardiomyocytes, muscle cells or neurons in microarrays and microcavity arrays.
A third objective in this second part was to develop homologous recombination and protein transfer for optimizing biological resources to be implemented in large-scale screens, focusing on newly developed tools, endonucleases. This aimed at engineering cells to make them most adequate for use on industrial HTS platforms. Engineering of the cells to be tested for toxicant effects using specifically designed tools would allow optimizing the assays by providing cells with purposely chosen properties that make them more fitted with test requirements.
A fourth objective of this second part of the project was to ensure “clean” reprogramming of somatic cells in order to create iPS cell lines devoid of any remnant of the transgenes originally used for triggering stemness. Clean protocols for the reprogramming of iPS cells were deemed an absolute requirement for the implementation of those cell lines in standardized assays for industrial purposes.
Similar to the last objective of the first part of the project, the last one in the second part was the production of ready-to-screen cells for further use in the program after differentiation, thorough quality control and documentation. This prepared for direct use of these biological resources in subsequent steps toward industrial-scale testing. Besides the actual production of the most relevant cells in terms of quantity and quality, particular emphasis was placed in the control of the relevance of these ready-to-screen cells with regards to the requirements of high throughput screening, i.e. demonstrate responses to tests that are robust, sensitive and specific.

The third part of the project explored toxicity pathways “on the bench”. Starting at half-term of the program, the third part of the program aimed at making use of the obtained biological and technological resources for developing “at bench scale” at least one cell-based assay of a toxicity pathway. After checking for relevance, i.e. observing the test signalling pathway in pluripotent stem cells derivatives, a test assay was to be designed and implemented at low scale, up to results allowing prevalidation.
The first objective was the demonstration of the existence and reliability of toxicity pathways in the biological resources developed from pluripotent stem cells. Relevance of the prepared resources for analysing toxicity pathways was planned through the identification of the signalling pathways of interest in cells to be tested, and their triggering by a known toxicant in conditions that are compatible –in terms of dose and duration of exposure, in particular– with those of an efficient assay.
A second objective was to design at least one assay to be tested further “on the bench” (in academic-scale conditions), based upon one identified toxicity pathway and associated biomarkers in pluripotent stem cells derivatives. Design of a prototype assay “on the bench” was planned upon the computed model defining the toxicity pathway, its biomarkers and endpoints as observed in the available derivatives of pluripotent stem cells. This involved the discovery of the protein components of the toxicity pathway and how the pathway is altered by test chemicals. On that basis, the project implied engineering specific gene constructs that would allow visualizing and measuring in quantitative manners any perturbation elicited by toxicants in the toxicity pathway of interest.
A third objective was the implementation “on the bench” of the designed assay for exploring the targeted toxicity pathway. Implementation of the prototype assay at academic scale was seen as a most determinant step to prepare for transfer of the technology to industrial HTS platforms.
The last objective aimed at ensuring prevalidation of the assay and documenting test methods as implemented “on the bench” according to ECVAM criteria for validation under good laboratory practice (GLP) conditions. Prevalidation and normalization of the assay “on the bench” by the specialist partner (ECVAM) was envisioned as the final step before implementation on industrial-scale platforms. There was clearly no time for a full validation of one prototype assay within the framework of the 5 year-long program, but we thought that there could be enough time for prevalidation and prospective normalization on the basis of the results of the “assay on the bench”.

The fourth and last part of the *SCR&Tox* Project aimed at operating the promotion of the selected cell-based assay up to the industrial scale. This required technology transfer as well as methodological adaptation and refinement in order to reach the proof of concept that pluripotent stem cells derivatives can show instrumental for testing a toxicology pathway in a relevant, efficient, extended and normalized assay on an industrial platform.
The first objective was the transfer of the technologies of the assay developed “on the bench” toward use on appropriate platforms for industrial-scale implementation through establishment of all standard operating procedures (SOP) and associated instructions for biological resources. Technology transfer to industry platforms was a major endeavour of the consortium as a whole, since it required combining all expertise and using biological and technological resources obtained during the Project. This in particular implied establishment of standard operating procedures and instructions requested for provision of the biological resources. All characteristics of the biologic material in industry conditions were determined using basic parameters allowing for formal “cell batch releases”. Quality Controls were assessed in parallel, providing for formal “quality control reports”.
A second objective was to refine protocols designed “on the bench” in order to adapt them to the industrial platforms, with particular emphasis on miniaturization and standardization. Adaptation and refinement were required for all analysis methodologies in order to make them suitable for a large-scale application. Flexibility cannot exist in industrial platforms that are entirely based upon robotic systems which will strictly deploy exact protocols. Transfer of the assay successfully developed in an “academic” set-up to the industrial robotic platform, therefore, required specific adaptation and refinement.
The third and last objective of this fourth part was the demonstration of the robustness, specificity and sensitivity of the prototype assay for testing toxicology, with particular emphasis on reproducibility of results in assays exploring repeated dose applications of potential toxicants.

Demonstration of the reliability and effectiveness on industrial platforms of at least one assay was the last objective of the *SCR&Tox* project. In order to be successfully reached, it would have required the full development of the prototype assay based upon an engineered cell line that could be transferred to the industrial platforms.

3. Main S&T results

WP1. Biological resources

1.1 Banking of undifferentiated hPSCs

Human pluripotent stem cells offer a unique opportunity to establish *in vitro* human cell culture models of *in vivo* tissue cells of any type. The availability of quality controlled hESC and iPSC lines to meet the needs of SCR&Tox partners and more widely for Seurat-1 partners is valuable to ensure that partners can work from the same reference seed stocks of research cell lines which have met agreed QC and characterization criteria.

Whilst a individual hPSC lines can provide useful tools for toxicology studies, variation has been observed between pluripotent cell lines, both within and between groups of hESC and iPSC lines. In addition, it is also highly valuable to have multiple lines generated by similar technologies available to enable selection of lines with the best capabilities for development of representative human cell-based systems for toxicology research. The use of techniques which do not involve integration into the genome, in theory avoiding genetic disruption, are considered important to provide cells systems that will provide responses most close to responses seen in native tissue cells.

Results

This task comprised banking of caucasian and three non-caucasian hESC lines, all of which passed standard QC testing and banking of hiPSC lines (“classical” and “clean”). All banking was completed according to partner needs following circulation of a questionnaire to partners. Generation of a reference hiPSC line for comparison of protocols was also performed.

Low passage stocks of “clean” iPSC lines were established using 3 different state of the art non-integrating reprogramming techniques (episomal vectors, modified mRNA transfection and co-transfection with miRNA and modified mRNA). These lines were banked and stocks subjected to quality control and characterisation testing including expression of surface markers (TRA-160, TRA 1-80, SSEA-4&1), expression of self-renewal genes, a pluripotency assay, karyology, mycoplasma and sterility testing. Safety testing for serious human blood born pathogenic viruses was also completed.

A subset of these lines was subject to extended characterisation in order to explore requirements of reference cells. This included additional analysis of pluripotent capacity by expression of lineage commitment genes and preliminary studies of response of the lines to a generic toxic challenge (hydrogen peroxide). Extended pluripotency assays showed a subtle range of lineage commitment profiles amongst the lines although all were characterized as “pluripotent” by routine methods applied to QC of cell banks. One line NIBSC 7 produced by the mi/mRNA method used, was closer in profile to the “reference” hESC line (H9) than the others. Further studies of this group of lines sought their response to oxygen radical challenge by analyzing expression of genes associated with apoptosis, cell cycle state and nrf-2 expression. Some inter-line variability was observed.

1.2 Amplification of quality-controlled undifferentiated stem cells

One of the main objectives of the SCR&Tox project was to build the necessary biological resources needed for supporting the second part of the work program. This included the mass cell production (automation) of undifferentiated pluripotent stem cells, as an important challenge for the final transfer

of an assay on industrial platforms that may eventually require billions of cells to be produced from each cell line.

Results

Optimal conditions for mass cell production of human pluripotent cells have been established and working cell banks of undifferentiated were produced. This has included the development of the necessary conditions for cell cultures of PSCs (either hESCs or iPSCs), automated at all the stages: culture, amplification and freezing. Cellartis DEF-CSTM feeder-free culture system has been successfully applied for the proof of principle production of feeder-free cell banks of hES cell lines. The feeder-free culture system has furthermore been tested for the expansion of hiPS cell lines.

A protocol has been established to amplify IPS cells in single cells and feeder free culture conditions on the CompactSelect automated cell culture platform. The amplification process allowed 130,000 fold amplification in 22 days. Those IPS cells were used to implement automated differentiation into MPCs, also on the CompactSelecT platform. After 16 days (4 automated passages) and 260 fold amplification, MPCs conserved their characteristics and could be amplified 600 fold during 12 days, giving rise to a theoretical master cell bank of 2,000 cryotubes, at 10^6 cells/mL.

A fully automated system has been developed for cryopreservation and banking of large batches of pluripotent stem cells, while controlling several cryobiological parameters. This technique allows the generation of consistent and standardized cryopreserved batches of cells, as checked by post-thawing quality control. The Cryomed system allows freezing by injecting liquid nitrogen in controlled conditions. The automation and distribution of cell cultures is possible by using the Fill-It robot, this module is capable to distribute a suspension of cells mixed to a cryoprotector into 96 vials, simultaneously and in less than 30 seconds.

QCs have been especially studied in order to obtain a set of tests that both comprehensively explore all relevant parameters and are in parallel workable within the framework of an industrial use. The following Figure and Table summarize results obtained by the network.

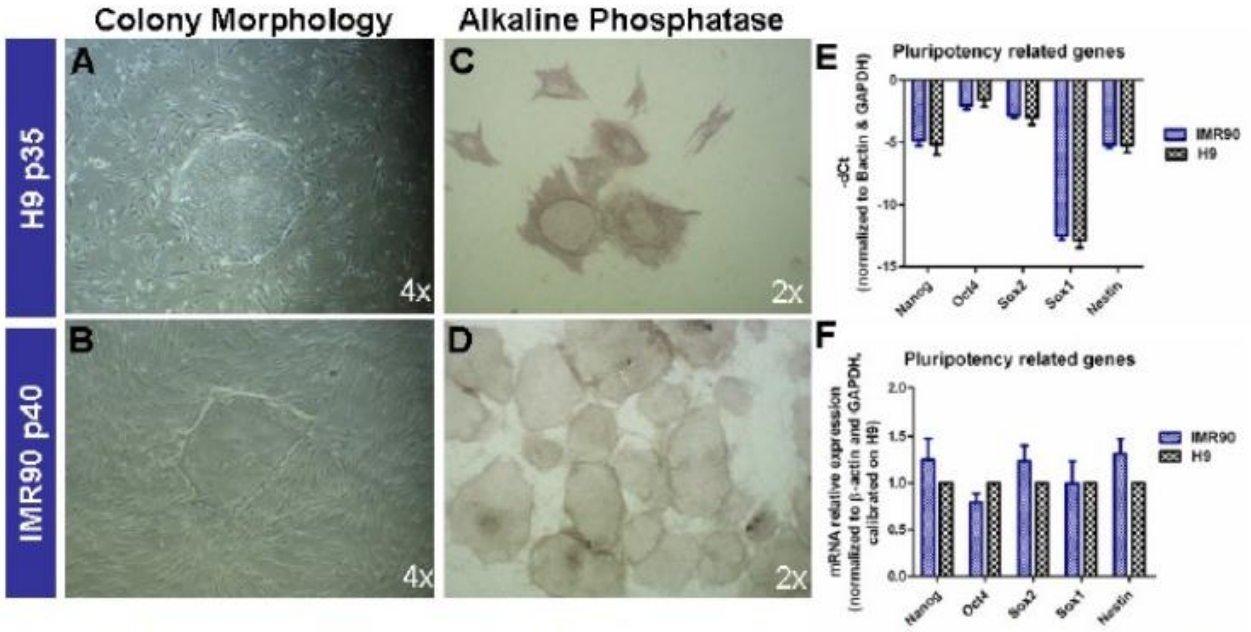


Figure: Colonies morphology, alkaline phosphatase and qPCR analyses. **(A, B)** Representative phase-bright images of undifferentiated colonies (H9 p35 and IMR90 p40) (4x magnification; **(C, D)** representative images of AP stained colonies (same samples (2x magnification); **(E, F)** Bar graphs reporting qPCR analyses of Nanog, Oct4, Sox2, Sox1 and Nestin, normalized to B-actin and GAPDH (-ΔCt, in **E**) and of the same genes normalized to B-actin and GAPDH and then calibrated to undifferentiated H9 cells (ΔΔCt method, in **F**). Mean of 3 independent analyses ±S.E.M.

Table Quality control analyses, expected results and proposed preliminary thresholds for undifferentiated PSCs (hiPSCs compared to hESCs, H9) characterization.

Type of analysis	Expected results			Preliminary threshold values
Analysis of Colonies Morphology by cell microscopy and digital photography	Colonies should be round shape, large nucleolus and not abundant cytoplasm; flat, tightly-packed colonies.			≥ 80% NB: when passaging, discard of morphologically differentiated colonies has to be performed.
Alkaline Phosphatase (AP) (with BCIP/NBT)	Undifferentiated colonies should be positive for AP activity.			≥ 80%
IC analyses (qualitative analysis and HC-imaging platform, Cellomics)	Undifferentiated colonies should be positive for:		Oct4	≥ 80%
			SSEA3	≥ 80%
			Tra1-60	≥ 80%
			Sox2	≥ 80%
Flow Cytometric analyses (BD FACSAria)	Undifferentiated cells should be positive for:		SSEA4-Alexa647	≥ 80%
			Tra-1-81-PE	≥ 80%
	Undifferentiated cells should be negative for:		SSEA1(CD15)-Pacific Blue	≤ 5%
RQ-PCR analyses, using the ΔΔCt method. Housekeeping genes: GAPDH and Bactin.	Significantly high expression levels of pluripotency related genes, as compared to undifferentiated H9:		Nanog	ΔCt ≤ 6
			POU5F1 (OCT4)	ΔCt ≤ 3
			Sox2	ΔCt ≤ 3.5
	Significantly low expression levels of ectoderm related genes, as compared to undifferentiated H9:		Nestin	ΔCt ≥ 6
			Sox1	ΔCt ≥ 13
TaqMan Human Stem Cell Pluripotency Array (Applied Biosystem), using the ΔΔCt method. To be run every 10 passages	Undifferentiation/pluripotency related genes should result significantly expressed. Significance is calculated by using a 1-tailed paired t-test comparing undifferentiated H9 cells at different passages and IMR90 cells at different passages VS the H9 lowest available passage number. Germ layer specific genes should be undetectable or not significant. Significance is calculated by using a 1-tailed paired t-test comparing undifferentiated H9 cells at different passages and IMR90 cells at different passages VS the H9 lowest available passage number.			
EBs formation (analyses @ day 0/2/4/6/10/14): RQ-PCR analyses of 3-germ layers related genes, using the ΔΔCt method ($2^{-\Delta\Delta Ct}$).	Comparing EBs at day6 to undifferentiated cells (day0), germ layer specific genes should result significantly expressed.	Ectoderm related genes	Sox1	$2^{-\Delta\Delta Ct}$ ≥ 3 (at day6)
			Pax6	$2^{-\Delta\Delta Ct}$ ≥ 10 (at day6)
			Nestin	$2^{-\Delta\Delta Ct}$ ≥ 2 (at day6)
		Endoderm related genes	α-fetoprotein (AFP)	$2^{-\Delta\Delta Ct}$ ≥ 400 (at day6)
			Cytokeratin 18 (KRT18)	$2^{-\Delta\Delta Ct}$ ≥ 4 (at day6)
		Mesoderm related genes	Brachyury (T)	$2^{-\Delta\Delta Ct}$ ≥ 40 (at day6)
			Atrial natriuretic factor gene (NPPA)	$2^{-\Delta\Delta Ct}$ ≥ 15 (at day6)

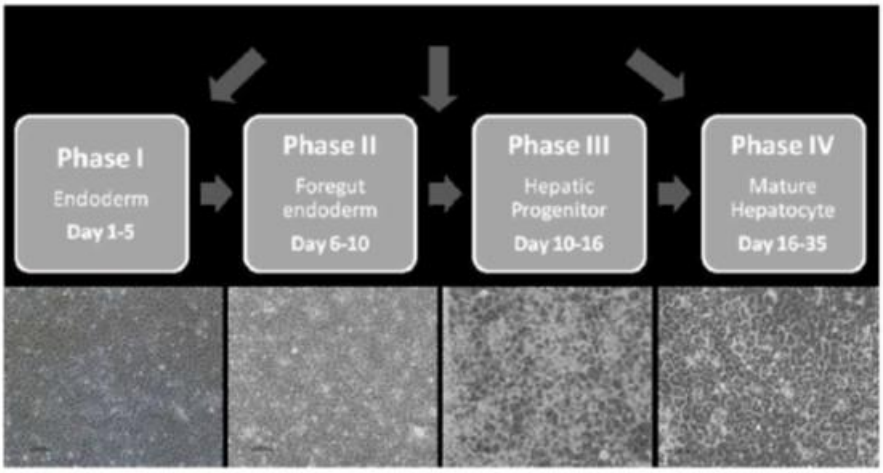
1.3 Differentiation protocols

SCR&Tox aimed to develop protocols for the differentiation of hPSCs to provide models of five different cell types (hepatocytes, neuronal cells, cardiomyocytes, keratinocytes and muscle precursor cells) that have significance for screening in drug safety testing and a variety of other applications.

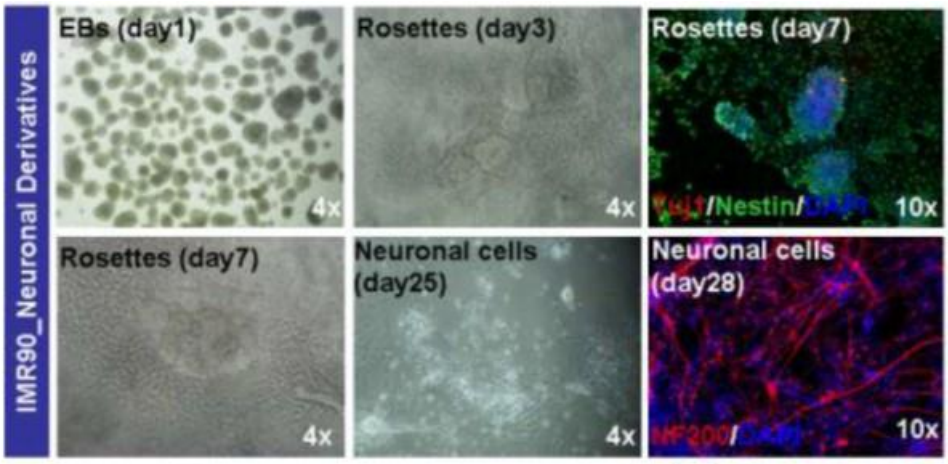
Results

Differentiation protocols were successfully developed for models of five different cell types (hepatocytes, neuronal cells, cardiomyocytes, keratinocytes and muscle precursor cells). General conclusion for each cell typed is as follows:

- In the case of protocols to establish models of hepatic cells, protocols were developed for hESCs grown on feeder cells and feeder-free and were optimized to provide robust differentiation of a range of hiPSC lines into high purity (>90%) functional hepatocyte like cells. Representative examples are shown below of the four successive phases of differentiation observed *in vitro* along the hepatocytic lineage.

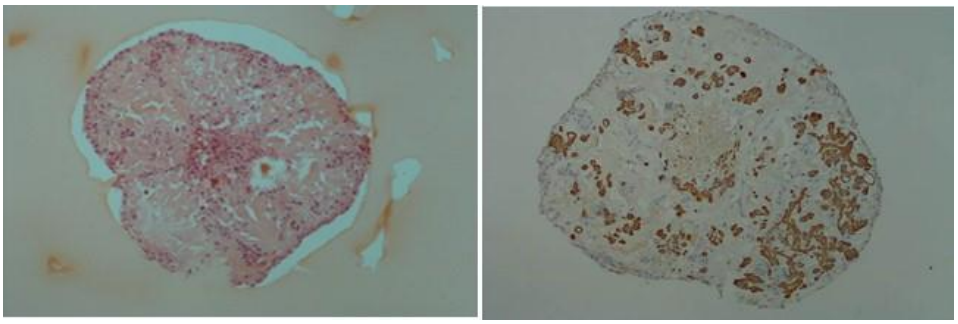


- Neuronal differentiation protocols were developed for pre- and post-mitotic neurones and also specialised neuronal cell types. These appear scalable for industrial application in drug screening. The generation of pure cultures of postmitotic neurons remains challenging. Nevertheless, it is also important to note that in the human brain there is a heterogeneous population of different types of neurons and supporting glial cells, and for this reason mixed populations of cells better represent the *in vivo* situation and may provide valuable complex culture systems which can be exploited in the future.

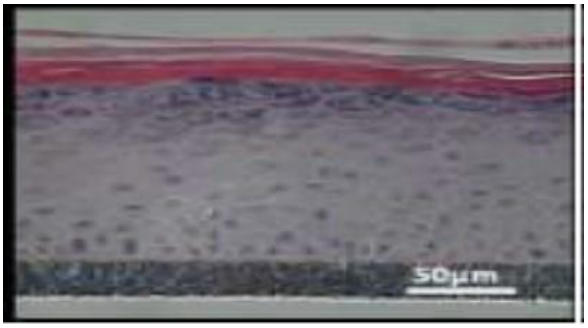


Various illustrations of different stages of differentiation along the neuronal lineage are shown above for one iPS cell line.

- The progress reported in the development of purified ventricular cardiomyocytes could provide a means of delivering significant improvements in these cell types, and also provides a potentially valuable approach for a range of other cell types. The illustrations below demonstrate the successful differentiation of iPS cells into ventricular cardiomyocytes, using a 3D cluster technique (left, H&E staining, right, immunohistochemistry staining for myosin).



- The feeder-free protocol for generation of basal keratinocytes reported within the framework of the program will facilitate the industrial application of this cell type in drug screening. A representative example is shown below of an epidermis reconstructed *in vitro* following seeding of basal keratinocytes differentiated out of hES cells on a synthetic matrix.



- Homogenous populations of mesodermal precursor cells of the myogenic lineage can also now be produced at scale from both hESC and iPSC lines. This protocol enables the differentiation of hESCs into a homogeneous population of mesodermal progenitors after 3 weeks of culture on a gelatin coating dishes and in an appropriate medium. In order to improve the yield of this process, we proceeded in several optimizations of the culture media. Notably, the addition of two cytokines (FGF2 and ascorbic acid 2-phosphate) significantly improved the rate proliferation of the progeny. In the optimized conditions, the obtained population presents a doubling time of approximately 30 hours and can be maintained for more than 35 cumulated divisions. This protocol was successfully transposed to iPSCs.

1.4 Production of ready to use cells for toxicology testing

Automated production of early stage mesodermal and neural stage precursor cells from cryopreservable hPSC suspension cultures has been established. For early mesodermal precursor cell expansion a 1000 fold expansion was achieved over 16 days whilst retaining stable phenotypic marker expression levels. The automation of early neural precursor expansion could be initiated from cryopreserved suspension cultures of hPSCs.

Results

The scalable automated production of late neural precursors from hESCs was also established, and furthermore, automated differentiation into motor neuron progenitor phenotypes was shown to be at

least as efficient as standard manual methods. In this case the capability to monitor the differentiated cell state was also established using an hESC line transfected with a reporter gene activated in the motor neuron lineage.
Scalable production of fully differentiated hPSCs was established for neural and hepatic cell types. The hepatic system was reported to have achieved T225 flask (225cm3 culture surface) scale.
Automation to set up 96 and 384 multi-well micro-plates of single genotype early stage cells was achieved for mesodermal precursor cells using the Bravo (liquid handling)/BenchCel (plate manipulation) system. Process optimisation for cell seeding achieved significant improvement in inter-plate consistency. This system also provided culture stability for 2 weeks. A system for automated manufacturing single genotype 96 well plates of late stage neural cells from hESCs was also developed in the CellHOST system yielding cells with evidence of neuronal functional maturation.
Automated preparation of multiple genotype plates of early stage cells were also successfully established for mesodermal precursor cells and neural precursor cells in the Bravo/BenchCel and CellHOST systems respectively. Both systems were demonstrated with multiple hESC and iPSC lines processed simultaneously. The mesodermal system showed stability of the final cell types for two weeks indicating capability for repeat dose testing in other workpackages.
Altogether, this part of the workpgrogram has provided scalable and automated manufacturing systems for industry scale studies of toxicology in mesodermal, neural and hepatic models. Both single and multiple cell lines can be handled automatically and in the case of the neural cell system this is achievable from thawing of a source vial of cells to harvesting or testing fully differentiated cells. In addition, it has provided comparative experience in use of a number of automation systems.
Another important outcome from this work is that it provided potential new technical procedures which could accelerate progress towards industry ready protocols. In particular, it has been demonstrated that undifferentiated hPSC lines can be expanded and cryopreserved as spherical aggregates in suspension and in addition that neural precursors can be preserved successfully and used in an automated system. These methods could provide important opportunities for more flexible planning of the manufacturing process and enable shorter assay timelines for industry users. In addition, stability demonstrated in one system and qualified over 2 weeks, paves the way for systems that will enable evaluation of repeat dose testing.

WP2. Technological resources

2.1 Profiling and functional characterisation of test cells

Work package 2 of the SCR&Tox consortium aimed at providing all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways.
The first task aimed specifically at providing the SCR&Tox consortium with technologies for profiling cell and gene expression, as well as to explore the cell functions. The task was further divided into three groups namely:
a) The characterization of gene and protein profiles and functional analysis of stem cell derivatives
b) Design and implementation of functional genomic and proteomic methodologies to analyse dynamically stem cell derivatives and finally,
c) Characterization of electrogenic properties of neural and cardiac stem cells derivatives.

Results

Implementation of transcriptomics,

Regarding the task to design and implement technologies for defining cell profiles of gene expression, corresponding to the differentiation of definite endoderm cells and hepatic progenitors in three-dimensional cultures, the findings in this study suggested that directed differentiation of DECs and HPs in dynamic 3D perfusion culture provides a promising approach to effectively derive functional hepatic cells. In contrast to conventional 2D culture, the 3D perfusion culture systems induced more functional maturation to hESC-derived hepatocytes. Both the identification of the key players in hepatocyte differentiation, and the validation of toxicity models based on stem cells-

derived systems, provide an essential mean for the *in vitro* evaluation of drugs and hence the substitution of animal based-systems.

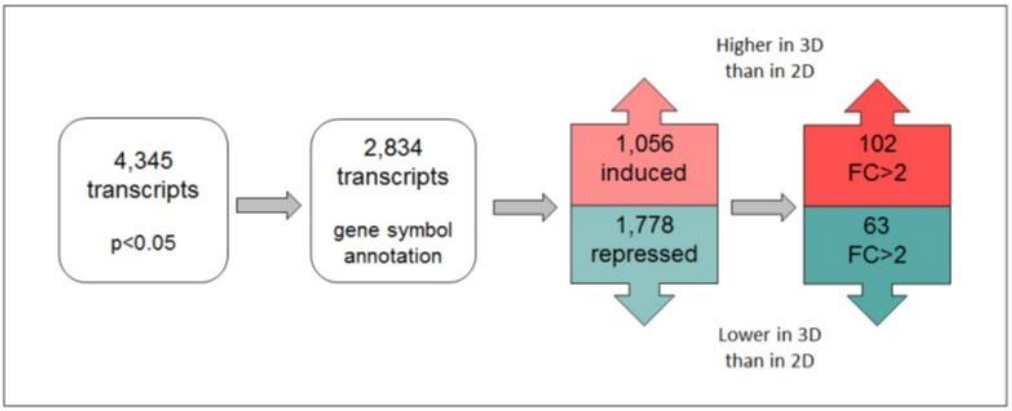


Figure Induced and repressed genes in 3D versus 2D.

Regarding hepatotoxicity, we have also investigated the viability, steatosis, phospholipidosis, and changes in gene expression of spheroid cultures of primary human hepatocytes following chronic dosing (7 and 14 days) with aflatoxin B1, amiodarone, APAP, chlorpromazine, troglitazone and ximelagatran. Further it has been compared with HepaRG and hIPS-Heps.

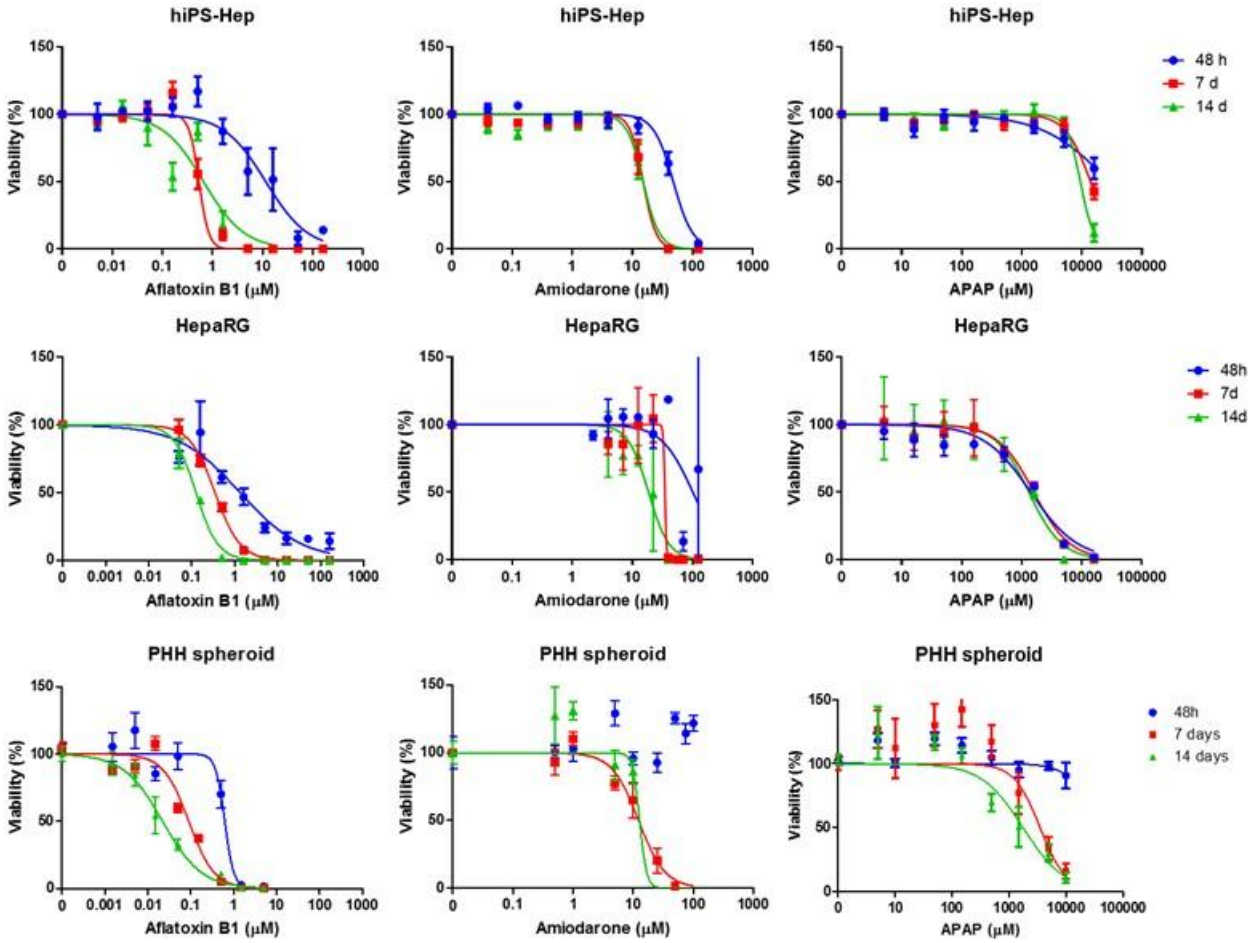
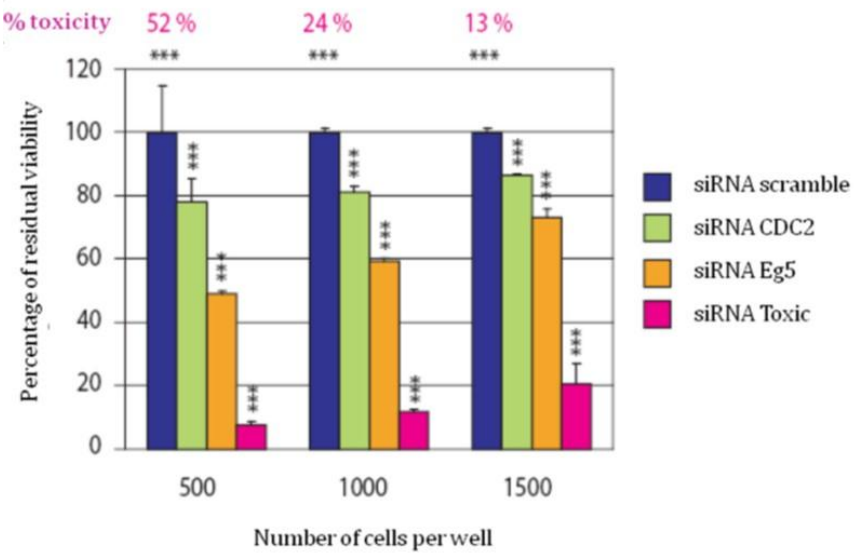


Figure Chronic toxicity of aflatoxin B1, amiodarone and APAP. Each cell type was dosed with the indicated compound every 2-3 days and viability was measured at 48h, 7 days and 14 days.

Regarding the task to design and implement technologies for optimizing the use of Mesodermal Progenitor Cells for their use in the Simvastatin subacute chronic muscle toxicity evaluation, we found that the differences in gene expression of the MPCs treated with simvastatin 1 □M for 2 days taken together with the decrease of cell viability from the same time period, suggests that such dose of simvastatin may exert toxicity by alterations at the transcriptional level. Both the identification of the key players in hepatocyte differentiation, and the validation of toxicity models based on stem cells-derived systems, provide an essential mean for the *in vitro* evaluation of drugs and hence the substitution of animal based-systems.

Implementation of interferomics Development of optimal conditions for siRNA transfection in one hPSC progeny (MPCs) has allowed us to implement an RNAi modifier screen in order to explore repeated dose toxicity mechanisms and the possibility to apply this genome-wide technology to hPSC progenies. The MPCs derived from hESCs or hiPSC can be efficiently transfected with siRNA, in 96 and 384 WP, manually and with a liquid handling automat. Further, development of a model of simvastatin repeated dose myotoxicity in MPCs established from hPSC and identification of 3 toxicity profiles. MPCs placed into a repeated dose toxicity state upon simvastatin pretreatment can be transfected as efficiently with siRNA as populations that did not receive any pretreatment and without excessive toxicity.



Implementation of interactomics

By using new human protein-protein interaction network, we aimed to identify functional and topological signatures of simvastatin-induced toxicity patterns. We have built a new human protein-protein interaction network (*i.e*. interactome) from public sources. This interactome is considered as up-to-date and of good quality. It is composed of 73,212 protein-protein interactions linking 11,636 distinct human proteins. Then transcriptomic analysis of cells exposed 2 to 17 days to high and low doses of simvastatin was performed. The results reveal clear difference between acute and chronic treatment at the level of genes and proteins. They can also be linked to cell phenotype (morphology and proliferation).

Study	Differential expression	Mean betweenness	When relevant, p-value (U-test) and conclusion
D2 SIM1	up	1.08 10-4	
	down	1.169 10-4	Significantly > human interactome (4.47 10-4)
	all	1.095 10-4	
	up	8.36 10-5	

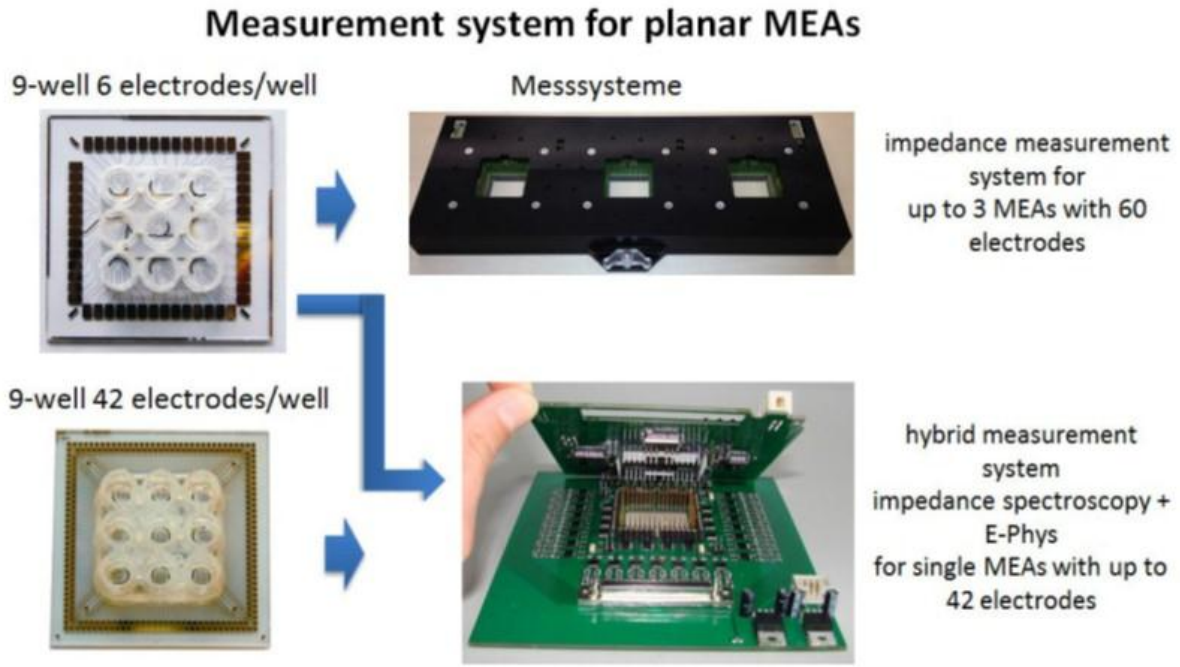
D17 SIM1	down	3.38 10-5	
	all	6.57 10-5	
D17 SIM0.4	up	1.75 10-4	
	down	/	
	all	1.75 10-4	

Table The betweenness distribution of the proteins from D2 (acute toxicity) SIM1 down genes is significantly higher than the betweenness distribution in the human interactome. This is not the case for repeated dose toxicity (D17) for the same concentration of Simvastatin (1 μM)

Implementation of bioelectronics

Advanced and novel bioelectronic-based stem cell monitoring system as well as functional bioelectronic monitoring system for pluripotent stem cells as well as thereof derived cell lineages and differentiated cell types, the read-out amplifiers and multiplexers as well as the planar multielectrode array (MEA) has been deveoped adapted and optimized based on the specifications needed for cardiomyocytes and neural cells.

Microarrays. we were able to adapt and optimize our bioelectronic measurement platform for the feasible monitoring of pluripotent stem cell characteristics. Therefore, we evaluated the optimum electrode size, configuration, geometry and, MEA substrates and conductive / semi-conductive electrode materials etc.. Finally, we could demonstrate the discrete detection of non-directed differentiation processes in hiPS cultures. Moreover, we could demonstrate the convincing suitability of our planar MEA measurement system and technology platform for real time and online recording for the functional analysis of pluripotent stem cell derived neuronal networks in the context of maturation. Based on the established system we initiated and intensified several collaborations where we already provided our self-developed and produced MEAs to the consortium partners.



Microcavity arrays.

For the establishment of a microcavity array based hCMC bioelectronic monitoring system, the MCA as well as the amplifier and multiplexer devices had to be characterized by applying reference

compound analysis using stem cell derived hCMCs, and had to be optimized for an optimum signal quality. The multimodal recording system had also to be tested and optimized for long-term measurements, especially to fulfill the demands on RDTT (repeated dose toxicity testing). The established MCA measurement system could be adapted and optimized for the feasable bioelectronic analysis of human cardiomyocytes (CMCs). The use of the MCA technology showed superior advantages over commonly used planar MEA based analysis, especially in the context of long-term monitoring with improved signal stability that is a prerequisite for RDT testing. Exclusive data records can be made regarding the efficacy and (bio)availability of active pharmaceutical ingredients including cytotoxic side effects. First silicone based MCAs were provided to the consortium partners and the advantages of the technology could also be evaluated and improved by end-users. A novel MCA design was developed for the optimized and parallel testing of compounds. First prototypes of the novel 2 x 2 well MCA (concerning novel cavity sizes adapted to the cardiomyocyte subtype clusters) could be produced and optimized with regard to signal quality and stability. So the novel MCAs according to adapted micro-cavity sizes could be used in our novel hybrid measurement system to achieve a multimodal bioelectronic read-out that can be combined with photonic analysis.

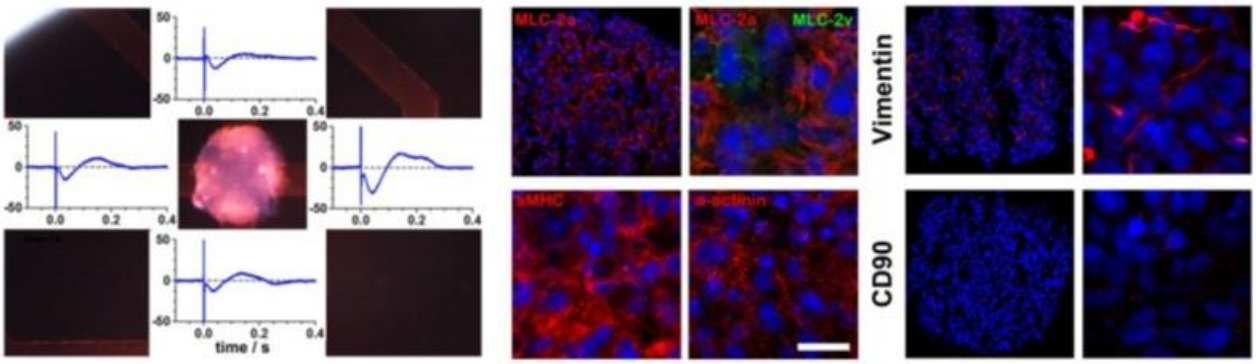


Figure MCA-based field potential recording on hCMCs. The use of the MCA technology for the direct field potential recording on hCMCs was evaluated (left). The immediate positioning of a single MCA without further incubation is sufficient to obtain feasible action potential derived field potential signals on all 4 electrodes. For correlation the measured hCMCs were fixed and characterized by immunocytochemistry with regard to cardiac (subtype) markers and non-cardiac cells.

Figure Design and production of a 2x2 multiwell MCA for bioelectronic monitoring of hCMC.

Implementation of bioelectronics.
In order to obtain a bioelectronic measurement system that can be used in combination with stem cell derived cells e.g. neuronal and cardiomyocyte 2D and 3D-cultures, the analogue and digital measurement pathways have to be tested and adapted/optimized. Especially for electrophysiological recording of cardiomyocyte clusters but also for neuronal network cultures signal sensitivity as well as signal-to-noise ratios had to be investigated for a feasible data analysis. Therefore, also recording and analysis software had to be adapted and optimized with regard to a feasible and automatable data acquisition and processing system that can be easily up-scaled.
A further focus was the integration of different bioelectronic detection methods to obtain a high content monitoring platform. Our self-developed and in the project provided hybrid measurement system including impedance spectroscopy and electrophysiological recording had to be evaluated and optimized for the use of stem cell derived cell cultures, especially in the context of repeated dose toxicity testing.

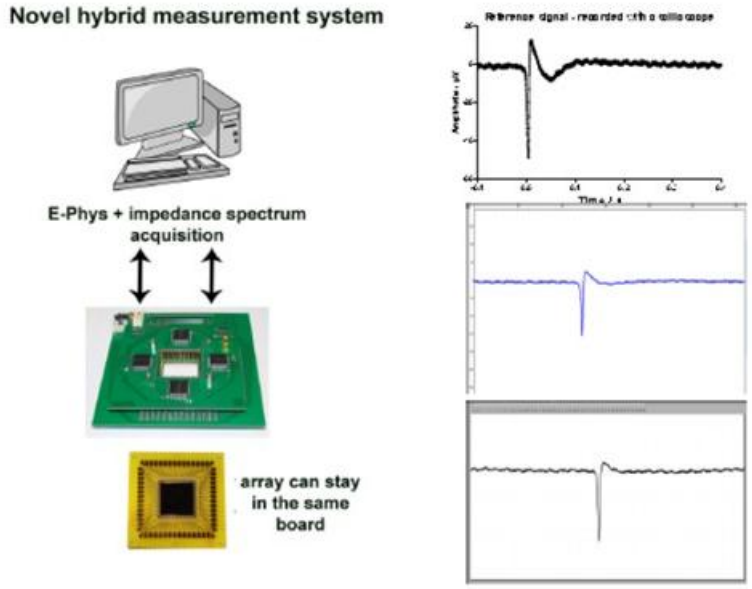


Figure Testing of hES-derived cardiomyocyte clusters a self-developed hybrid measurement system. In comparison to a hCMC derived reference signal (top), the recorded stream with a conventional amplifier (middle) as well as the stream recorded with our novel hybrid measurement system (bottom) are shown.

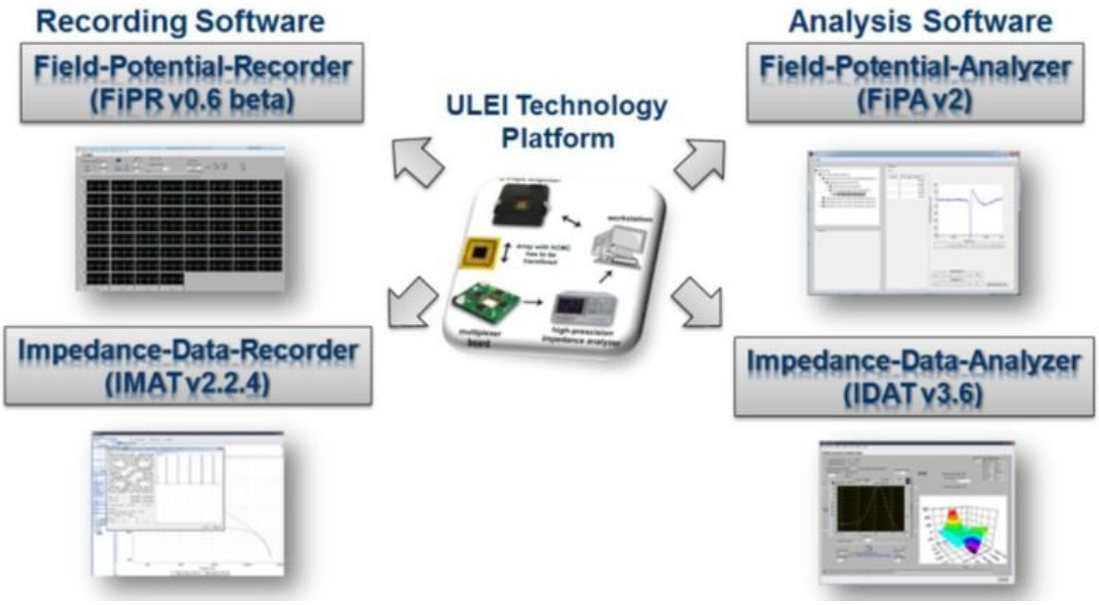


Figure Recording and analysis software for the bioelectronic measurement platform for both, the impedance spectroscopy measurement system and the electrophysiological recording system.

Based on our well established measurement systems for electrophysiological recording and impedance spectroscopy we adapted and optimized both systems with regard to the novel microelectrode-based planar arrays and microcavity arrays that were adapted and newly developed within the *SCR&Tox* project. To use and operate the developed bioelectronic measurement systems in a feasible and end-user friendly way the software packages had to be adapted and optimized for the specific needs and characteristics of stem cell and stem cell derived cell cultures. With the adapted and optimized data recording and analysis software packages for electrophysiological recording and impedance spectroscopy on microelectrode-based planar arrays and microcavity arrays with up to 384 channels, our established systems can be used to monitor and analyze the stem cell derived cultures like cardiomyocytes and neurons but also muscle cells and hepatocytes/hepatocyte like cells. Since our bioelectronic measurement systems are label-free and non-invasive they perfectly match the needs for long-term monitoring in the context of chronic toxicity and RDTT.

2.2 Genetic engineering of test cells

The members of the consortium have successfully developed and validated methods and protocols for in a robust and reproducible manner genome engineer human pluripotent stem cells in an exact and precise manner. Further, two different methods to reprogrammed somatic into induced pluripotent stem cells have been established. The reprogramming methods are non-integrative (foot print free). iPS lines have been generated with these technologies.

Results

TALENs homologous recombination.

Nucleases (meganuclease and TALEN) have been successfully used to integrate repair matrices into iPS genome, in several different loci. We have established a process, from the early design of nucleases and matrices to the harsh selection procedure where we on a routine basis can achieve homologous recombination in human iPS and ES cells. Efficiencies were, however, low.

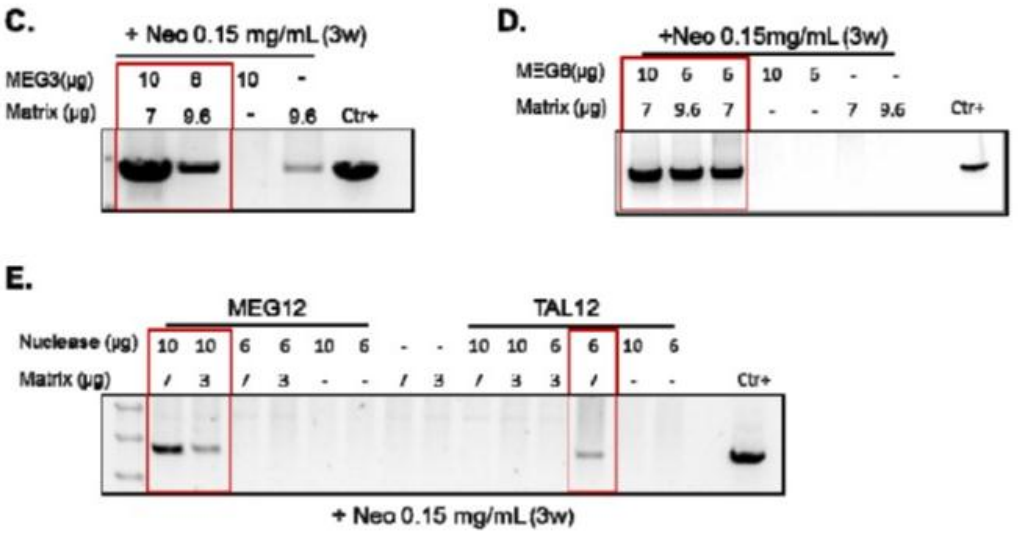


Figure: Targeted integration with the meganucleases MEG3 (C), MEG6 (D.), MEG12 and TAL12 (E.) in iPS cells

Gesicles as a tool to deliver gene constructs to stem cells.

Gesicle production relies on the coexpression of VSV-G, a viral glycoprotein, and a plasmid coding a protein of interest (POI) in a producer cells (HEK cells). Gesicles are next release from the supernatant of producer cells and can be collected/concentrated/stored. The objective was to create a robust gesicle-producing system for the transfer of transcription factors. These factors being nuclear and carrying nuclear localization signal, their incorporation in Gesicles budding from the membrane of producers cells might be challenging due to a poor incorporation in budding gesicles.

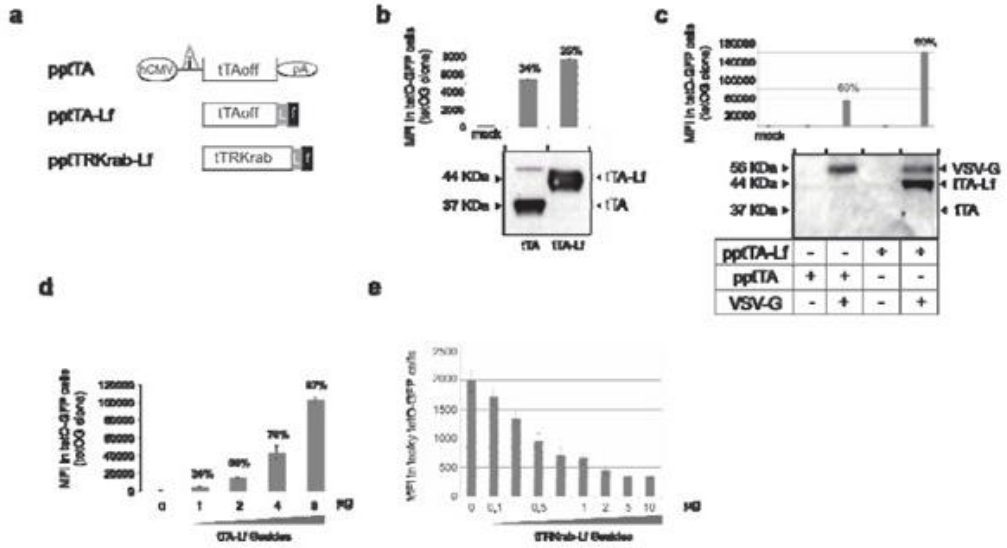


Figure Gesicle-mediated delivery of TetR-regulators in human cells

iPS with reprogramming genes.

Objective of this sub-deliverable was to create “clean” iPS cells, primarily by demonstrating the feasibility of the nuclease-based targeted integration into human fibroblast cells and to develop a robust and reproducible process of reprogramming. As a back-up of the nuclease-based reprogramming option and taking into account the recent advances in this area, it was decided to asses an enhanced version of the episomes reprogramming process as published by Yamanaka’s group in 2011 (Okita *et al.*, 2011). Adult fibroblasts were therefore transfected and cultured as described previously. Fibroblasts from five different healthy donors, and with appropriate donor consents, were reprogrammed by employing the episome-based technology. The episomes were delivered via electrical based transfection, employing either an AMAXA or a microporator (Neon). Both type of machines gave rise to similar results. The cells were reprogrammed under feeder free conditions employing DEF-CS. When the emerging colonies were big enough they were picked and expanded individually under the same culture conditions. The depletion of the episomes was monitored via a qPCR approach. At the stage of cell banking, episomes could no longer be detected within the reprogrammed cells.

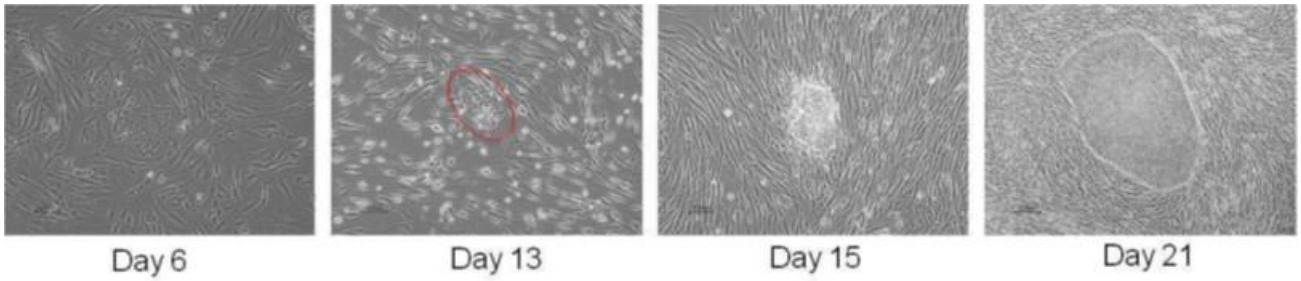
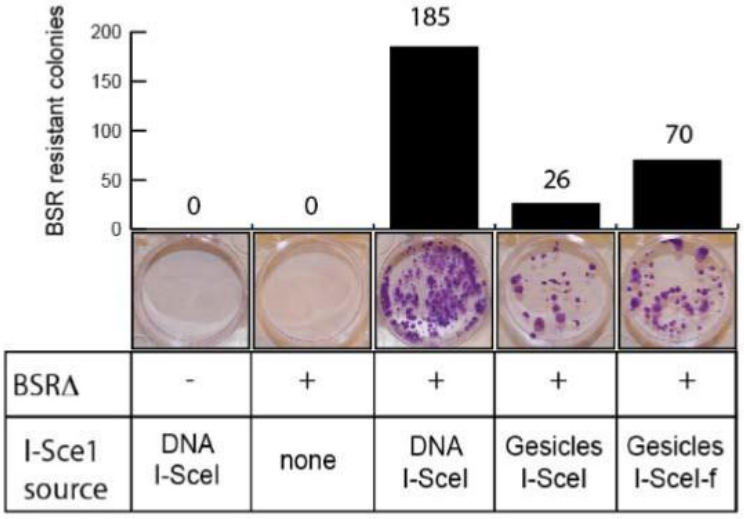


Figure: Morphology of the iPS-like colonies observed with the episome-based reprogramming methodology

Demonstration of Gesicle-vectorisation of a meganuclease.

I-Sce-1 gesicles were produced and analysed by western Blot to validate the protein incorporation. Monitoring different independent batches of Gesicles, we estimated that a given batch of ISCE-1 gesicles contains 1-2 units per 10ul of product.



iPS lentivector for temporary engineering.
Molecular feasibility of the meganuclease-based reprogramming strategy was successfully demonstrated into the 293H cell line. In order to transfer this process into primary fibroblast cells, twelve different meganucleases targeting twelve distinct loci into the human genome were assessed. Their expression and functionality (targeted mutagenesis) were demonstrated. A reporter matrix was also correctly integrated into the MEG6 locus in the two fetal fibroblasts cell lines used within the study. However neither an integration of the reprogramming matrix, nor reprogramming events were observed so far with this methodology.
The episome-based back-up strategy was, therefore, selected rather than the endonuclease-mediated reprogramming, on the basis of the positive results presented above.

Supplementary work. In light of ongoing discussion on challenges of identifying diploid iPSC lines without genetic mutation due to the reprogramming process and also on standardisation of stem cell based assays within *SCR&Tox* and at the Seurat-1 Stem Cell Standardisation Group, further work has been completed by one partner.
iPSC line NIBSC8 was generated using Stemgent's mRNA/miRNA reprogramming kit from the cell line MRC9 on Vitronectin. In the *SCR&Tox* programme it had been difficult to identify iPSC lines which were diploid and free of genetic mutations which might affect the performance of these cells differentiated for systemic toxicity assays. The additional line generated using mRNA technology appears to have a stable diploid genetic makeup in the majority of cells. It thus offers a further potential candidate of unaltered wild-type biology for use in assay development.

2.3 Production of optimized ready-to use cells for toxicology testing

The aim for that task was to design and implement technologies for introducing gene constructs into human pluripotent stem cells that can facilitate cell selection at a particular differentiation stage or promote differentiation into a specific cell phenotype. The focus of the work has been on two different cell types, stem cell derived hepatocytes and stem cell derived cardiomyocytes. For keratinocytes and neural cells the consortium already had very robust differentiation protocol or valuable reporter cell lines available so no efforts were spent on engineering work for these two cell types.

Results
By using TALEN based genome engineering technology we first derived human pluripotent stems cells which if differentiated into hepatocytes will overexpress the important upstream genes PXR-RXR and CAR-RXR, respectively. To further enhance the functionality of maturing hepatocytes derived from hiPS cells, three additional plasmid constructs were engineered. Two different approaches were considered, either to overexpress certain transcription factors that are known from literature to play a key role during differentiation regulation (i.e. HNF4alpha and HNF6), or to

overexpress a transporter protein involved in the CYP-metabolism (i.e. OAT1B1). However, analysis of the performance of the gene modified cells did not prove that more functional hPSC-derived hepatocytes were achieved.
As described above (WP1), improved differentiation of hepatocytes has been obtained through changes in cell culture techniques, in particular using 3D.
As to the gene engineering of cadiomyocytes we initially made a reporter line generated using hESC-line SA002 and a NCX1-GFP construct. During evaluation of this transduced line, the GFP signal was detected in differentiated CMs. However, the GFP expression appeared quite weak. In order to improve the outcome we therefore, as an alternative, generated another reporter line using a cTnT-GFP construct. In that case, the GFP signal was strong and readily detected in differentiated CMs originating from the transduced cell line.
As described above (WP1), differentiation of fully differentiated ventricular cardiomyocytes has been obtained during the framework of the *SCR&Tox* program through changes in cell culture techniques, in particular using 3D.

WP3 - Assay development

This part of the program has been changed because the withdrawal of the partner in charge of the production of a genetically-engineered cell line for the selected toxicity pathway precluded full completion of the program as scheduled. The iPS clones with ARE-Luc construction were produced and distributed to the relevant partners but with a significant delay.
The tasks described below show, therefore, two types of activities related to the development of assays for exploring toxicity pathways. First, the progress made despite the absence of the engineered cell line is presented for the selected pathway, which concerned the NRF2 system. Second, other assays successfully developed using non-engineered cell lines are presented.

3.1. Profiling and functional characterization of cells with reference to the selected toxicity pathway

The aim of this task was to establish an assay using human pluripotent stem cells derivatives for one chosen toxicity pathway, relevant to repeated dose toxicity evaluation. The complexity of repeated dose toxicity involves a number of different target organs and a whole range of different pathways. These pathways are often referred to as toxicity pathways since their activation is triggered when cellular adaptive and defense mechanisms are being challenged by toxic insult. Obviously, pathways of toxicity that lead to adversities *in vivo* vary as they might be specific for different cell types. However, there is also strong evidence that the different manifestations can be triggered by the perturbation of the same pathways. It is widely accepted that oxidative stress contributes to a variety of target organ toxicities induced by different classes of chemicals and generation of reactive oxygen species (ROS) is one of the measured endpoints, widely used for oxidative stress evaluation. Therefore in this project Nrf2 pathway activation has been selected as a key event highlighting the oxidative stress as well as cellular defense mechanisms for assessing the hazard of chemicals across different cell types.

Results

The NRF2 model

Nrf2 target genes encoding phase II detoxification enzymes and antioxidant proteins such as NAD(P)H quinone oxidoreductase-1 (NQO1), glutathione *S*-transferases (GSTs), glutamate-cysteine ligase and heme oxygenase-1 (HMOX1) were studied as readouts for Nrf2 pathway activation evaluation. These target genes expression has been evaluated using neuronal, cardiomyocytes and keratinocytes models derived from hiPSCs in the control cultures and after the exposure to the chemicals that activate Nrf2 signalling pathway.
The hiPSCs IMR-90-derived neuronal cultures responded to rotenone treatment by up-regulation of the antioxidant enzymes NQO1 and SRXN1 at the protein level in a concentration- and time-dependent manner.
Dose-response curves were determined in mature neuronal culture derived from hESCs treated for up to 14 days with two reference compounds; rotenone, a well-known inhibitor of the mitochondrial complex I and AI-1 (ARE-Inducer-1), a synthetic molecule, a specific activator of the NRF2 pathway

and established IC_{50} values based on Alamar Blu reduction. Regarding the gene expression analysis after exposure to test compounds, low level of exposure to rotenone at 20 nM and 100 nM changed the expression of the neural genes, in particular of BetaTubulin, synapsin GABA and glutamate transporters as well as NRF associated genes (KEAP1, SRXN1, NQO1 and SRXN1) differently at different time points. At day 14 all the neural markers were down-regulated but the expression of NQO1 and SRXN1 was increased. HMOX1 instead returned near the control level in the two highest concentrations while it was upregulated at 1 nM indicating not only a dose-response effect but also a time-dependent effect. After 14 days of AI-1 exposure the neural genes expression was down-regulated only at the highest concentration. At the not cytotoxic concentrations the neural genes maintained levels similar to the untreated control but the NQO1, SRXN1 and HMOX expression was significantly increased. The data indicate that the system can discriminate specific neurotoxic effects from unspecific cytoxic effects and NRF2 pathway can be specifically activated in this test system.
To assess the toxicological relevance of the Nrf2 pathway we first evaluated its basal and inducible activity in lt-NES and lt-NES-derived neurons of different maturation stages. The protocols established yield stable, proliferative, and tripotent RGL-NSC and lt-NES populations. They enable the generation of terminally differentiated subtype-specific cells of the glial and neuronal lineage, respectively, at high purities via distinct self-renewing intermediate populations, providing scalable and standardized hPSC-derived cellular resources for a variety of neurotoxicological studies. The generated lt-NES-based Nrf2 luciferase reporter cell line provided evidence that this system is amenable to dose-specific Rotenone and AI-1-mediated modulation and thus might serve as platform for neurotoxicity assay development. QRT-PCR analysis of the Nrf2 downstream revealed increased expression levels with the progression of neuronal maturation.

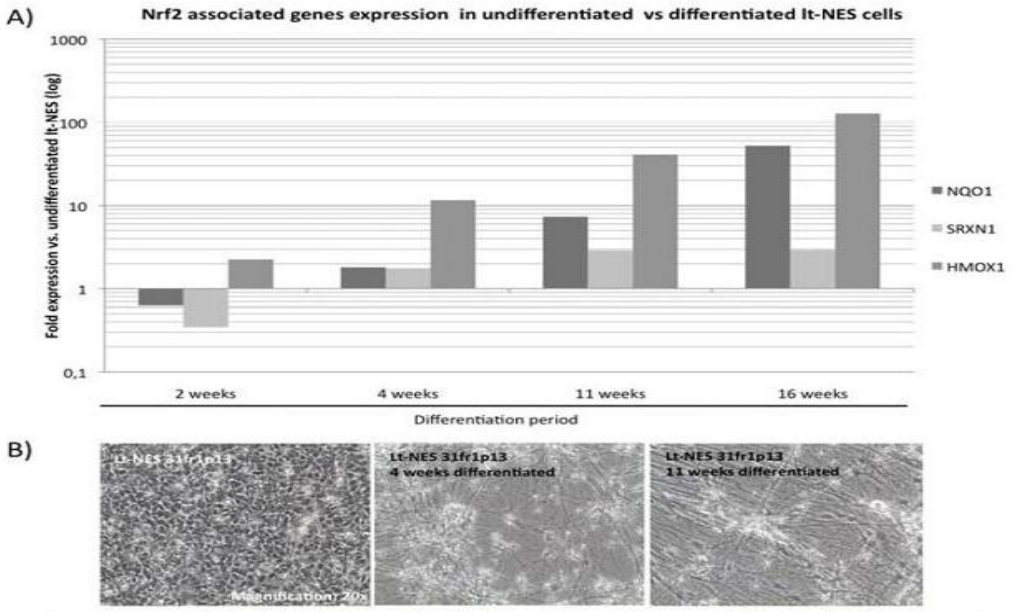


Figure Basal expression levels of Nrf2 downstream target genes increased with progressing neuronal maturation of lt-NES cells. 31fr1 lt-NES cells were differentiated by growth factor withdrawal from the culture medium for up to 16 weeks and RNA was collected at the indicated time points**. (A)** QRT-PCR analysis of the expression of the Nrf2 downstream target genes NQO1, SRXN1, and HMOX1 in comparison to undifferentiated lt-NES cells (equal to 1). Data are normalized to GAPDH RNA levels. **(B)** Representative bright field images of the cells at the indicated maturation stages.

In accordance with the qRT-PCR results, dose-response curves for repeated-dose toxicity revealed an increased resistance of more mature neurons to Rotenone as determined by AlamarBlue assay. Furthermore we could show that Nrf2 pathway activation is strongly increased in response to Rotenone treatment upon complete removal of antioxidants from the cell culture medium. Co-culture experiments with our RGL-NPC-derived astrocytes indicated that the glia cells in lt-NES-derived neuronal cultures are a main contributor to the Nrf2-response.

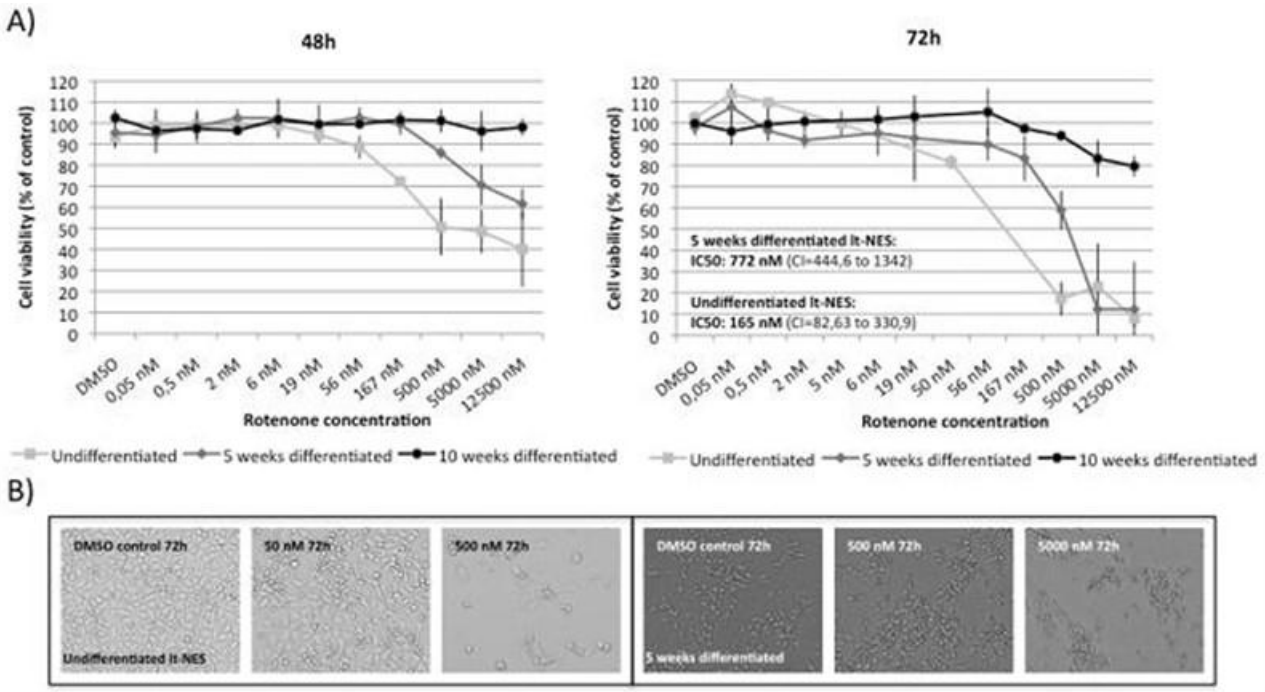


Figure: Susceptibility to Rotenone treatment decreased with increasing time of neuronal differentiation of lt-NES cells. Undifferentiated lt-NES cells and lt-NES cell-derived neurons differentiated for 5 and 10 weeks were treated with a Rotenone concentrations between 0,05 nM and 12500 nM for up to 72h. Cell were treated with the corresponding DMSO concentration as a solvent control. **(A)** Cell viability was measured by AlamarBlue assay after 48h and 72h and is depicted as percentage relative to untreated cells (equal to 100%). Data are presented as means ±SD (n=3, consisting of 3 technical replicates each). IC50 values for 72h were calculated by performing dose-response analysis by nonlinear regression curve fit using GraphPad Prism 6. **(B)** Representative images after 72h of treatment under the indicated conditions. Images were acquired with a CellaVista imaging platform.

In order to exploit the Nrf2 pathway as an indicator of cell stress-associated events we generated an lt-NES reporter cell line for Nrf2-mediated oxidative stress response. To that end, hiPSC-derived lt-NES cells were transduced with a lentiviral vector carrying a luciferase reporter gene under the control of a single copy of the ARE-element of the human AKR1C2 gene (ARE-Luc; Givaudan Schweiz AG).

Bioelectronic measurement platform was an optimal system for the long-term monitoring of cell functionality of both cardiomyocytes and neurons. The obtained results highlight the capabilities of this non-invasive, highly-sensitive, electrophysiology screening platform developed for the real-time analysis of acute as well as chronic effects of active or toxic compounds so applicable for both, toxicology and safety pharmacology studies.

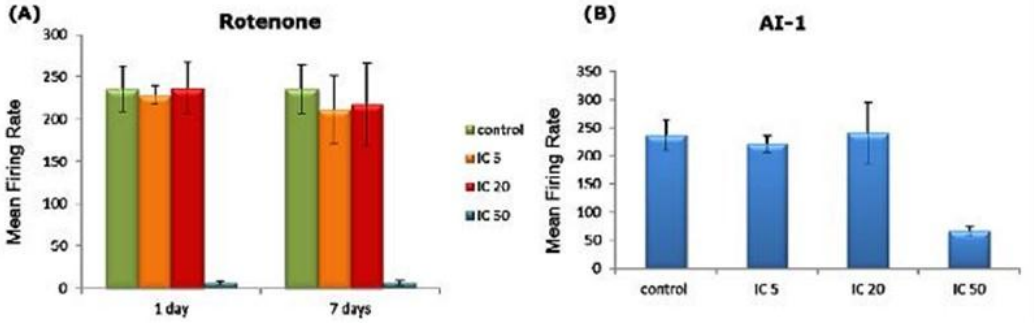


Figure: Representative graphs of mean firing rates (spikes/min) of IMR90-derived neurons after 3 weeks of differentiation and after exposure to: (A) to Rotenone at IC5, IC20, and IC50 concentrations for 1 and 7 days and (B) to AI-1 at IC5, IC20, and IC50 concentrations for 1 day. Data are given as mean ± S.D. of 3 independent experiments.

Nrf2qRT-PCR analysis of Nrf2 pathway target genes were also performed using cardiomyocyte cultures established from the long-term stable hiPSCs derived hCMC model. After verifying that Nrf2 and its target genes were present in the developed 3D cardiomyocyte culture model, a chronic toxicity experiment over 28 days were performed and effect of doxorubicin was analyzed. It has been shown that concentrations of 0.1 µM and 1 µM Doxorubicin lead to a decrease of the contraction rate. In contrast, the impedimetric monitoring showed no significant decrease for 0.01 µM and 0.1 µM Doxorubicin until day 21 but at day 28 a slight decrease was observed.

3.2. Assay development of toxicity tests

Several other *in vitro* cell models of toxicity have been used in order to characterize responses of specific cell phenotypes to various toxicants.

Results

Comparison between the effect of doxorubicin treated 3D cardiomyocyte cultures derived from the hES cell line SA002 with 3D cardiomyocyte cultures derived from the hiPS cell line IMR90C01 was performed. An acute effect could be detected by a decrease of SOD2 but also an increase of NQO1. Both mitochondrial proteins are targets of Nrf2 signalling indicating oxidative stress. Furthermore, long-term application of doxorubicin led to a significant gene expression changes at 1 µM as well as 0.1 µM at later time points for IMR90C01. Additionally, mRNA-expression studies of genes encoding for structure cardiac proteins were performed for TNNT2 and MYL7, both belonging to the contractile cytoskeleton of cardiomyocytes.

A comparison was also carried out between human iPSCs-derived hepatocytes developed in the network and established cell-based systems such as HepaRG and human primary hepatocytes in 3D spheroids. Long-term hepatotoxicity testing has been performed by global gene expression analysis. The different cell systems, iPSCs derived hepatocytes, HepaRG and spheriods were exposed to IC10 doses of four hepatotoxic drugs, amiodarone, troglitazone, aflatoxin 1B and ximegalatran for 48h, 7 days and 14 days. For each time point cells were fixed and stained with fluorescent probes for steatosis, phospholipidosis and nuclei followed also by image based high content analysis.

In parallel, we performed the comparison of toxicity of paracetamol (APAP, metabolism dependent toxicity) and staurosporin (apoptosis) in stem cell derived hepatocytes, HepaRG cells and HUH7 cells. Furthermore, six compounds with defined metabolic activation pathways or toxicity pathways were selected to be tested in HepaRG cells and contrasted with results from iPS cell derived hepatocytes and primary human hepatocytes. The results show that acetaminophen caused toxicity in all cell system. Interestingly, toxicity in Cryo-heps was caused by necrosis while the toxicity in the other cells are apoptosis as indicate by caspase activation. Only primary human hepatocytes and HepaRG cells formed the reactive metabolite NAPQI from paracetamol as indicated by the formation of the cysteine cognate. Staurosporin caused apoptosis in all three cell systems as indicated by caspase activation. The EC50s values of the long term dose response experiments were determined. The data indicate that primary human hepatocytes in spheroid cultures are more sensitive to the test compounds.

One model that was fully developed within the framework of the *SCR&Tox* program used mesodermal precursor cells of the myogenic lineage in order to explore simvastatin-induced toxicity to muscle cells. Employing MPCs we tested several statins (cerivastatin, atorvastatin, pravastatin, fluvastatin, simvastatin and lovastatin) following acute dose toxicity experiments and based upon *in vitro* toxicity results and pharmacokinetic data, we selected simvastatin as a good representative of the toxicity of this pharmacological class.

In order to set-up an experimental model of repeated-dose exposure, MPCs were exposed to simvastatin, renewed every other day in the culture medium, for 17 days. Three different doses of simvastatin were chosen based on the results of the 48 h acute toxicity dose-response curve: the highest dose that did not affect cell numbers (0.4 µM), the lowest dose that induced a cell loss at plateau level (2.5 µM) and an intermediate dose (1 µM).

The cellular mechanisms leading to the chronic reduction of cumulated MPC number under the 1 µM simvastatin treatment were explored by analyzing in parallel cell death and cell proliferation at different time points following repeated-dose exposure. Once adjusted to the level of basal cell death

occurring in control conditions, there was very little, if any, additional cell death under the 1 µM treatment. In contrast to the absence of direct induction of cell lethality, cell proliferation was significantly affected by simvastatin 1 µM at all time-points studied. The chronic cytostatic effect of simvastatin 1 µM was reversible when the drug was withdrawn from the culture medium after 8 days of exposure.

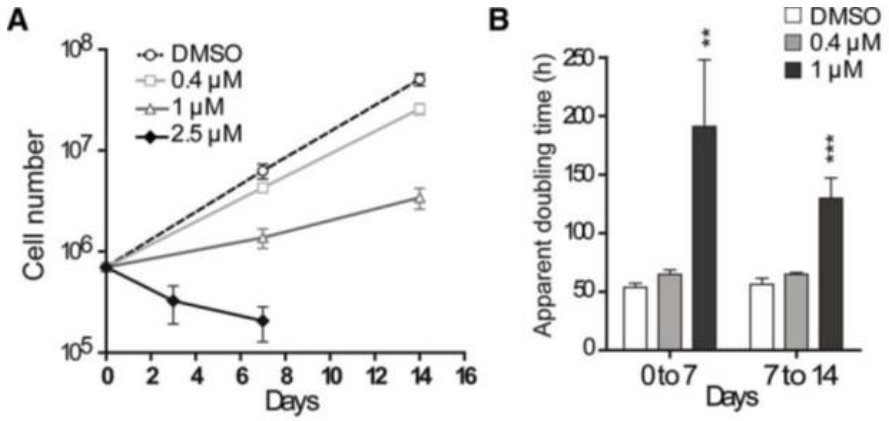
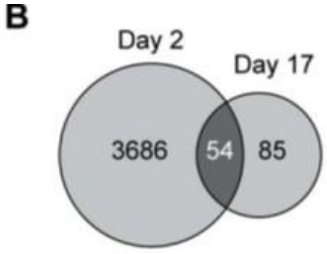


Figure: Characterization of simvastatin-induced acute and repeated-dose exposure responses in mesodermal precursor cells

A whole genome transcriptomic analysis was performed on MPCs treated with simvastatin 1 µM either for 48 h with a single dose exposure or with repeated-dose exposure for 17 days. A massive perturbation in gene expression followed the acute single dose exposure, with a total of 3,740 differentially expressed genes when compared to DMSO treated controls ($|FC| \geq 2$, $p \leq 0.05$). However, the prolonged drug exposure dramatically decreased the number of differentially expressed genes to 139 after 17 days of treatment. After the repeated-dose exposure of 17 days, gene expression changes were mainly related to tissue morphogenesis, cell adhesion, and regulation of differentiation and of muscle development.

A

	Day 2	Day 17
Total number	**3740**	**139**
Upregulated	3617	93
Downregulated	123	46
Maximum Fold Change		
Upregulated	77,47	7,01
Downregulated	-6,03	-3,78



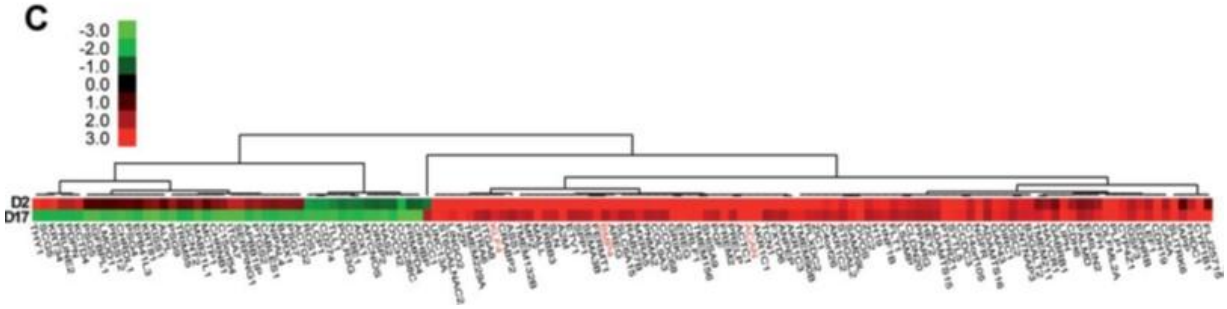


Figure: Differentially expressed genes in mesodermal precursor cells in response to simvastatin 1 mM repeated-dose exposure. (A): Number of differentially expressed genes following treatment with simvastatin 1 mM as compared to DMSO (jFCj_2; p<.05). (B): Venn diagram comparing number and distribution of differentially expressed genes following treatment with simvastatin 1 mM during 2 and 17 days, respectively. (C): Heatmap representing color-coded hierarchical clustering of FC expression values from microarray analysis, after 2 days or 17 days of simvastatin 1 mM treatment, for the list of genes significantly modulated by the repeated-dose exposure of 17 days.

Altogether, that study demonstrated the value of derivatives of human pluripotent stem cells by showing, in particular, that repeated-dose exposure to simvastatin may elicit an adverse cytostatic effect on cells of the myogenic lineage in the absence of cell death. This long-term effect was associated with molecular changes that strongly differed from those induced by acute exposure of cells to the same dose of the drug, thus revealing powerful adaptive mechanisms.

3.3. Validation of selected toxicity test

This part of the program could not be undertaken, due to the lack of an engineered cell line for the NRF2 construct following the premature –and unannounced- withdrawal of the partner in charge of the production of the line.

WP4. Technology transfer to industry platforms

4.1 Standard operating procedures for industrial scale assessment of the selected toxicity test

SOPs have been transferred for all cell lineages.

Human pluripotent stem cells differentiated efficiently into hepatocytes *in vitro*, but the resulting final cell population generated was considered to be still not functional as adult human freshly isolated hepatocytes.

In the case of cardiomyocytes, hPSC differentiated well into this cell *in vitro*. Although a relatively high yield of cardiomyocytes was generated with specific protocols, the resulting final cell population still contained a fraction of non-cardiomyocytes. A purification step for selecting the differentiated cardiomyocytes from mixed cell populations was suggested, in order to engineer cells expressing GFP under the control of a cardiomyocyte specific promoter, and subsequently use a FACS to purify the cells.

An SOP was successfully developed for the cultivation of It-NES cells (stable neuronal intermediates) in 96-well plates (which were then cryopreserved) in order to meet the challenges of industrial-scale neurotoxicity screening tests.

An SOP was first transferred for the manual production of keratinocytes after iPSC-differentiation, and another for keratinocyte amplification and the *in vitro* production of reconstructed 3D epidermis. Then SOPs have been established for industrial-scale automated differentiation and amplification of keratinocytes from iPS cells using the Compact SelecT automated platform. The efficacy of the keratinocyte production depends, however, on the iPS cell line.

An SOP has not been successfully delivered for skeletal muscle.

Other operational SOPs transferred to industry partners included: seeding and differentiation of keratinocytes in 2D. Inherent metabolic capability of the iPS keratinocytes was investigated by exposure to four compounds (EROD, PROD, BROD and MROD), which are reduced in the presence of CYP1A and 1B, CYP2B, CYP3A and CYP1A2, respectively. It was determined that when CYP3A was present, BROD activity was reduced (when compared to the control), thus demonstrating the presence of inherent BROD metabolic activity, which showed that members of the CYP3A family are inherently present in the iPS keratinocyte cell line.

Time course of cell responses.
The objective of this part was to determine the optimal exposure period to a test agent of the iPS Keratinocyte cell line under investigation. DNCB-treated iPS keratinocytes showed large shifts in the cytotoxic profile, according to the concentration tested, which were not observed after exposure to the other compounds (salicylic acid and EGDA). The qPCR data revealed increases in HMOX1 expression at 48 and 72 hours after exposure to EGDA and DNCB, and increases in NQO1 expression after 72 hours.

Cell viability, attachment.
An unexpectedly slow growth rate of the iPS-derived Keratinocytes supplied, compromised the planned establishment rate of 3D cultures and limited 3D epidermis production (due to the loss of cell viability over time), leading to a more rapid usage of the cell bank supplied. Working on primary Keratinocytes (HPEK and NHEK), we confirmed that the combined method was effective in generating 3D epidermis and also demonstrated the robustness of the protocol transferred for the 3D-epidermis construct. Regarding the experiments performed in 2D on the iPS keratinocytes and primary keratinocytes (HPEK and NHEK) on T75 flasks or on 96 well plates, the necessity of culturing the cells in collagen coated flasks and plates was clearly shown.

Growth curve.
The average population doubling (PD) of the 4603 iPS keratinocytes was about 97.0 hours, which was in agreement with the QC values (60 to 100 hours). The average PD determined for the HPEK and NHEK cell lines was about 44.8 and 69.6 hours respectively.

Positive and negative controls.
We used 1% DMSO as a negative control following the instructions of the experimental procedure developed by Givaudan and governed by OCDE Test guidelines. Acceptance criteria of those tests had to be applied to the positive control trans-Cinnamaldehyde (*t*-CA) and the test chemical results. The adaptation of this assay on iPS Keratinocytes demonstrated that *t*-CA was relevant for use in the iPS Keratinocyte assay even the cell line line used did not contain the Luciferase construct. Similar work was performed on iPS neuronal cells.

QC specific to HTS.
QC measures were required to define when an assay is within the expected variation and when it is not. The limited biological resources did not allow us to investigate HTS and this sub-deliverable was not achieved. Thus QC steps were performed on cells available: flow cytometry for cell culture confirmed the population purity, H&E staining demonstrated the well-defined structure of the 3D-epidermis and immunofluorescence staining with Keratin 10 and 14, for horizontal and vertical differentiation, respectively, was conclusive. Cell viability was confirmed in the barrier function test, and satisfactory IC50 values were obtained.

4.2 Transfer of the selected toxicity test to industrial scale platforms

This part of the program was severely affected by the lack of an engineered cell line for the NRF2 construct.

Technology transfer of the assay.
The objectives of this part were to demonstrate a satisfactory transfer of the cells and assays to the WP4 partners.

The SOPs for seeding, differentiating iPS keratinocytes and culturing 3D epidermis for this cell line were transferred following successful training. Since the monolayer keratinocyte assay would be more applicable than the 3D epidermis it had been suggested that the assay on keratinocytes in 2D should be performed. As the KeratinoSens™ assay had already been validated in-house at CiToxLAB, Covance hosted I-Stem for a training program on the assay procedure using the KeratinoSens™ assay. Bonn University transferred the lt-NES iPS derived neuronal cells and protocols to Covance, who successfully performed the experiments according to the supplied protocol. No specific assay was transferred but University of Bonn had previously presented data on a number of genes downstream of NRF2 pathway among which NQO1 and HMOX-1, analyzed at Covance with iPS keratinocytes and primary keratinocytes and KeratinoSens cell line at CiToxLAB.

Protocols refinement.
The aim of this part was to document any modification of the standard operating procedures designed and provided by the other partners.

Two additional protocol-endpoints were established with the lt-NES iPS neuronal cells: the determination of relative LDH values in comparison to maximum LDH value (method of analysis

reflecting the cytotoxic effect of the test chemical) and the total GSH quantification following exposure of the cells to chemicals.

Concerning the keratinocytes assay performed with the iPS keratinocytes, it was decided to investigate the contribution of inflammatory cytokine effects following the exposure of the cells to chemicals. Three cytokines were selected (IL-6, IL-8 and IL-10) and the results obtained demonstrated that the chemicals tested did not affect the release of those cytokines.

4.3 Proof of concept of selected toxicity test implemented on industrial-scale platforms

This part of the program could not be performed, due to the lack of the appropriate engineered cell line to be tested.

4. Potential impact and the main dissemination activities and exploitation of results

The *SCR&Tox* project was organized in order to offer a systematic scientific and technological approach towards improving our search for an efficient and reliable assessment of the toxicity of chemicals, whether they be pharmaceutical compounds or cosmetic ingredients. This was deemed necessary within the general framework of a shift in the focus of regulatory toxicology from empirical assessment in whole animals and clinical trials to *in vitro* assays based on human cells. This shift in scientific paradigm can be associated with major foreseeable impacts, namely on the overall value of toxicity evaluation, on the price of drugs and cosmetic products and on the societal acceptance of activities in the health industry.

As concerns the value of toxicity evaluation, it is widely considered nowadays that whole animal testing is associated with a number of false negatives and false positives due to interspecies variations. False negatives, due to lack of sensitivity, is sending potentially valuable drugs to the waste basket, with social and industrial consequences. False positives, resulting from lack of specificity, may lead to hasty and costly withdrawal, and sometimes to human morbidity and mortality. The *SCR&Tox* project was designed to bring the proof of concept at industrial scale of the possibility to overcome previous problems by turning to the most rigorous scientific approach of toxicity pathways. Even though the final demonstration could not be brought, due to unfortunate delays linked to the unexpected withdrawal of a key partner, the results obtained are highly consequential in scientific terms and will help promote the shift in focus in the entire toxicology community.

Predictive toxicology has become, over the past twenty years, one of the most expensive components in drug and ingredient discovery, and some analyses consider that it accounts for 20% of the entire cost of the final product. The continuous expansion of regulatory requirements and the pressure against *in vivo* animal testing are continuously increasing those costs. In vitro cell-based assays, because they rely on unlimited biological resources and large-scale technological approaches well before development of the products up to clinical trials, will allow for major reduction in this heavy bill. The versatility of the human cells and their theoretical ability to accommodate any functional read-out will be major advantages in that direction.

Last, but not least, the life sciences as a whole have faced strong attacks over the past decades from advocates of animal welfare, with particular focus on the industrial use of animals for toxicity testing, in particular in the cosmetic industry. Cell-based assays of toxicity pathways are no promise for a complete elimination of animal testing in the pharmaceutical industry –in particular because it is beyond reach to test in cells either ADME (absorption, distribution, metabolism and elimination) or

DMPK (drug metabolism and pharmacokinetic). However, cell-based assays will unavoidably reduce considerably the number of animals as the brunt of the toxicity testing will be borne by *in vitro* assays and animal testing will come as a complement only for those issues when relevant.

SCR&Tox has met the strategic objectives that it had set more than 5 years ago. The depth and breadth of the consortium has enabled a substantial impact on developing the essential knowledge and platform technologies. A key feature of *SCR&Tox* was its industrial dimension by incorporating 6 biotech companies but also two CROs that are directly the main stakeholders of the program in the large pharmaceutical and cosmetic industries. Emphasis to this connection was further given through the organization of an “industrial and regulatory consultative body” in which representatives of major companies were convened and invited to discuss, on a regular basis, concepts and results of the Project. It is our contention that all the research carried out by *SCR&Tox*, whether it be addressing basic questions or else promoting assay development to industrial scale, has provided the foundation for exploiting this area of biotechnology for wealth and job creation across Europe.

Even though the program has not been able to complete the development of new designs for predictive toxicology at major stakeholder sites, as originally planned, it has delivered all the components of such a design. We have, indeed, established biological resources for those new designs. The five cell lineages along which human pluripotent stem cells have been differentiated either fully or up to a relevant intermediate precursor, are now available tools for *in vitro* drug testing. In parallel, we have developed technological means and methodologies to use those resources in assays that allow analysing toxicity pathways at the molecular level. Among them, a particular notice should be made of the bioelectronics approaches that have been developed in a quite original manner by the network. Bioelectronics would, in our collective view, be a major addition to the current array of methods that analyse cell responses to toxicants.

Using those biological and technological resources developed by the network, we have been able to demonstrate in several assays for specific toxicants applied to a variety of cell phenotypes that drug toxicity could be reproduced and quantified. We have shown that repeated dose toxicity elicited molecular changes that were very significantly different from those that accompany an acute response, even for the same dose of a toxicant. This result, by itself, is a major warning for the field and an important contributor to the trend toward a change, which has been promoted since the mid 2000’s and has contributed to the launch of the call to which the SEURAT super-consortium was a response. In addition, we have used the paradigms and resources developed in the *SCR&Tox* program to reassess the myopathic toxicity of statins and suggested that, contrary to what had been proposed before, direct cell lethality, a cytostatic effect of statins may be responsible for the chronic myalgias that affect many patients who take statins regularly. This result underscores the clinical relevance of the *in vitro* approaches that were at the core of the *SCR&Tox* project.

During the elaboration of the program, we had identified collectively four domains in which impact could be anticipated and we can now indicate for each of them the impact of the work performed during the *SCR&Tox* program.

(a) Tangible advances in the scientific approaches to

i. *pluripotent stem cells provision and differentiation into derivatives along different lineages of interest*. This has been fully carried out and transfer of the SOPs to industry partners has demonstrated the value of this work for the two lineages tested.
ii. *gene and protein engineering as a way to provide cells with additional properties in view of an industrial use*. The techniques have been developed and demonstrated on the bench, although the unfortunate withdrawal of the partner in charge of that development (Cellectis SA) before the transfer of the technology was done did not allow us to establish its value at the industrial scale.
iii. *gene and protein profiling as well as exploration of dynamic functions at those two same levels as well as using bioelectronics in excitable cells*. This part of the program has provided major improvement to the array of analyses that can be used for studying drug toxicity *in vitro*. As referred to above, a special mention is worth making for bioelectronics, which has revealed extremely informative.

(b) European industrial innovation and competitiveness *by bringing proof of concept of predictive toxicology testing at industrial scale on the basis of cell-based assays using human pluripotent stem cell derivatives*. Cell-based assays have been quite successfully developed up to transfer of resources and technologies to industry partners. This has clearly opened a path for reaching the goal that was originally set to the network. The delay introduced by the use of a contingency plan in order to overcome the difficulty related to the withdrawal of a key partner in the middle of the program has not, however, allowed us to demonstrate fully the value of our paradigms. This will have to be completed beyond the time schedule of the *SCR&Tox* program.

(c) European healthcare policy and regulation *by entering into the validation process in vitro testing to replace or reduce animal experimentations in predictive toxicology for pharmaceutical compounds and cosmetic ingredients*. For the same reason described in the previous paragraph, this impact could not be fully reached. One assay that was designed in order to meet all challenges of pre-validation by ECVAM has been partially developed, though, and only awaited the cell resources (the NRF2-responsive engineered iPS cell line) required for its full exploration. All preliminary analyses have been done, and the path toward pre-validation is now open with the delayed provision, at the end of the time schedule of the program, of the needed cell line.

(d) Specific training experiences for the next generation of toxicologists *involved in regulatory toxicology*. It was quite important to us to make scientific advances usable at industry scale. Scientific progress by itself does not guarantee that impact will happen. For actual impact to occur, scientific advance must be coupled and integrated with an appropriate strategy to promote the new findings up to the appropriate setting, and, in the particular case of predictive toxicology, that means industrial platforms sufficiently scaled to welcome large libraries of compounds and ingredients as well as existing chemicals. The new findings and developments should be made understandable to the potential beneficiaries in ways that are normal and actionable to them. For this reason *SCR&Tox* has dedicated its Training and Dissemination Workpackage 5 essentially to “technology transfer”, in order to provide industrial R&D personnel with the necessary training to handle biological and technological resources required for implementing cell-based assays as developed by the consortium. A number of internal and external training sessions have been organized during the *SCR&Tox* program, with specific emphasis placed on cell reprogramming for the production of iPS cell line (25 external teams trained), the differentiation of cells into phenotypes of interest for drug screening (4 internal teams trained, including those of the two CROs), and automation/miniaturisation of cell bioproduction (three platforms developed and the two CROs trained).

5. Partners involved and coordinator's contact details

Part. #	Participant organisation name	Organisation short name	Country	Principal Investigator
1A	Institut National de la Santé et de la Recherche Médicale	Inserm	France	Marc Peschanski
1B				Vincent Lotteau
2	Universitaetsklinikum Bonn	UKB	Germany	Oliver Brüstle
3	Takara Biotechnology Europe (former Cellectis AB)	Cellartis	Sweden	Catharina Ellerström
4	Cellectis SA *(until 31. December 2013)*	Cellectis	France	David Sourdive
5	CXR BIOSCIENCES LIMITED	CXR	UK	Cliff Elcombe
6	Universität Leipzig	ULEI	Germany	Andrea Robitzki
7	CiToxLab	CIT	France	Roy Forster
8	Covance Laboratories Ltd	Covance	UK	Julie Clements
9	Commission of the European Communities - Directorate General Joint Research Centre	JRC	Italy	Anna Price
10	AstraZeneca AB	AstraZeneca	Sweden	Tommy B. Anderson
11	Karolinska Insitutet	KI	Sweden	Magnus Ingelman-Sundberg
12	Avantea srl	Avantea	Italy	Giovanna Lazzari
13	Health Protection Agency HPA *(until 31. March 2013)*	NIBSC-HPA	UK	Glyn Stacey
14	Inserm-Transfert SA	IT	France	Christiane Dascher-Nadel
13	Department of Health *(starting 1. April 2013)*	MHRA-NIBSC	UK	Glyn Stacey

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6. Project logo and public website

www.scrtox.eu