1. Publishable summary

Embryonic Stem cell-based Novel Alternative Testing Strategies

The aim of the ESNATS project is to develop a novel toxicity test platform based on embryonic stem cells (ESCs), in particular human ESC (hESCs), to streamline the drug development R&D process and evaluation of drug toxicity in clinical studies, reduce related costs and thus to not only increase the safety of patients but also to reduce the number of animals due to earlier detection of adverse effects.

ESNATS addresses current shortcomings in toxicity testing:

- A major part of safety testing takes place late in the research and development cycle, implying protracted experimentation involving high numbers of animals and generating significant costs.
- Some *in vitro* assays rely on cells lines of malignant origin or primary cells that are hard to standardise and limited in terms of quantity, homogeneity and genetic diversity.
- Existing assay systems based on primary animal and human cell lines do not reliably represent the physiological situation of cells in native tissue.

To reach the project goals, within a time frame of five years, a battery of toxicity tests is developed using ESC lines subjected to standardised culture and differentiation protocols. Tests cover both pluripotent ESCs as a model for early-stage human embryos, cells in defined stages of development as well as further developed cells, including gamete and neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches are used to determine predictive toxicoproteomics and –genomics signatures. The individual tests are then integrated into an overall testing strategy. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture are developed and scalability aspects of test systems are considered during development. In a later stage of the project, the predictivity, quality and reproducibility of the test strategy is evaluated in a “proof of concept” study.

The results of ESNATS are expected to have an impact at several levels:

- On pharmaceutical R&D, by providing a new technology which will facilitate screening and early decision-making of candidate drugs, and in the long-term might contribute to a more rational and effective drug development process;
- On public health, by contributing to the production of safer drugs at lower cost which will be available much quicker;
- On European stem cell research, by providing new technologies for stable hESC culture, improved protocols for hESC differentiation and a world leading toxicogenomic database.

Furthermore, the new testing rules under the European Regulation of Chemical Substances (REACH) require extensive toxicological safety testing of both existing and new chemicals which can also include drug intermediates. The ESNATS project provides valuable information for risk identification in regulatory toxicology. Alternative testing strategies are highly needed in this field of work to limit the number of animal tests required to comply with the REACH framework.

The work to be performed in ESNATS is divided into four main research areas (see Figure 1), each one representing a sub-project (SP). These SPs are complemented by central work packages (cWPs) which cover transversal scientific aspects of the project (see Figure 1). In addition, the project plan foresees central WPs for the management of the consortium, as well as for training and dissemination work.

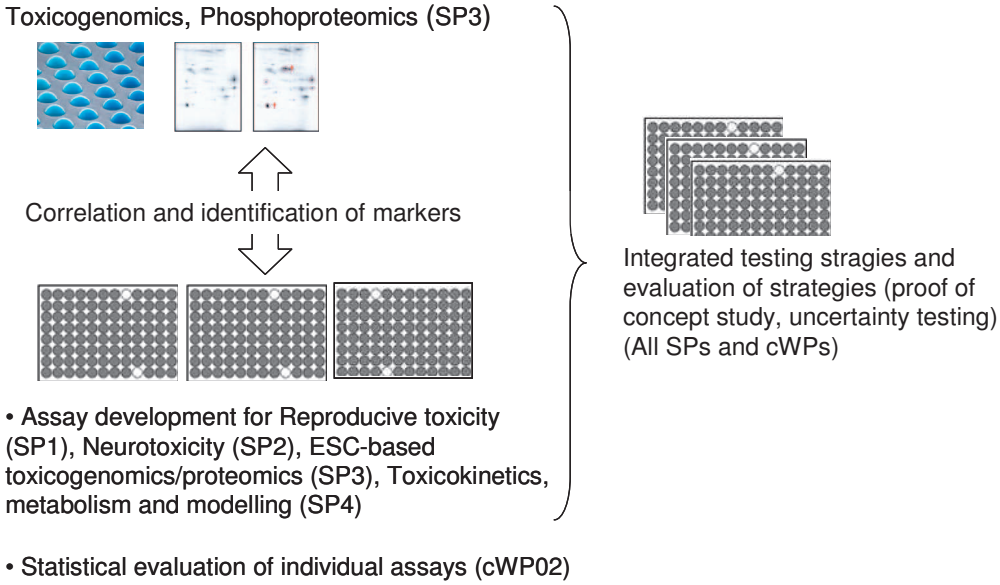

Figure 1 ESNATS main research areas

The ESNATS project has now completed the first two project years. Work in this period focused mainly on the preparation of the cell systems as the basis for the test development which will be fine-tuned into readily usable test systems in year 3. Year 4 and 5 will be mainly dedicated to the combination of the test systems in an overall testing strategy and the proof of concept study, including through blind tests.

In the first project year, the ESNATS partners initiated the set up of the *in vitro* assays for reproductive toxicity and neurotoxicity, focusing on the establishment of the underlying ESC systems, including work on ESC maintenance and differentiation into the developed target cells. Standard operating procedures (SOPs) were developed for several cell systems to standardise differentiation of hESCs into target cells.

Standard methods for RNA sample generation and processing were developed in order to minimise variability due to sample handling and quality control protocols for hESC cultures were developed to ensure reliability/reproducibility of the cell culture systems. A database for online tracking of samples and associated data was developed.

Culture conditions of primary mouse and human hepatocytes were optimised in order to be able to integrate them as metabolising systems into the testing strategies with ESC derived cells. Work started on physiologically-based pharmacokinetic (PBPK) modelling.

A list of test substances was drafted by the SPs and a first recommendation of selected test substances was made by the Steering Committee, an advisory panel composed of representatives from industry and regulatory bodies. The Steering Committee also provided industrial and regulatory specifications for assay development.

The user requirements specification for equipment to automate standard protocols was drafted and passaging methods were identified and evaluated for their applicability to automation.

Training courses were carried out by the ESNATS partners on phosphoproteomics, microarray expression analysis and ethical aspects, in order to share a common understanding of issues at stake. A workshop on statistics was organised together with the Predict-IV project.

The ESNATS public website was set up and the ESNATS flyer was edited and distributed at the first public event, organised by the Univerity of Newcastle upon Tyne in April 2009.

In the second project year, the ESNATS partners maintained their focus on setting up the *in vitro* toxicology test assays. **ESC culture and differentiation systems were further developed for use in reproductive, developmental and neural toxicity testing (see Figure 2)**. In particular, the differentiation of stem cells into a wide range of cells and tissues was optimised, including neural cell types and engineered neural tissues, for cardiomyogenesis and for (mouse) ESC-derived spermatogenesis. SOPs for additional cell systems were developed, including for two- and three-dimensional neural tissues and for two- and three-dimensional trophoblast.

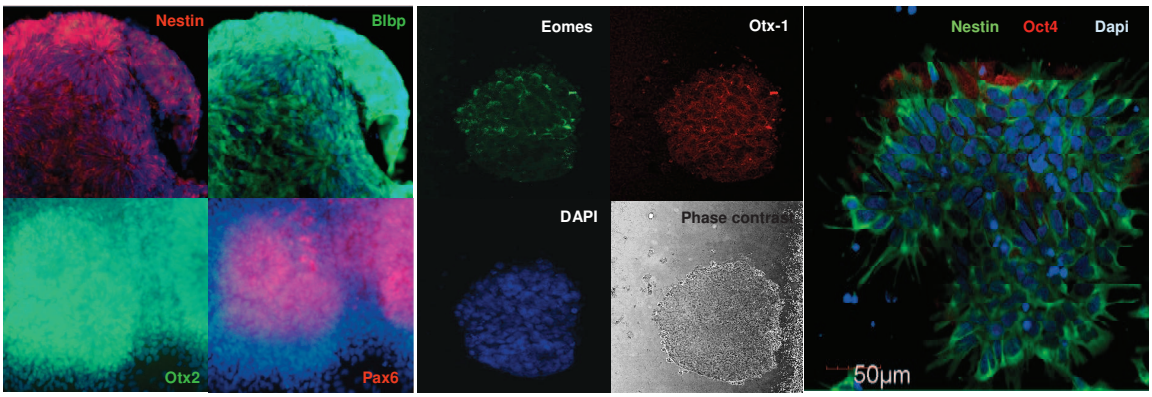

Figure 2 Cellular model systems developed in ESNATS. Left: Expression of markers in neural rosettes. Nuclei are stained blue (Hoechst dye). Source: G. Lazzari. Middle: Marker expression in the standardised trophoblast model system. Source: P. DeSousa. Right, Marker expression in HES1-derived neural progenitors. Source O. Wiser.

The ESNATS proteomics and genomics subproject (SP3) has been analysing sample material prepared by partner laboratories to characterise cell models and identify toxicological signatures. All samples are documented in the online database for sample and associated data management and then processed and analysed by the toxicogenomics and –proteomics facilities. **Proteomics and genomics signatures for selected test substances in individual assays were identified**. *In vitro* assays will be systematically challenged with extended sets of test substances to enable definition of comparative toxicity signatures. To host the resulting data and permit analysis according to user-defined criteria, a web-based and password-protected tool for genomics analysis was developed and made available to all partners. Similarly, a database for the collection of dose-response data and online tools for their analysis was developed and made available.

In the area of metabolising systems, dedifferentiation and loss of metabolic activity, one of the major hurdles of using primary hepatocytes as a metabolising system has been investigated and the most critical mechanisms responsible for dedifferentiation were identified, enabling approaches to better maintain the differentiated state. **Stem cell-derived metabolically active cells have been generated** but major differences remain as compared to primary hepatocytes and further improvements are necessary. An integrated approach of *in vitro* testing and PBPK modelling to be used to predict *in vivo* effect levels for reproduction toxicity and neurotoxicity has also been developed.

The list of test substances for test development has been extended and finalised based on the input of the SPs, which was reviewed by the Steering Committee and a **first recommendation of selected test substances was provided**.

In the area of automation and scale-up, enzymatic dissociation systems were evaluated as a prerequisite to scaling up of hESC culture. Based on the user requirements specification, **a concept for a medium-scale robotic system to automate standard ESC culture has been developed**. Regarding banking, a comparative study on feeder-based and feeder-free systems is being completed.

The **first ESNATS summer school** was held in Zermatt, Switzerland and was a great success with many doctoral students, postdocs and senior scientists attending. In addition to presentations and posters by ESNATS partners, keynote lectures enhanced the scope of the summer school. A second ethics training session was held in Zermatt as well.

The ESNATS public website was regularly updated and an ESNATS **poster with results from year 1** was prepared and distributed for use by partners. Updates on consortium activities and progress were regularly disseminated in the ecopa newsletters.

Figure 3 shows the main milestones achieved in the first two years of the project as well as the major milestones planned in the coming three years.

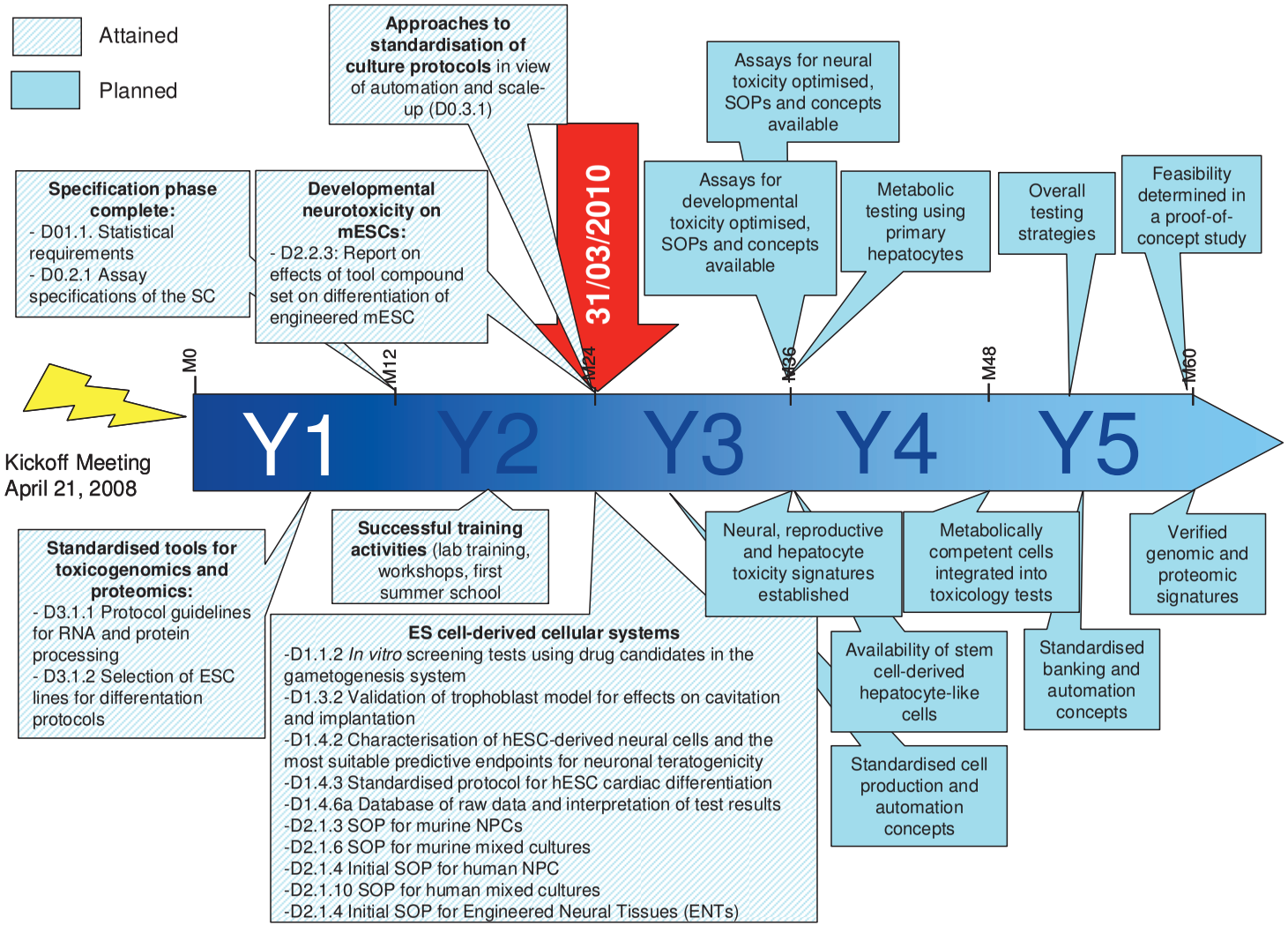

Figure 3: Main milestones achieved in the two first years and major milestones in the next three years of ESNATS

Over the next three years, cell systems will be fine-tuned and adjusted to become readily usable test systems to be challenged with full sets of reference substances. A specific task force has been set up in the project which aims to define the overall test strategy and to identify the test systems on which the project will focus. The overall integrated testing strategy to be applied in the two last years in the project will be set up and validated, including the selection of the most suitable hESC-derived *in vitro* test systems (looking at criteria such as reliability, status of the protocol, availability of *in vivo* data etc.), identification of targets to be predicted by the assays, definition of the appropriate readouts as well as the combination of these, such as toxicogenomics, proteomics, functional readouts etc.

ESNATS partners

ESNATS addresses its objectives in a multidisciplinary collaboration of leading European researchers in alternative testing, toxicology, ESC research, genomics, modelling, and automation. The consortium includes representatives from regulatory bodies, the pharmaceutical industry and ethical advisors to provide guidance and ensure applicability of the developed test systems.

The ESNATS consortium is composed of the following organisations:

- Universität zu Köln – Universitätsklinikum (Jürgen Hescheler, Agapios Sachinidis)
- Commission of the European Communities – Directorate General Joint Research Centre JRC (Susanne Bremer)
- University of Newcastle upon Tyne (Karim Nayernia)
- Université de Genève (Karl-Heinz Krause)
- Forschungsgesellschaft für Arbeitsphysiologie und Arbeitschutz e.V. (Jan Hengstler)
- European Consensus Platform on 3R Alternatives to Animal Experimentation (Bernward Garthoff)
- The Automation Partnership (Cambridge) Limited (Rosemary Drake)
- OÜ Quretec (Jaak Vilo)
- ProteoSys AG (André Schrattenholz)
- Université de Liège (Luc Grobet)
- Cellartis AB (Raimund Strehl)
- Cell Cure Neurosciences Ltd. (Charles Irving)
- Universität Konstanz (Marcel Leist)
- National Biological Standards Board (Glyn Stacey)
- Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek – TNO (Miriam Verwei)
- The University of Edinburgh (Paul deSousa)
- Vrije Universiteit Brussel (Vera Rogiers)
- Technische Universität München Klinikum Rechts der Isar (Andreas Nüssler)
- ARTTIC (Annette Ringwald)
- The School of Pharmacy, University of London (Andreas Kortenkamp)
- N.V. Organon (Sjeng Horbach)
- Läkemedelsverket / Medical Products Agency (Ira Palminger-Hallen)
- H. Lundbeck A/S (Nina Ostenfeld)
- In Vitro Testing Industrial Platform (Erwin Roggen)
- Bundesinstitut für Risikobewertung (Sara Adler)
- Edinethics Ltd (Donald Bruce)
- Gottfried Wilhelm Leibniz Universität Hannover (Ludwig Hothorn)
- F. Hoffmann-La Roche, Ltd. (Nicole Clemann)
- Avantea srl (Giovanna Lazzari)

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