Theme V:
Replacement and Reduction in Basic Research

Session summaries for Theme V

Session V-1

Novel Technologies and Approaches to Allow Hazard Assessment of Chemicals, Cosmetics, and Drugs with Animal-Free Methods

Co-Chairs: Marcel Leist, University of Konstanz, Germany
Yasuo Ohno, National Institutes of Health, Japan

Many alternative methods to replace animal experiments have been validated and accepted by legal bodies, e.g. the OECD (Organization for Economic cooperation and development). Even more such methods are under development or already in use for non regulatory applications or to provide proprietary knowledge base. The next step for the use of the different available *in vitro* methods is to combine them into integrated testing strategies (ITS) to get closer to the overall goal of an "*in vitro* based risk evaluation process." We introduce here a concept that might lead to a testing scheme when properly analyzed, combined and weighed. Originally these promising, innovative technologies were developed as stand-alone approaches, presented at the 8th World Congress on Alternatives and Animal Use in the Life Sciences, held in Montreal September 2011 in Theme V: Replacement and Reduction in Basic Research in Session V-1, Novel methodologies and their potential *in vitro* application for drug development and safety assessment.

In the area of "*in vitro* modeling," Altamira developed a mechanistic rationale for the prediction of skin irritancy effects implemented in a workflow process to predict a molecule's potential for skin irritancy based on *in vivo* legacy data. The combination of rule-based and regression modeling on structural features and physicochemical descriptors relevant to the underlying mechanisms of action were used to develop this predictive scheme, which improves the reliability of skin irritancy estimations and provides user-friendly implementation for toxicologists.

As a point of departure in an *in vitro* risk assessment approach, two- and three-dimensional cell systems play a major role in generating *in vitro* concentration-response information and determination of the POD for *in vitro-in vivo* extrapolation.

The chair of *in vitro* Toxicology from the University of Konstanz presented a 2D cell system of conditionally immortalized neuronal precursor cells able to differentiate into neurons by the addition of tetracycline. This model system of human neurons has applications in neurotoxicity testing. Epigenetic changes have been shown in neuro-degeneration studies of Alzheimer's and Parkinson's disease. Using endpoints like neurite outgrowth and pathway of toxicity (POT) screening, further characterization of these diseases has been accomplished.

In a high throughput screening manner, more complex data may be generated by a 3D novel platform for automated production and screening applying scaffold-free, organotypic microtissues, introduced by Insphero. This technology allows the production of tumor and primary microtissues in a scaffold-free hanging-drop culture with a size uniformity of <10%. A whole screening process using reference compounds was performed in comparison to classical monolayer cultures, underscoring the different behavior of both cell-culture models.

The identification of POT (pathways of toxicity) may be enhanced by a 3D model for organotypic *in vitro* human epithelial models (EpiAirway,EpiDerm-FT) with engineered toxicological reporter functions presented by the MatTek Corp. This model provides more realistic, *in vivo*-like structures, barrier properties, metabolic functions, and dosing capabilities, plus the added feature of engineered toxicological reporter functions. NFκB reporters linked to either GFP or luciferase were found to be activated about 5-fold above background by treatment of the organotypic models with TNFα. This tool may provide the bases for mechanistic toxicity screening assays.

The natural variation in human genetics and environment has always been a problem, which might be solved using data ana-

lytic techniques borrowed from computer science and statistics. The ANU & MAWA Trust found relevant and unique patterns within highly variable human populations by using combined pattern recognition data mining and knowledge discovery applying recursive partitioning and Support Vector Machines (SVMs). These and other bioinformatics techniques allow the modeling of multi-dimensional data and the identification of "patterns" associated with a disease outcome. Such patterns, or profiles, can then be summarized as a set of "rules" that allow the clustering of human data associations with an outcome.

Each of the technologies presented is a stand-alone method, valuable for its specific purpose. Combined in a scheme, they are able to fill knowledge gaps, which could lead, finally, to an *in vitro* risk evaluation approach without the use of animals.

Session V-2

Systematic Reviews of Animal Experiments

Co-Chairs: Marlies Leenaars, Radboud University Nijmegen Medical Centre, Netherlands
Michelle Hudson, FRAME, UK

The aim of the session was to explore why systematic reviews and meta-analysis of animal studies are urgently needed, how they might be conducted and which hurdles there are to overcome. The first speaker, **Merel Ritskes-Hoitinga**, outlined the medical origin of systematic reviews and how they have now become a gold standard in that field. She described many of the advantages of applying the reviews to animal experiments, including transparent synthesis of evidence, improving the quality of future studies and the possibilities for replacing further studies. There has been a great deal of work to develop strategies and tools (i.e., animal search filters for PubMed and Embase, a step-by-step search guide and a Gold Standard Publication Checklist) to facilitate reviews of this nature and Merel explained these along with some of the problems that had arisen during the process, in particular the difficulties associated with the variability of the types of experiments and the quality of the reports related to them.

The second speaker, **Emily Sena**, followed this with a very interesting account of the practical application of systematic reviews. She described a selection of her findings from reviews of various fields of neurological disease research involving animal studies. Her work shows that systematic reviews and meta-analysis are useful tools in quantitatively estimating the impact of study quality on the outcomes of animal research. The results revealed that problems with publication bias and insufficient blinding and/or randomization in studies have led to overestimations of efficacy giving rise to translational failure when it comes to clinical trials.

The final presentation in the session was given by **Nathalie Percie du Sert**, who again highlighted some of the problems that are revealed by systematic review, in particular the quality of the reporting of animal studies that can hinder meta-analysis. She described how she had utilized systematic review and meta-analysis to examine preclinical data to evaluate and refine animal models and to also identify which is the most appropriate model to answer the research question. As a possible solution to the problem of poor quality reporting Nathalie discussed the development of the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines, which describe the minimum information that all scientific publications reporting *in vivo* research should contain. They have been endorsed by many journals and funding bodies.

The discussions and questions which followed each presentation where extremely informative and indicated that there was a great deal of support for the concept of systematic review. They revealed that in order to progress towards greater implementation scientists will need to work together to identify when it is most effective to conduct such reviews and to develop tools to streamline the process.

Session V-3

Cell Culture and Tissue Engineering

Co-Chairs: François A. Auger, University of Laval, Canada
Gladys Ouédraogo, L'Oréal, France

The success (usefulness) of alternative methods depends on the quality/relevance of the methods (models + endpoints).

This session highlighted several features that need to be taken into account when using cells and tissues:

1. The need for well characterized, stable, standardized, and biologically relevant models. This was illustrated by L. **Aschauer** (Innsbruck, Austria) by functionally characterizing renal epithelial cells (*Temporal transcriptomic alterations during renal epithelial monolayer formation*).
2. The need for a toolbox of models/methods to choose from, depending on endpoint/mechanism. A variety of biological models are now available: primary cells, cell lines, stem cells (embryonic, IPS) reconstructed tissues, spheroids, MEMS (*Microelectromechanical systems*). Different culture conditions also are available: static, dynamic, microfluidic devices, bioreactors, nanodevices, media, gas, temperature, humidity. Depending on the purpose, it is important to check the physiological relevance of the cell/tissue type. Several presentations covered these points:
   - **Y. Sakai** (Tokyo, Japan): *Direct oxygen supply to liver-derived cells using oxygen permeable membranes*; more physiological responses were obtained when hepatocytes were cultured using the oxygen- permeable membranes.
   - **S. Constant** (Plan-Les-Ouates, Switzerland): *Assessment of acute, long-term and chronic respiratory toxicity using a long shelf-life 3D model of the human airway epithelium (MucilAir™)*; the model has an amazingly long shelf life, making it useful for chronic and repeated dose testing.
   - **M. Tondreau** (Québec City, Canada): *A human tissue-engineered vascular substitute with a functional vasa vasorum*. This model can be used to investigate the pharmacology of the vascular system *in vitro*.

Cell culture and tissue engineering are multidisciplinary fields where several areas meet: biology, (bio)material science (for scaffolds), microelectronics, optics, chemistry, medicine.

To achieve successful model development, it is useful to bring together these multidisciplinary stakeholders

Session V-4

Refinement and Reduction in the Use of Genetically Engineered Animals

Co-Chairs: Fernando Benvides, University of Texas, USA
Frederik Dagnaes-Hansen, University of Aarhus, Denmark

The main topics covered during Session V-4 were as follows:

First, Dr **Scott Fahrenkrug** (University of Minnesota, USA) described novel methods for creating genetically engineered (GE) animals, focusing on genome editing in livestock. Using nucleases, large-animal (e.g., pig and cow) models and animals with enhanced welfare traits can be produced (i.e., cows without horns). By this method, GE animals can be created without excessive breeding, contributing to both refinement and reduction. As the methodology has no species barriers, it opens the possibility for refining better animal models of human diseases. The second speaker,

Dr **Fernando Benavides** (MD Anderson Cancer Center, USA), discussed the importance of the genetic background in the use of GE mice and rats. Establishing the genetic modification (i.e., transgene or targeted alleles) on a well-defined inbred background by backcrossing (developing congenic strains) may involve a large number of animals and take considerable time. Through marker-assisted backcrossing (also known as "speed congenics") a remarkable number of animals and time can be saved. Also, considerable refinement can be achieved if adequate genetic quality controls are established and correct strain and gene nomenclature is used.

Third, Dr **Hélène Héon** (Centre Hospitalier de l'Université de Montréal, Canada) explained how proper breeding schemes, colony management, and training of staff and scientists could improve the welfare of the GE animals and contribute to refinement and reduction. Appropriate breeding planning and record keeping may reduce both the number of animals as well as the costs. Dr. Héon mentioned that the use of pictures and videos on the intranet or in the animal facilities showing abnormal phenotypes in the GE animals could avoid unnecessary suffering, as early intervention could be accomplished.

Finally, Dr **Mitra Cowan** (Centre Hospitalier de l'Université de Montréal, Canada) discussed initiatives to implement and promote refinement and reduction in the production and maintenance of GE rodents. As the director of a transgenic core facility, she presented many examples for refinement, like the use of non-surgical embryo transfer techniques, the use of spermatogonia-mediated transgenesis, the use of buccal swabs replacing tail biopsies, and the consultation of well-established databases (e.g., Mouse Genome Informatics) to avoid the production of duplicated GE models. To conclude, she mentioned that cryopreservation of embryos and sperm is becoming increasingly popular because of the cost and space savings, but it will also reduce the number of animals to be bred and maintained.

The speakers answered many questions during the session, contributing with a very productive discussion.

Session V-5

Developments in stem cell research as the basis for sustainable availability of differentiated human cells and tissues

Co-Chairs: Vera Rogiers, Brussels, Belgium
Jeff Biernaskie, Calgary, Canada

The session opened with Dr **Biernaskie** who described his recent work demonstrating the existence of a dermal stem cell within mammalian skin. These multipotent stem cells (termed skin-derived precursors or "SKPs") reside in hair follicles and function to induce hair follicle formation but also act as a reservoir of dermal cells to replenish the dermis to maintain homeostasis. He suggested that these cells are a renewable source of cells that potentially can be used to engineer improved skin equivalents for *in vitro* experimentation.

If exposed to appropriate *in vitro* conditions, SKPs also can generate functional neural crest derivatives such as Schwann cells, which he showed can myelinate axons using an *in vitro* co-culture system. Biernaskie provided several examples of how SKPs represent an accessible source of "patient-specific" stem cells (derived from skin biopsy) for high-throughput pharmacological screens or toxicological testing. Due to their accessibility, SKPs may be particularly important "sentinel cells" for pharmacological effects on normal stem cell behavior and are also currently being used as a control in drug screens against "tumor initiating cells" and for studying developmental abnormalities related to the neural crest.

Second, Dr **Leist** described his work developing protocols for differentiation of ES cells toward a neural lineage. He provided evidence for both neuronal and glial specification from ES cells and highlighted their potential value as an *in vitro* model system to assess toxicological effects on 1) neuronal and glial specification, 2) maturation and late neuronal development, and 3) early neuroectoderm formation. He also provided intriguing data for induction of neural crest cells from ES cells, which provides a renewable source of cells to assay neural crest function, as well as migration and differentiation of NC derivatives including neurons, Schwann cells, melanocytes, and others.

Third, Dr **Hescheler** asked whether production of teratomas in immunodeficient mice should still be regarded as the "gold standard" assay for pluripotency and whether alternative (*in vitro*) assays can be utilized as an equivalent predictor. He provided several alternatives to the mouse teratoma assay including: directed differentiation of ES and iPS cells into organotypic cells, expression of pluripotency-associated markers (i.e., TRA-1-60, DNMT3B, REX1), and epigenomic footprints (such as DNA methylation, and histone modifications). The conclusion was that indeed each of these assays is capable of addressing one or several aspects of pluripotency and that, if they can be standardized within the scientific community, these alternative tests will be a sufficient substitute. An important comment was made following the presentation, suggesting that in order to enact this change, attitudes needed to change at both the scientist level and journal editors/reviewers, since use of this assay is commonly required for publication.

Mr **Kiyokawa** presented his work showing derivation of functional human cardiomyocytes from iPS cells. He described a comprehensive 38-day protocol from human iPS cells to cardiomyocyte phenotype and showed that iPS-derived cardiomyocytes expressed phenotypically appropriate ion channels. Moreover, he showed convincing electrophysiological evidence that these cells are bona fide cardiomyocytes and are responsive to pharmacological blockade as well as induced triggers of arrhythmia.

An important point was raised suggesting that indeed several laboratories/companies are currently working to produce different types of cardiomyocytes using a variety of strategies. Genetic stablility and frequency of unwanted cells types that are generated using each differentiation protocols also are important concerns. It was suggested that universal standards may be required in order to objectively evaluate the efficacy of each protocol across laboratories or companies.

Dr **Vera Rogiers** described her work demonstrating that skin-derived precursor cells (SKPs) can be isolated from human skin biopsy samples and represent an accessible (potentially autologous) source of multipotent cells that can generate functional hepatocytes. huSKPs were characterized as having high expression of cd90, and cd105 expression with low expression of pluripotency genes. SKPs are exposed to a 24-day differentiation procedure that induces formation of liver progenitors followed by expression of hepatocyte markers albumin, connexin32, cebpα and HNF1α. Generation of functional hepatocytes was confirmed by transplanting SKP-derived hepatocyte progenitors to the uPA+/+ SCID mice, in which they generated mature hepatocytes *in vivo*.

Interestingly, Rogiers described the development of an elegant procedure for rapid generation of decellularized liver. Unlike previous reports, the procedure can be done in 60

mins and preserves the natural composition of extracellular matrix proteins (laminin, fibronectin, collagen I) as well as growth factor islands within the organ. This provides a natural 3-dimensional scaffold that can be seeded with human cells to study liver development, regeneration, and toxicology *in vitro*, representing a potentially valuable alternative to animal use.

Overall Summary

Overall, the session highlighted the diversity of uses (diagnostic, pharmacological screening, and clinical application) that stem cells provide. Each presentation focused on a different type of stem cell (adult somatic stem cells, iPS cells, and ES cells) and highlighted various advantages and disadvantages of each. The overall take-home message was that stem cells offer a renewable and robust alternative to many of the diagnostic assays that currently utilize animals. A particularly compelling example was use of lineage-specific gene expression analyses and *in vitro* directed-differentiation assays as a highly sufficient alternative to the (traditional) teratoma assay used to confirm stem cell pluripotency. Finally, although it was clear that use of animals to validate *in vivo* functionality remains essential, further understanding of stem cell biology (specifically with regard to each type) will certainly enhance the utility of stem cells as a viable replacement/alternative in basic research.

Session V-6

Animal Reduction Through the Better Use of Mechanistically Based Translational Animal Disease Models

Co-Chairs: Michel Tremblay, Goodman Cancer Research Centre, McGill University, Canada
Andrew Bennett, FRAME Alternatives Laboratory, University of Nottingham, UK

The session began with an invited presentation from Dr **Sean Ekins** (Collaborations in Chemistry and Department of Pharmaceutical Sciences, University of Maryland) entitled *Computational Models for Predicting Human Toxicities*. Dr Ekins opened by explaining that the expense involved in buying both hardware and software for bioinformatic analysis has declined dramatically over the past decade. This, in concert with the emergence of open access software and databases, has made the use of computational modeling in toxicity much more widespread. The requirements of such initiatives as REACH and Toxcast mean that vast numbers of compounds need to be screened. If this is not to result in a concomitant rise in animal testing, then computational modeling must play a major role due to the limited throughput available with *in vitro* models.

Dr Ekins stressed the importance of the following approaches in the search for ADME/Tox approaches: (Quantitative Structure Activity Relationship, pharmacophore needs good *in vitro* data relating to protein); (Homology models, docking, crystallography); (Assumes current knowledge is complete – flexibility to add rules); *Pathway Reconstruction* (Combining similarity to known ligands and regulatory and metabolic pathways. Assumes databases of human biology are reasonably complete. Systems biology – linkage of empirical and computational.).

Dr Ekins presented several examples of computational predicitivity, of which several were particularly notable, including which prediction of docking and activating compounds for the pregnane x receptor as part of the Toxcast initiative and the demonstration that modeling predicted that drugs that cause rhabdomyolysis act via off target binding and inhibition of the carnitine transporter hOCTN2.

In the next presentation, Dr **Rosemary Broome** (Medimmune) described the simple and highly effective cooperation between Medimmune and Stanford University. Scientists at Stanford were able to use the trachea from ferrets being used at Medimmune for asthma research to reduce the overall animal usage. The presentation also highlighted the consideration of species suitability for both biomedical and drug discovery research – the ferret trachea is by far the closest species to humans in terms of physiology and response to irritation. This prompted discussion concerning the suitability, or otherwise, of using dogs as a second species in toxicological testing. The consensus was that the use of dogs is indiscriminate and often scientifically inappropriate.

The third presentation, by Professor **Heemskerk** (Maastricht University), described the development of a flow chamber test to replace animal research on arterial thrombosis and bleeding *in vivo*. The flow chamber device uses collagen and a variety of other physiologically relevant proteins involved in thrombus formation to produce an *in vitro* model that could be scaled to replace the current animal and transgenic models.

Dr **Norman Peterson** (Medimmune) gave the final presentation, offering his thoughts on the use of more physiologically relevant animal models in drug discovery. Dr Peterson described the importance of using a variety of species in order to predict both efficacy and toxicity, and he emphasized the need to choose the correct species, depending upon the drug target/metabolic action of the compound. Dr Peterson also discussed the use of "humanized" transgenic mice in which the human gene for a given drug target replaces that of the rodent model. In theory, this model – if more relevant to humans – will reduce the number of animals/species required for testing. However, Dr Peterson went on to describe the importance of genetic background and indicated that "humanized" transgenics would need to be made using a variety of mouse strains in order to be predictive of human response. Discussion focused on the fact that the requirement to use multiple transgenic strains, while potentially increasing the relevance and predictability of the data, would lead, in fact, to an increase in the numbers of animals required.

Session V-7

Relevance, Reproducibility and Robustness – the Other Three Rs Important to Science and Animal Welfare

Sponsored by the Scientists Center for Animal Welfare

Co-Chairs: Jeffrey Everitt, GlaxoSmithKline Pharmaceutical R&D, USA
Joanne Zurlo, Center for Alternatives to Animal Testing, USA

Jeffrey Everitt from GSK introduced the session and talked about the need in the pharmaceutical industry to address the 3Rs with a scientific approach that balances the needs of the experimental objectives with animal care and use. He stressed that scientists are most receptive to considering the 3Rs from an animal welfare perspective and from a scientific perspective. His take-home message was to advise project and program teams in the pharmaceutical industry to use a mix of *in silico*, *in vitro*, *ex vivo*, and *in vivo* techniques to achieve scientific objectives.

Julie Huxley-Jones from GlaxoSmithKline presented the first talk entitled, "Picking the right species." She emphasized the point that, when and where animal research is necessary to understand the efficacy and safety of a new therapeutic, it is essential to know that the protein targeted by a drug in the preclinical model is similar to the human. Not only should the protein and its activity be similar, but how, when, and where it binds a drug, as well as the downstream effects of the binding should be similar. Interesting examples for animal model selection indicated that well designed mechanistic and disease models may be highly important for some drug targets but quite irrelevant for others. The example studies demonstrated an impact on drug discovery investigation plans, as well as on- and off-target (secondary) pharmacology and effects on the interpretation of safety findings. The key take-home point was that utilization of these new molecular approaches for drug discovery projects can dramatically increase the relevance of animal research and reduce sub-optimal research practices.

William Pennie from Pfizer, Inc. introduced his talk by reflecting that the incorporation of predictive toxicology approaches in early preclinical drug development will be important for reducing the attrition rate within the industry. To identify potential toxic compounds and guide medicinal chemistry efforts in Pfizer, targeted *in vitro* assays have been integrated with structure activity-relationship rules and physicochemical properties to predict *in vivo* effects. In profiling drug-like molecules, Dr. Pennie's lab has considered multiple mechanistic endpoints (cell death, apoptosis, promiscuity against secondary targets, mitochondrial endpoints, etc.) in cell lines of different tissue origins (e.g. transformed cell line of hepatic or cardiac origin, or using stem-cell derived specific cell lineages). In building predictive models, particular challenges exist in the careful annotation of the training data set and building databases of well-annotated and accessible data; this is an area often overlooked by organizations striving to build predictive capability. Finally, the need to balance an understanding of transporter interactions, metabolism, and intended dose of the compound are all shown to have an effect on predictive model performance. In this regard Dr. Pennie's lab has built computational models that incorporate dose prediction as well as chemical properties in an attempt to guide early medicinal chemistry to safer chemical space.

Myrtle Davis from the Division of Cancer Treatments and Diagnosis of the National Cancer Institute, NIH (USA) focused her talk on the use of mechanistic toxicology as a means to enable refinement of toxicology study designs and integration of *in vitro* screening. It was proposed that application of what we understand about mechanisms of toxicity would allow the development of focused *in vitro* screens that are connected to *in vivo* outcomes. Examples were presented that showed how the "reverse" translation of insights derived from adverse effects (derived from clinical experience) and data from mouse models (that mechanistically connect these adverse effects to targets and pathways) can facilitate development of biologically targeted toxicology screens and biomarkers. The key point was an expectation that more frequent translation of mechanistic data will contribute to modernized preclinical toxicology study designs that employ fewer terminal endpoints and resemble the designs of Phase I safety studies in humans.

Joanne Zurlo wrapped up the session, commenting on the sophistication of the methods currently being used in drug discovery. The pharmaceutical industry is in the forefront of incorporating cutting edge, mechanistic methods that dramatically reduce the numbers of animals in the process. The speakers illustrated how new *in silico* and *in vitro* methods are incorporated into drug discovery and work in an integrated way with whole animal studies to develop more relevant pharmaceuticals.

Session V-8

Presentation of Go3R Search Engine with Hands-On Trials on PC Terminals

Organisers: Barbara Grune, Federal Institute for Risk Assessment, Germany
Ursula G. Sauer, Scientific Consultancy – Animal Welfare, Germany/Technische Universität Dresden, Germany
Michael R. Alvers, Transinsight GmbH, Germany

The hands-on Go3R workshop instructed all those involved in the planning, authorization and performance of animal experiments in using Go3R, the first knowledge-based Internet search engine for alternative methods to animal experiments. Go3R "understands" the meaning of search queries by making use of 3Rs-specific expert knowledge captured within a 3Rs-specific ontology. An ontology is a hierarchically structured network of terms and their synonyms, such as toxicological endpoints, 3Rs methods, and cell lines.

Online literature search queries with Go3R, searching in the Pubmed and Toxline data bases, are performed on the right hand

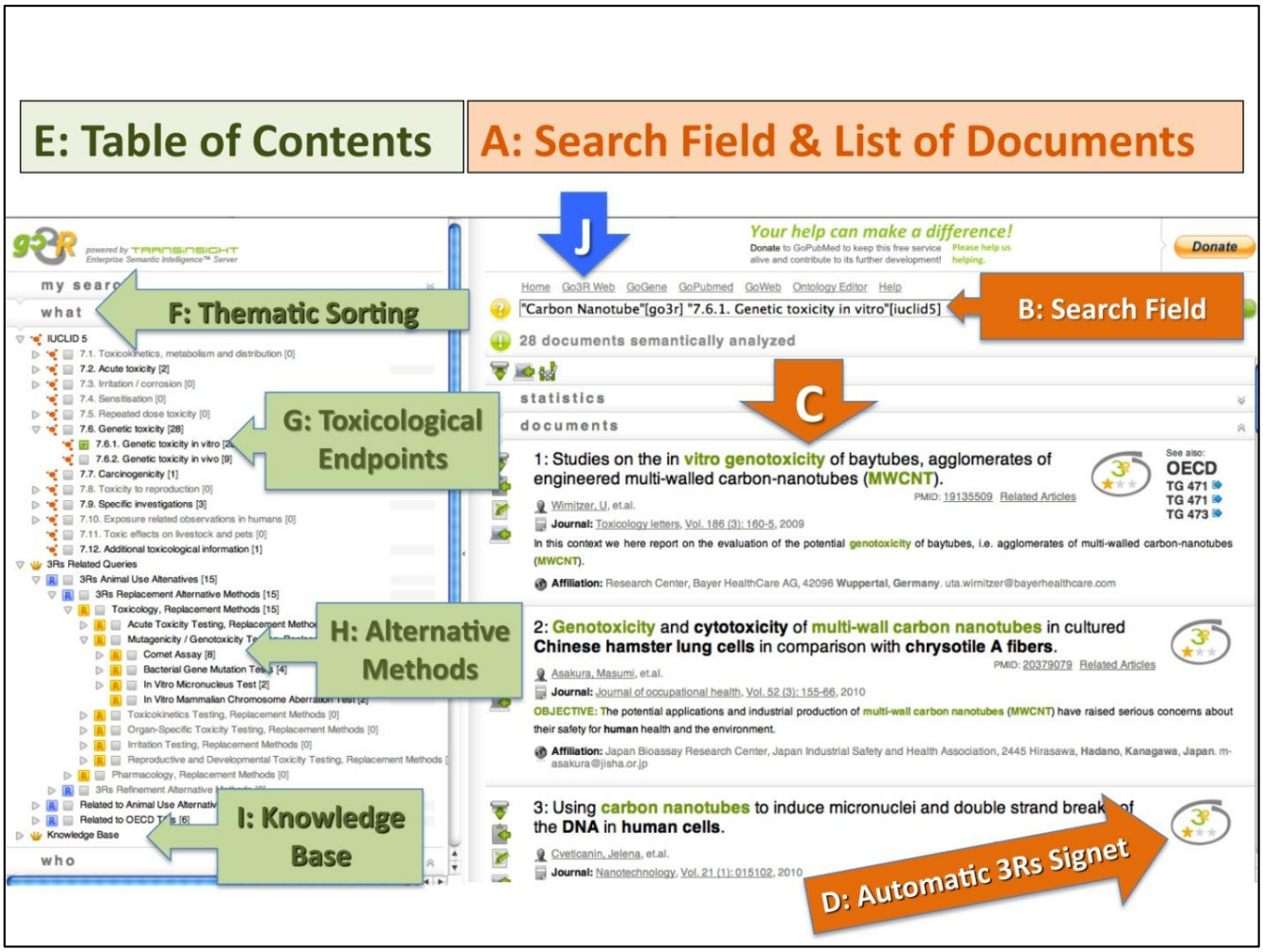

Fig. 1: Online literature search queries with Go3R (www.go3r.org)
Online literature search queries with Go3R (www.go3r.org) are performed on the right hand side of the website (A) making use of the search field (B). Search results are presented as a list of documents (C) using an automatic 3Rs signet (D) to mark documents with likely 3Rs relevance. The left hand side of the website (E) presents an "intelligent table of contents," which can be used to quickly sort the documents retrieved. The section "what" (F) enables thematic sorting, e.g. in accordance to toxicological endpoints (G) or alternative methods (H). Further topical sorting can be done using the knowledge base (I). Additionally, "Google" searches in 3Rs relevant websites can be performed using "Go3R Web" (J).

side of the Go3R website (Fig. 1A) by typing search queries into the search field (B). While typing, Go3R suggests auto-completing search terms making use of Go3R specific terms. When searching with these Go3R terms, the search engine makes use of the ontology's structure and terminology to expand the request, e.g., with relevant synonyms and subordinate terms. Search results are presented as a list of documents (C) using an automatic 3Rs signet (D) to mark documents with likely 3Rs relevance – based upon statistical calculations of the vocabulary used in the documents.

The left hand side of the website (E) presents an "intelligent table of contents" that allows quick thematic sorting of the documents retrieved. By clicking on the respective rows of the section "what" (F), the search result is sorted, e.g. in accordance to toxicological endpoints (G) or alternative methods (H). More intricate sorting of search queries can be achieved by making use of the knowledge base, which provides the entire ontology (I). Documents also can be sorted in accordance to the names of authors, their affiliations, specific journals or the time of publication (sections "who," "where," "when," of the "intelligent table of contents" – not shown in Figure 1). Furthermore, "Google" searches in 3Rs-relevant websites can be performed using "Go3R Web" (J), thereby retrieving information on alternative methods that have not been published in peer reviewed journals.

Go3R continues to be developed further, enabling a determination of the availability of 3Rs-relevant information in a fast and comprehensive manner. It is available free of charge at: www.Go3R.org.