様式第１９

学 会 等 発 表 実 績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」
機関名　国立医薬品食品衛生研究所

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
iPS細胞由来肝細胞を用いた薬剤毒性評価技術の最前線	石田誠一	CPhI Japan 2015（国際医薬品原料・中間体展）	2014,4	国内
iPS細胞由来肝細胞の創薬応用の現状とその有効活用のための周辺技術	石田誠一	日本組織培養学会 第87回大会	2014,5	国内
iPS細胞由来肝細胞を用いた医薬品安全性評価	石田誠一	動物用ワクチン-バイオ医薬品研究会	2014,9	国内
ヒト iPS 細胞由来肝細胞の技術的課題	石田誠一	CBI学会2014年大会	2014,10	国内
Evaluation of Human iPS cell-derived Hepatocytes for the Application to ADME/Tox Tests in Drug Development	Ishida S, Kubo T, Kuroda Y, Kim S, Sekino Y.	CBI学会2014年大会	2014,10	国内
肝臓の代謝酵素誘導評価法の確立	石田誠一	第11回医薬品レギュラトリーサイエンスフォーラム　ヒトiPS細胞を利用した安全性薬理試験法の実現に向けて	2014,12	国内
Utility of iPS cells for drug metabolizing enzyme expression	Matsunaga T.	29th JSSX Meeting - 19th North American ISSX joint meeting	2014,10	国外
ヒトiPS細胞由来小腸幹細胞の至適培養法の開発	壁谷知樹，岩尾岳洋，小玉菜央，中村克徳，松永民秀	第66回日本生物工学会大会	2014,9	国内
ヒト iPS細胞から効率的かつ安定に肝臓を分化誘導する方法の開発	白木伸明	第１１回医薬品レギュラトリーサイエンスフォーラム『ヒトiPS細胞を利用した安全性薬理試験法の実現に向けて』　日本薬学会レギュラトリーサイエンス部会	2014.12	国内
幹細胞から様々な機能細胞を分化誘導する試み	白木伸明	第37回日本分子生物学会	2014.11	国内

ヒト多能性幹細胞におけるS-アデノシルメチオニンの重要性	津山 友徳、白木 伸明、白木 恭子、小幡 史明、三浦 正幸、粂 和彦、遠藤 文夫、粂 昭苑	第37回日本分子生物学会	2014.11	国内
A cost effective intestinal epithelial differentiation system from human iPS cells	Ogaki S, Morooka M, Otera K and Kume S.	Key Forum	2014.9	国外
S-adenosyl methionine is crucial for maintaining human ES/ iPS cells	Tsuyama T, Shiraki N, Kume S.	Key Forum	2014.9	国外
Easy purification of human iPSC-derived immature intestinal epithelial cells	6) Otera K, Ogaki S, Kume S.	Key Forum	2014.9	国外
多能性幹細胞から消化器官を創る	粂昭苑	New Insights of Molecular Genetics on Growth Disorders	2014.7	国内
Chemical genetic identification of signals that control late-stage pancreatic beta cell differentiation.	Kume S.	ISSCR	2014.6	国外
Signals that control differentiation of pluripotent stem cells into pancreatic beta cells	Kume S	Tissue Engineering Regenerative Medicine International Society	2014.6	国外
Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells	Shiraki N, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H, Kume K, Endo F, Kume S.	第12回幹細胞シンポジウム	2014.5	国内
薬物動態評価系への応用を目指したヒトES/iPS細胞由来小腸上皮細胞の作製	小澤 辰哉、高山 和雄、櫻井 文教、立花 雅史、川端 健二、水口 裕之	第37回日本分子生物学会	2014,11	国内
ヒトiPS細胞の状態遷移におけるDNAメチル化可変領域の解析	三木卓也，脇谷晶一，阿久津英憲，梅澤明弘，西野光一郎	第8回日本エピジェネティクス研究会	2014,5	国内

Generation of committed neural progenitors from human fibroblasts by defined factors	Miura T, Sugawara T, Fukuda A, Tamoto R, Umezawa A, Akutsu H.	2th Annual Meeting of ISSCR	2014,6	国外
薬物吸収時の小腸の代謝および輸送の定量的解析	樋坂章博	第5回杉山研究室（理研）公開シンポジウム	2015,2	国内
Systematic Assessment of Intestinal Metabolism and Degree of Inhibition in Drug-drug interactions caused by Inhibition of CYP3A	Nakamura M, Koh S, Hisaka A, Suzuki H.	American Society for Clinical Pharmacology and Therapeutics (ASCPT) 2015 Annual Meeting	2015,3	国外

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Opportunities to integrate new approaches in genetic toxicology	Zeiger E, Gollapudi B, Aardema MJ, Auerbach S, Boverhof D, Custer L, Dedon P, Honma M, Ishida S, Kasinski AL, Kim JH, Manjanatha MG, Marlowe J, Pfuhler S, Pogribny I, Slikker W, Stankowski LF Jr, Tanir JY, Tice R, van Benthem J, White P, Witt KL., Thybaud V.	An ILSI-HESI workshop report. Environ Mol Mutagen	2014	国外
Human small intestinal epithelial cells differentiated from adult intestinal stem cells as a novel system for predicting oral drug absorption in humans	Takenaka T, Harada N, Kuze J, Chiba M, Iwao T, Matsunaga T.	Drug Metab Dispos	2014	国外

Histone deacetylase inhibitors promote hepatic differentiation of human induced pluripotent stem cells into hepatocyte-like cells.	Kondo Y, Iwao T, Yoshihashi S, Mimori K, Ogihara R, Nagata K, Kurose K, Saito M, Niwa T, Suzuki T, Miyata N, Ohmori S, Nakamura K, Matsunaga T.	PLoS One	2014	国外
Selective culture method for hepatocyte-like cells differentiated from human induced pluripotent stem cells	Kondo Y, Yoshihashi S, Mimori K, Ogihara R, Kanehama Y, Maki Y, Enosawa S, Kurose K, Iwao T, Nakamura K, Matsunaga T.	Drug Metab Pharmacokinet	2014	国外
An efficient method for differentiation of human induced pluripotent stem cells into hepatocyte-like cells retaining drug metabolizing activit	Takahashi S, Kamada N, Matsubara T, Gonzalez FJ, Akutsu H, Miyagawa Y, Okita H, Kiyokawa N, Toyoda M, Umezawa A, Nagata K, Matsunaga T, Ohmori S.	Drug Metab Pharmacokinet	2014	国外
Differentiation of human induced pluripotent stem cells into functional enterocyte-like cells using a simple method	Iwao T, Toyota M, Miyagawa Y, Okita H, Kiyokawa N, Akutsu H, Umezawa A, Nagata K, Matsunaga T.	Drug Metab Pharmacokinet	2014	国外
多能性幹細胞（ES細胞，iPS細胞）の利用	松永民秀，岩尾岳洋	薬剤学実験法　必携マニュアル -Pharmaceutical ScientistのためにーII 生物薬剤学	2014	国内

Definitive endoderm differentiation of human embryonic stem cells combined with selective elimination of undifferentiated cells by methionine deprivation	Tsuyama T, Shiraki N, Kume S	Human Embryonic Stem Cells, 3rd Edition	in press	国外
Hepatic differentiation from human iPS cells using M15 cells, in "iPS Cells: Generation Characterization and Differentiation -Methods and Protocols	Umeda K, Shiraki N, Kume S.	Methods Mol Biol	2014	国外
Hepatic differentiation from murine and human iPS cells using nanofiber scaffolds	Yamazoe , Shiraki N, Kume S.	Methods Mol Biol.	2014	国外
Profiling of embryonic stem cell differentiation	Shiraki N, Ogaki S, Kume S.	Rev Diabet Stud.	2014	国外
Generation of insulin-producing beta-like cells from human iPS cells in a defined and completely Xeno-free culture system	Shahjalal HM, Shiraki N, Sakano D, Kikawa H, Ogaki S, Baba H, Kume K., Kume S.	J. Mol. Cell Biol.	2014	国外
Methionine metabolism regulates maintenance and differentiation of human pluripotent stem cells	Shiraki N, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H., Kume K, Endo F, Kume S.	Cell Metab	2014	国外
ES/iP細胞を用いた内胚葉細胞（膵、肝、小腸）への分化誘導	白木伸明　粂昭苑	iPS細胞研究最前線一疾患モデルから臓器再生まで　医学のあゆみ	2014	国内
ES細胞を用いた発生分化の研究と再生医学への応用	坂野大介　粂昭苑	『特集　器官の発生と再生の基礎』　公益財団法人金原一郎医学医療振興財団(医学書院)生体の科学	2014	国内
メチオニンの代謝はヒトのES細胞およびiPS細胞の未分化維持および分化を制御している	白木伸明　粂昭苑	First Author's	2014	国内

calcium-dependent protease as a potential therapeutic target for Wolfram syndrome	Lu S, Kanekura K, Hara T, Mahadevan J, Spears LD, Oslowski CM, Martinez R, Yamazaki-Inoue M, Toyoda M, Neilson A, Blanner P, Brown CM, Semenkovich CF, Marshall BA, Hershey T, Umezawa A, Greer PA, Urano F.	Proc Natl Acad Sci U S A	2014	国外
Generation of pluripotent stem cells without the use of genetic material	Higuchi A, Ling QD, Kumar SS, Munusamy MA, Alarfaj AA, Chang Y, Kao SH, Lin KC, Wang HC, Umezawa A.	Lab Invest	2015	国外
A novel in vitro method for detecting undifferentiated human pluripotent stem cells as impurities in cell therapy products using a highly efficient culture system	Tano K, Yasuda S, Kuroda T, Saito H, Umezawa A, Sato Y.	PLoS One	2014	国外
A practical guide to induced pluripotent stem cell research using patient samples	Santostefano KE, Hamazaki T, Biel NM, Jin S, Umezawa A, Terada N.	Lab Invest	2015	国外

Ataxia telangiectasia derived iPS cells show preserved x-ray sensitivity and decreased chromosomal instability	Fukawatase Y, Toyoda M, Okamura K, Nakamura K, Nakabayashi K, Takada S, Yamazaki-Inoue M, Masuda A, Nasu M, Hata K, Hanaoka K, Higuchi A, Takubo K, Umezawa A.	Sci Rep	2014	国外
The contribution of epithelial-mesenchymal transition to renal fibrosis differs among kidney disease models	Inoue T, Umezawa A, Takenaka T, Suzuki H, Okada H.	Kidney Int	2015	国外
Notch inhibition allows oncogene-independent generation of iPS cells	Ichida JK, TCW J, Williams LA, Carter AC, Shi Y, Moura MT, Ziller M, Singh S, Amabile G, Bock C, Umezawa A, Rubin LL, Bradner JE, Akutsu H, Meissner A, Eggan K.	Nat Chem Biol	2014	国外
Stem cells bond our organs/tissues and engineering products	Toyoda M, Umezawa A.	Circ J	2014	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

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Review

Opportunities to Integrate New Approaches in Genetic Toxicology: An ILSI-HESI Workshop Report

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Genetic toxicity tests currently used to identify and characterize potential human mutagens and carcinogens rely on measurements of primary DNA damage, gene mutation, and chromosome damage *in vitro* and *in rodents*. The International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) Committee on the Relevance and Follow-up of Positive Results in In Vitro Genetic Toxicity Testing held an April 2012 Workshop in Washington, DC, to consider the impact of new understanding of biology and new technologies on the identification and characterization of genotoxic substances, and to identify new approaches to inform more accurate human risk assessment for genetic and carcinogenic effects. Workshop organizers and speakers were from industry, academe, and government. The Workshop focused on biological effects and technologies that would potentially yield the most useful information for evaluating human risk of genetic damage. Also addressed was the impact that improved understanding of biology and availability of new techniques might have on genetic toxicology practices. Workshop topics included (1) alternative experimental models to improve genetic toxicity testing, (2) Biomarkers of epigenetic changes and their applicability to genetic toxicology, and (3) new technologies and approaches. The ability of these new tests and technologies to be developed into tests to identify and characterize genotoxic agents; to serve as a bridge between in vitro and in vivo rodent, or preferably human, data; or to be used to provide dose response information for quantitative risk assessment was also addressed. A summary of the workshop and links to the scientific presentations are provided. Environ. Mol. Mutagen. 00:000–000, 2014. © 2014 Wiley Periodicals, Inc.

Key words: genetic toxicity; mutation; epigenetics; genomics

INTRODUCTION

Genetic toxicology testing of chemicals for regulatory agency approval relies on in vitro and in vivo tests that measure primary DNA damage, gene mutation, and chromosome damage for identification of potential human mutagens and carcinogens. The majority of these tests have been in use for more than 30 years. During that time, our understanding of molecular biology has increased exponentially, particularly in areas such as epigenetics, noncoding RNA, genetic structure, and mechanisms responsible for the maintenance of DNA integrity. In addition, technological advances have been made within and outside the field of genetic toxicology (e.g., 3-D tissue and organ cultures; high throughput methods; flow cytometric analyses; gene expression analysis; imaging) that could permit the measurement of multiple parameters for different effects on the genome that lead to mutation or cancer. It is recognized that many of these new procedures could be of use in developing or interpreting genetic toxicity test results, enabling a better understanding of the mechanism of action of chemicals, and improving extrapolation to potential effects in humans.

Despite these advances, the regulatory testing battery has remained relatively unchanged. Most countries require new chemicals, including drugs, pesticides, industrial chemicals, and food additives, to be tested in (1) an in vitro test for gene mutation in bacteria, (2) an in vitro cytogenetic or micronucleus assay with mammalian cells or an in vitro mammalian cell gene mutation assay, and in some cases, (3) an in vivo test for chromosomal damage using rodent hematopoietic cells. The measurement of genetic damage is frequently assessed as one of the key events in the progression of cancer, although toxicologists recognize that a wide spectrum of additional human health effects, including neurological disorders, birth defects, and mitochondrial diseases, may also result. These tests are generally not used in a quantitative manner (e.g., to provide information on relative potency) but only to provide binary, yes/no, information on the genetic hazard of the test substance. It is clear that for genetic toxicity testing to progress beyond this yes/no level, new test procedures or techniques will be needed that could bridge the gaps between in vitro data, in vivo rodent data, and human data for hazard characterization and quantitative risk assessment.

The International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI-HESI) Project Committee on the Relevance and Follow-up of Positive Results in In Vitro Genetic Toxicity (IVGT) Testing[1] was formed in 2008 to assess the current state of genetic toxicity testing, address issues related to the performance of the assays in use at that time, and evaluate new testing techniques and technologies that hold promise for improving on the predictive performance of the tests. Additionally, efforts are underway to develop new approaches for quantitative analysis of the test data [Gollapudi et al., 2013; Johnson et al., 2014] and improve hazard and risk assessment for humans.

An initial workshop was convened in May, 2008 to review and assess methods that could be used in place of, or in addition to, this test battery for identification and characterization of genetically active substances [Lynch et al., 2011]. In that workshop, a number of new and emerging in vitro and in vivo test methods were evaluated for their potential utility for incorporation into existing testing schemes.

[1]Steering committee for this Workshop: M. J. Aardema, L. Custer, B. Gollapudi, M. Honma, M. Manjanatha, S. Pfuhler, L. Stankowski, Jr., J. van Benthem, P. White, K.L. Witt, V. Thybaud, E. Zeiger.

The subsequent April 2012 Workshop[2], reported herein, was developed to consider the impact that our improved understanding of biology and new technologies might have on the identification and characterization of genotoxic substances, to define ways of bridging genetic toxicology to other disciplines, and to identify potential synergies that would result in new approaches to inform more accurate human risk assessment for genetic and carcinogenic effects. The workshop organizers identified tests and test endpoints that are being investigated by a number of laboratories and which may have promise for improving the identification of potential genetic (and epigenetic) effects, and which may be used in the future (and in most cases, upon further development) to replace or supplement the test procedures currently in use. To accomplish these goals, the Workshop brought together expertise from both within and outside the discipline of genetic toxicology, and experts in risk assessment from regulatory agencies were invited to participate and offer perspectives on how the new procedures and technologies, including some still in the research phase, might be applied to address mutagenic modes-of-action in carcinogenesis and to inform quantitative assessment of mutagenic risk.

The genetic and epigenetic effects and the technologies that were selected as topics for the Workshop were those that the Organizing Committee believed would potentially yield the most useful information relevant to human hazard and risk assessment for genetic damage. The participants were also asked to consider the impact that the improved understanding of biology and the availability the new techniques might have on genetic toxicology practices. Among the general issues discussed were the ability of these new tests/technologies to be developed into tests to identify and characterize genotoxic agents; whether the test could provide information on in vivo genetic damage or serve as a bridge between in vitro and in vivo rodent, or preferably human, data, or be used to provide dose response information for quantitative risk assessment. To help better put the new technologies into perspective, the presenters were each asked to prepare a SWOT (**S**trengths; **W**eaknesses; **O**pportunities; **T**hreats) analysis as part of their presentations, which would then be discussed in detail by the Workshop participants (see Tables (I–III)). To maximize the interaction among the Workshop participants, time for discussion was allotted after each presentation, and extended periods of discussion were provided at the end of each session.

There were 108 Workshop participants from regulatory agencies, government research laboratories, industry, and academe, including presenters. A condensed summary of the workshop along with links to key scientific presentations is presented in this report. In addition, this report also documents the attempts of the genetic toxicology community to advance the field by embracing/integrating the advances in various allied scientific disciplines over the past 30 years.

[2]Genetic Toxicology: Opportunities to Integrate New Approaches. April 24–25, 2012. Crowne Plaza Hotel, Silver Spring, Maryland. http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3596.

WORKSHOP PRESENTATIONS

Overview (V. Thybaud, Sanofi; B. Gollapudi, The Dow Chemical Co. [Cochairs])

The introductory comments to the Workshop participants provided an overview of the history of the HESI initiative, the structure of the HESI committee, including its various subcommittees and leaders, and the organizing committee, as well as the objectives of the Workshop, are available at: http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/2-IVGT-NAWkshpThybaud.pdf.

Session 1: Alternative Experimental Models to Improve Genetic Toxicity Testing (M. J. Aardema, BioReliance Corp.; S. Pfuhler, The Procter and Gamble Co.)

This session was designed to present and discuss recently developed biological models and test systems. Issues addressed included the adaptation of these models as tools for genetic toxicity testing and the best use of these models, whether as supplements or potential replacements to the genetic toxicity test systems currently in use. The relevance of these new models to animal and human exposure scenarios, metabolic capacity, etc., and their ability to inform and improve hazard identification and risk assessment decisions was evaluated.

Stefan Pfuhler (The Procter & Gamble Co.) described and discussed the use of 3-dimensional (3-D) tissue constructs for genotoxicity testing. The in vitro genotoxicity tests currently used are based on two-dimensional mammalian cell cultures. 3-D tissue constructs are logical follow-up tools for genotoxicity testing because they allow for more natural cell-cell and cell-matrix interactions, and show "in vivo-like" behavior for key parameters such as cell viability differentiation, morphology, gene and protein expression, and function. These constructs are available at various levels of complexity from simple (e.g., epidermal skin models with only one cell type) to highly complex (e.g., vascularized human liver model). 3-D skin models have been successfully established for testing for genotoxic properties of dermally applied compounds using micronucleus and Comet assays. Several liver culture systems were also presented and their potential use in genotoxicity testing was discussed. Details of this presentation can be viewed at http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/4%Pfuhler.pdf).

Seiichi Ishida (National Institute of Health Sciences) discussed the development of in vitro toxicity tests using hepatocytes differentiated from human induced pluripotent stem cells (http://www.hesiglobal.org/files/public/Commit

TABLE I. Session 1 SWOT Analyses: Alternative Experimental Models to Improve Genetic Toxicity Testing

Test/system	Strengths	Weaknesses	Opportunities	Threats
Three-Dimensional Tissue Constructs	Closer (than 2-D) to "in vivo" conditions. Technology is well-developed and 3-D models are commercially available. Cells are of human origin and function more organ-like in terms of cell viability, proliferation, differentiation, morphology, gene and protein expression. Large interest in assay, used by increasing number of laboratories in various locations. (Pre)validation exercise ongoing for 3-D skin.	More difficult to handle than 2-D. High throughput possible only for low-complexity models. Various levels of supporting data available, minimal to moderate. Used in limited number of labortories. Some models in early stage of development. Validation is needed for most models. Cost.	3-D constructs have the potential to serve as follow-up assays based on results in the traditional 2-D tests, especially when 2-D models cannot be used. Collaborative efforts recommended for relevant tissues for which such efforts are not currently ongoing (liver, lung). May be useful to bridge gap between in vitro and in vivo for quantitative risk assessment.	Validation of methods is very resource intense. Limited availability of tissue constructs. Potential issues with patents.
Stem cell-derived hepatocytes	Theoretically unlimited supply of human hepatocytes. Use of hepatocytes with different genetic backgrounds. Use of hepatocytes from patients suffering specific diseases. Simultaneous analysis of multiple drug metabolism cascade in one cell.	Complexity of differentiation process of stem cells into hepatocytes. Low reproducibility of differentiation process. Interlaboratory reproducibility needs to be defined. Early stage of development. Cost.	Replacement of human primary hepatocytes. Replacement/reduction of animal toxicity testing. Improvement of hepatotoxicity prediction. Evaluation of hepatotoxicity, prediction of metabolites, of induction of drug metabolism-related enzymes, and of drug-drug interaction. Supply of metabolites for other testing.	Complexity of differentiation process. Requirements of higher maturation of hepatocyte activities. Establishment of standards for hepatocyte qualification. Hepatocyte progenitor cell line: HepaRG. Ethical and regulatory issues if from human origin.
Humanized animal models	Allows for better characterization of human hazard and risk potential. Relevant to humans. Provides data on mode/mechanism of action. Allows comparison of rodent vs. human metabolism and toxicity pathways.	Involves animal use. Expensive to create and maintain. Low-throughput. Models human gene product in mouse environment.	Refinement of hazard and risk assessments: decreased uncertainty increased human relevance New technologies are decreasing the cost and expanding the model species. Can be used to further define "toxicity pathways" thereby facilitating development of in vitro assays based on MoA. Can be used to validate in vitro hypotheses.	Models not widely available. Models using different technologies may generate different results. Lack of acceptance of this technology for advancing human health risk assessments.

tee%20Presentations/IVGT/5-ISHIDAshorten.pdf). Pluripotent stem cells, including those from humans, have the potential to differentiate into tissue-specific primary cells, which could be used for genetic toxicity studies. Several kinds of stem cells are considered suitable. However, their use had been limited because of their availability and accessibility, and because of ethical issues regarding their source. The situation has changed since the establishment of the human iPS (induced pluripotent stem) cell line. Differentiation of iPS cells into hepatocytes could make possible an unlimited and uniform supply of a variety of cells with different genetic backgrounds.

Darrell Boverhof (The Dow Chemical Co.) described the construction and use of humanized animal models (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/6-Humanized_Models-DRB-FINAL.pdf).

Humanized models can be defined as animal models that carry functioning human genes, cells, tissues, or organs, and include transgenic animals that express human genes or immunodeficient animals that have been engrafted with human cells or tissues. The models have been applied in research areas such as hematopoiesis, innate and adaptive immunity, autoimmunity, infectious disease, cancer biology, regenerative medicine, pharmacology, and toxicology. Two applications are humanized metabolizing enzyme models and nuclear receptor models. These, and other, models have provided valuable insights into human-specific responses and modes of action that can be used to decrease uncertainties in human hazard and risk assessments. Although these models have traditionally been limited to the mouse, new technologies are facilitating the development of models in other species.

Session 2: Biomarkers of Epigenetic Changes and Their Applicability to Genetic Toxicology (B. Gollapudi, The Dow Chemical Co.; V. Thybaud, Sanofi)

It is generally recognized that epigenetic changes to the DNA, while not causing a change in the nucleotide composition or sequence, can lead to heritable phenotypic changes, including heritable changes in the newborn. This session was organized to discuss the potential interplay between chemically induced alterations to the genome (genetic toxicity) and changes to the epigenome leading to or exacerbating heritable effects. This session considered screening systems that can be designed to identify "epimutations" and to evaluate the potential for their transgenerational inheritance, and whether new, less invasive and hopefully transspecies biomarkers of exposure, susceptibility, and effect can be identified in order to contribute to the risk assessment process.

Peter Dedon (Massachusetts Institute of Technology) described studies on the dynamic reprogramming of tRNA modifications and their effects on the stress response (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/8-DedonHESIGentoxWorkshop.pdf). Complex interactions between tRNA, mRNA, and ribosomes control the rate and fidelity of translation. Contributing to this are the large numbers of genes encoding tRNAs, rRNA and proteins, in addition to different ribonucleoside modifications. The modified ribonucleosides in tRNA behave as a system, and are reprogrammed in response to different types of cell stress. When cells are exposed to toxicants there are dynamic shifts in the population of RNA modifications as part a step-wise mechanism of cell responses to the chemical stresses and damage, with signature changes for each agent and for different doses of each agent. These responses involve reprogramming of tRNA modifications that leads to reprogramming of ribosome structure in the translational control of cellular stress responses.

Jennifer Marlowe (Novartis) described studies of the potential use of epigenetics in toxicology (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/9-IVGT%20meeting_24Apr2012_JMarlowe_Final.pdf). Some of the earliest events preceding the development of overt pathologies, including those arising from exposure to environmental and pharmaceutical agents, involve perturbations of the epigenome. The application of epigenomic profiling technologies to drug safety sciences has potential for providing novel insights into the molecular basis of long-lasting cellular perturbations. Genome-wide epigenomic and transcriptomic profiling, combined with molecular histopathology, are valuable components towards investigation of temporal sequences of events, and therefore mechanistic understanding of the toxicity response. Integrated molecular profiling that can be performed includes analyses of mRNA, microRNA, DNA methylation, and proteins. The mapping of epigenomes from humans and other species will enhance our ability to interpret the biological significance of xenobiotic-induced epigenetic perturbations, and may provide novel biomarkers for susceptibility to adverse events.

Igor Pogribny (US Food and Drug Administration) addressed epigenetic traits as biomarkers of carcinogenesis (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/10-Pogribny_ILSI_2012.pdf). Numerous epigenetic responses that may be used as potential biomarkers for the molecular diagnosis of cancer and prognosis for survival or treatment outcomes have been identified. Epigenetic alterations may be used as early indicators in the evaluation of the carcinogenic potential of both genotoxic and nongenotoxic substances. Incorporation of epigenetic biomarkers into cancer risk assessment holds a number of advantages over traditionally used methods, such as evaluation of DNA damage, DNA adduct formation, or bacterial mutagenicity. These advantages include their earlier appearance, stability, target tissue-specificity, and applicability to both genotoxic and nongenotoxic agents. In addition, a greater number of detectable epigenetic changes than detectable genetic alterations are present after exposure to the insult.

Andrea L. Kasinski (Yale University) described studies on the use of microRNAs (mRNAs) for treatment of lung cancers (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/11–042412Kasinski.pdf). Because they are endogenous substances, toxicities associated with mRNA use are expected to be minimal. Different delivery agents for these regulatory molecules are being investigated, and studies are ongoing to better understand their therapeutic potential, notably in the context of cancer. Targeted therapies have shown encouraging results for subsets of lung cancer patients. Likewise, gene therapies using retrovirus and replication-impaired adenoviruses to reintroduce the tumor suppressive protein p53 have had compelling results, including tumor regression and stabilization.

TABLE II. Session 2 SWOT Analyses: Biomarkers of Epigenetic Changes and their Applicability to Genetic Toxicology

Test/System	Strengths	Weaknesses	Opportunities	Threats
Epigenetic control of cell phenotype	Assay is predictive of chemical exposures, including genotoxins. Analysis of RNA modification spectra provides insights into mechanisms of toxicity. Possible complementation to transcriptional and proteomic data for identifying genotoxic and nongenotoxic carcinogens. Relatively straightforward method for practitioners of analytical chemistry.	Analysis of RNA modification spectra in very early stages of development. Very limited data for toxicants. Relatively specialized method at this point. Probably not entirely predictive of carcinogenicity of a chemical or drug candidate. Interlaboratory validation will be needed.	Possible coordination of RNA modification analysis with other 'omic data sets. Application to in vitro screening for hazard assessment. Provides insights into mechanisms of action of toxicants. Possible biomarkers that are specific for non-genotoxic carcinogens. Allow distinction between genotoxic and nongenotoxic carcinogens.	Analysis of RNA modification reprogramming is in the earliest stages of scientific acceptance and application. Translation to a high-throughput method. Relating RNA modification patterns to specific toxicities.
Epigenetics	Provides comprehensive view of modifications or changes that may precede events that lead to phenotypic change or toxicity. Rich biomarker resource. Potential for assessment of species-specific responses in vivo and in vitro.	Large and complex datasets; need for customized bioinformatic tools. Huge number of changes observed many possible. mechanisms/outcomes High probability for nonspecific or irrelevant responses. Distinguishing stress responses from chemical-specific responses.	Biomarkers for use in acute and subchronic toxicity studies. Early prediction of genotoxic risk; inform or replace 2-year bioassay. Drug-induced vs. non-drug-induced tumors. Provide or support carcinogen mode-of-action determinations. May allow identification of preneoplastic changes.	Tremendous complexity with possibility of compound-specific effects rather than class-effects amenable to generalized biomarkers generation. Translation of findings from preclinical models/results to humans.
Epigenetic traits as biomarkers of carcinogenesis	Early appearance of epigenetic carcinogen-alterations. Target tissue specificity. Specificity for genotoxic and nongenotoxic carcinogens.	Cellular epigenome individual and dynamic variability not completely characterized. Models for safety assessment not yet developed. Endpoints and techniques for use in safety assessment not established. Interlaboratory reproducibility not determined.	Early prediction and identification of hazardous compounds before their dissemination into environment. Provide or support carcinogen mode-of-action determinations.	Complexity of cellular epigenomic landscape and epigenetic regulatory mechanisms. Correlating epigenomic changes between test animal species and humans.
MicroRNA analysis with lentivirus platform	This analysis provides data to progress towards more clinically relevant therapeutic applications. The lentivirus system used is commercially available and serves as a proof-of-concept to move forward.	Lentivirus platform explored may not be the best platform for human patient delivery.	Provides background for developing and evaluating better and more clinically relevant miRNA delivery mechanisms.	Lack of approval for use of lentivirus in humans for ethical reasons. Advancement of better, clinically relevant delivery agents needed. Multiple companies on board with the development phase. Patent protection and confidentiality agreements limit access.

Session 3: New Technologies and Approaches (M. Manjanatha, US Food and Drug Administration; K. L. Witt, National Institute of Environmental Health Sciences)

This session was organized to present technology-driven approaches to assessing genetic toxicity, including the potential mode-of-action leading to the effect. There are a number of new initiatives and advances in the field of toxicology, ranging from high resolution, high content imaging assays to high throughput approaches that allow the profiling of thousands of chemicals in multiple, diverse assays. It is important from both scientific and strategic points of view that genetic toxicologists understand the implications

TABLE III. Session 3 SWOT Analyses: New Technologies and Approaches

Test/system	Strengths	Weaknesses	Opportunities	Threats
Imaging for safety assessment	Longitudinal study design and minimal invasiveness. Acceptance of imaging by the medical community enhances its utility as a translatable pre-clinical biomarker. Can be highly quantitative. Minimally to noninvasive. The test animal or human subject can serve as its own control.	No standardized approach. Deficits in quantization. A powerful technology looking for an application for genetic toxicology and carcinogenesis.	Broad applications due to recent advances in resolution and standardization of approaches. Identification of early toxic or precarcinogenic changes in situ.	Expensive technology. Not universally available.
Tox21 strategy for detecting genotoxicants	Capability to test thousands of chemicals in human, or other species, cells. Very small amounts of compound needed for screening. Rapid generation of test data. Eliminates or greatly reduces use of animals. Large number of cell lines can be used. Provides MOA information.	High throughput screens currently lack means to provide bioactivation. Limited number of suitable genotoxicity assays currently available. Tested compounds limited to those that are DMSO-soluble, nonvolatile, stable in solution for a period of time, etc. No or limited, evaluation of metabolites. Expensive. Data processing complexity heavily software dependent.	Ability to develop approaches to assess differential susceptibility on a wide-spread scale. Ability to identify susceptible subpopulations using genomic assays. Ability to develop comprehensive profiles of chemical activity. Ability to develop SAR and chemical clustering models based on chemical activity. Potential to reduce the number of animals needed for toxicity testing.	Challenges in anchoring chemical profiles for genotoxicity-associated activity generated with these technologies to results from traditional tests for genotoxicity with sufficient accuracy to be acceptable to regulatory agencies. Biological relevance and reliability of the selected assays for risk assessment. Reproducibility across cell lines.
Genomic Signatures of Genotoxicity	Query large swaths of biological space in one assay. Provides pathway/mechanistic information.	In vivo genomics dependent on animals. Ability to interpret complex data. Variability (time-dependent, platforms, dose). Lack of mechanistic anchoring (need more correlative data).	High dimensionality allows for querying of large amount of biological space including genotoxicity and beyond (i.e., one assay to query all). Potential to rapidly identify a no-effect dose. Potential to reduce the numbers of animals needed to characterize toxicities.	Metrics (i.e., genes and pathways) are different from traditional endpoints. If it is not possible to change metrics (i.e. pathology to pathways), it will significantly hinder development in the regulatory arena. Reproducibility of results across testing platforms and between laboratories.

and impact of these new technologies and embrace those approaches that hold the greatest promise for improving genetic toxicity hazard and risk assessment.

William Slikker (US Food and Drug Administration) addressed new imaging techniques that could be used in safety assessment of chemicals (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/14-Slikker ImagingGenetoxIVGTconference041512.pdf). Although the use of imaging in the clinical setting is well established, the use of imaging for preclinical assessments is infrequent. Positron Emission Tomography (MicroPET) can be used to assess any target organ and deliver quantitative information in a minimally invasive manner and in parallel with other endpoint requirements. One advantage to this approach is that the animal can be used as its own control. A number of dose-response studies were performed of CNS responses to anesthetic drugs. The data from these studies support the concept that imaging is a viable approach for assessing nervous system status by serving as a minimally invasive and translatable biomarker. Similar imaging techniques may find use in the identification of precarcinogenic changes in organs of animals treated with carcinogenic chemicals or subject to cancer-inducing stress conditions.

Raymond Tice (National Institute of Environmental Health Sciences) described the Tox21 interagency (NIEHS, NHGRI, EPA, and FDA) studies on the responses of ~100 quantitative high throughput screens (qHTS), predominantly reporter gene

assays using a 1536-well format on an automated platform (Phase I) (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/15-Tice-ILSI-HESI_GeneTox.pdf). Among the qHTS assays were several that related to the detection of activities associated with some aspect of genotoxicity. Phase II of the program screened a 10,000 compound library against a set of nuclear receptor and stress response pathway assays, to detect potentially genotoxic compounds. Confirmation of genotoxicity of a small set of selected compounds will be performed using standard in vivo tests. The lack of xenobiotic metabolism in the qHTS assays is a major limitation, so protocols that use metabolically competent cells are being developed to address this limitation. Data from these assays will be compared against existing in vitro genotoxicity data. All the data generated through Tox21 are available through a variety of publicly accessible databases.

Scott Auerbach (National Institute of Environmental Health Sciences) discussed the effect of dose level and exposure duration on genomic signatures in response to chemical exposure (http://www.hesiglobal.org/files/public/Committee%20Presentations/IVGT/16-Auerbach-IVGT-2012-3.pdf). One potential approach to the problem of setting protective exposure limits for toxic responses is the use of in vivo toxicity testing coupled with toxicogenomic dose-response analysis. Typically, the most important piece of information acquired from these studies is the benchmark dose (BMD). A systematic assessment of no-observed-effect levels indicates that they change minimally going from subacute to chronic exposures. The application of genomics allows for a broad-based query of biological perturbations that is in line with the goals of safety testing, as it is an agnostic query of nearly all the possible changes at the level of the transcriptome. Results from studies of specific agents suggest that, depending upon the mode of action, genomic signatures can identify less sensitive, in the case of genotoxic agents, or more sensitive, in the case of nongenotoxic agents, BMD values when compared with the values from tumor response data.

DISCUSSION

The disciplines of genetic toxicology and genetic toxicity testing were originally designed and developed for hazard identification. This was based on the presumption that identification of genotoxic substances would allow society to avoid or minimize exposure to agents that induce mutations in somatic and germ cells, and thereby contribute to the efforts to prevent cancer formation and other adverse effects in exposed and future generations. These uses of genetic toxicity data have not changed significantly for more than 30 years. The identification of substances posing a genetic hazard would also contribute to the risk assessment and risk management decisions.

For the field of genetic toxicity testing to move beyond the traditional test endpoints and binning of test responses into "positive" and "negative" it will be necessary to either improve the existing assays or apply new approaches used in other biology domains, and to "think outside of the box." These new approaches would employ new experimental models and technologies and incorporate our growing understanding of the relevant biological mechanisms responsible for the development of a mutated cell and its contribution to adverse health effects (e.g., cancer).

This Workshop addressed how the new scientific discoveries and methodologies could allow for more effective and relevant screening of chemicals and other environmental substances for mutagenic risk and resulting health issues. There are a large number of test methods, testing approaches, and technologies that can potentially address these issues. However, because of time and other constraints, only a few could be addressed at the Workshop, and the lack of inclusion of other biological endpoints and techniques was not meant to imply that they would be less useful. Included among the methods discussed in the Workshop were those that measured the traditional genetic endpoints (e.g., micronuclei, DNA and chromosome breaks, and gene mutation), but more efficiently or in cell, tissue, or animal constructs that are deemed to be more relevant to human exposure than the traditional test systems. The integration of new, nontraditional methods concentrated on the relevance and use of epigenetic changes as biomarkers of heritable genetic damage or cancer without the underlying change in DNA sequence. In addition, new detection techniques amenable to high-throughput screening, and noninvasive in vivo approaches, were presented. It was recognized that not all of the models addressed at the workshop appeared to be directly applicable to genetic toxicology or cancer initiation. However, the structure of the workshop and the presentations served as much to introduce the researchers and developers to the issues and needs of genetic toxicology, as they did to introduce the genetic toxicologists to the newly emerging models.

Although there were a number of discussions and suggestions of how the various test procedures might be used as adjuncts or alternatives to the current test guidelines, it would have been premature to make such recommendations, given the experimental nature or lack of validation of some of these procedures. As a result, no specific recommendations were made with regard to the future use or role of these procedures or whether they should, or could, be integrated into the existing testing schemes or replace currently used methods. It was clear that, in the future, genetic toxicology scientists will have to use more complex, often patented, commercially available models and technologies.

The Workshop participants identified issues and knowledge gaps that will require more work and resources, and that would best profit from collaborations between genetic toxicologists and the new technology researchers. The conclusions of the presenters and the discussions during

and at the end of the Workshop are presented in the SWOT analyses (see Tables (I–III)) developed for the individual test approaches and assays and which were agreed to be a useful approach for evaluating the potential suitability of a process or test procedure.

Remaining issues and knowledge gaps identified by the Workshop included:

- The relevance of surrogate models, and the ability of these models to mimic human cells and whole body complexity, with respect to DNA repair, metabolic activation, and genetic stability;
- The relevance of the various identified end-points for human risk assessment and the ability to extrapolate the results from these models to humans based on protective mechanisms and genetic polymorphisms, including the identification of appropriate safety and uncertainty factors;
- The need to consider all key events, including epigenetic factors, that might contribute to DNA damage or modification and affect the organism's response to these DNA effects and DNA integrity;
- Whether and how multiparametric and more integrated test methods can help improve our understanding of genotoxic modes of action.

The presentations and discussions at this Workshop have helped to stimulate ongoing efforts to develop and refine additional methods of identifying genotoxicants and will provide valuable information for the future paradigm of genetic toxicology testing. An ongoing activity of the ILSI/HESI IVGT (now named the Genetic Toxicology Technical Committee) is to start from a "clean sheet" to determine what a new and improved testing strategy for the identification and evaluation of genotoxicants might look like, based on both the current practices, and on new and developing approaches. This new testing strategy would be developed to cover all aspects of genomic damage, incorporate the advances in systems biology, be relevant to human risk assessment and resource efficient, and move away from a standard battery approach to a more flexible testing strategy.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

The workshop organizing committee members (M.J.A., L.C., B.G., M.H., M.M., S.P., L.F.S. Jr., J.vB., P.W., K.L.W., and V.T.) were responsible for the development of the workshop, selection of the speakers, chairing the various sessions, and writing the section descriptions. The speakers (S.A., D.B., S.I., A.L.K., J.M., S.P., W.S., and R.T.) contributed expanded abstracts of their talks. ILSI/HESI staff members (J.K., and J.Y.T.) were responsible for the organization of the workshop and coordination of the writing assignments. All authors contributed to the final wording of the manuscript and in addressing the internal reviewers' comments.

Disclaimer: This document represents the consensus of the participants' views expressed as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

Conflicts of Interest: M.J.A. and L.F.S., Jr. are employed by BioReliance Corporation, which offers genetic toxicology testing services on a contract basis.

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Definitive Endoderm Differentiation of Human Embryonic Stem Cells Combined with Selective Elimination of Undifferentiated Cells by Methionine Deprivation

Tomonori Tsuyama, Nobuaki Shiraki, and Shoen Kume

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Abstract

Human embryonic stem cells (ESCs) show a characteristic feature in that they are highly dependent on methionine metabolism. Undifferentiated human ESCs cannot survive under the condition that methionine is deprived from culture medium. We describe here a procedure for definitive endoderm differentiation from human ESCs, in which human ESCs are subject to 10 days (d) differentiation combined with methionine deprivation between differentiation day (d) 8 to d10. Methionine deprivation results in elimination of undifferentiated cells from the culture with no significant loss of definitive endoderm cells, as compared to those cultured under complete condition throughout the whole culture period.

Keywords: Methionine deprivation, Endoderm differentiation, M15 cells, Selective elimination, Undifferentiated cells, Metabolic difference

1 Introduction

Human embryonic stem cells and/or induced pluripotent stem cells (hESCs/iPSCs) have been proposed as a cell source for drug discovery and cell replacement therapy. To this end, we established several protocols for stepwise differentiation of hESCs/iPSCs towards the definitive endoderm lineage (namely, the pancreas, liver, and intestine) (1–8).

One concern with hESCs/iPSCs-based approach is the differences in differentiation potentials among cell lines (9, 10). Certain cell lines are refractory to definitive endoderm differentiation and a substantial population of undifferentiated cells remain although other cell lines achieve high efficiencies of endoderm differentiation with the same differentiation protocol. The persistence of undifferentiated cells is undesirable because these cells might be inhibitory for further differentiation and might form tumor after transplantation.

Human ESCs/iPSCs are in a higher flux methionine metabolism, compared to definitive endoderm (11). Utilizing this metabolic difference, we developed a protocol to eliminate the remaining undifferentiated cells (11).

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This protocol is based on our previous culture system (coculture with M15 feeder cells in RPMI supplemented with 100 ng/ml activin A for 10 d) (4) with a slight modification in using methionine-deprived medium from d8 to d10. A protocol without using M15 cells is also described. 37 38 39 40 41

2 Materials 42

1. M15 cells (ECACC cell no. 95102517). 43 AU2
2. Culture Dish and Plate (60-mm, BD falcon, 353004)(90-mm, Nunc, 150350)(150-mm, Nunc, 168381)(96-well plate, Corning, 3595). 44 45 46
3. PBS (*see* **Note 1**). 47
4. 0.05 % trypsin/EDTA (Invitrogen, 25300-062), 0.25 % Trypsin/EDTA (Invitrogen, 25200-072). 48 49
5. EF medium. 50

DMEM (Invitrogen, 11995-075)	500 ml	51
FBS (Hyclone)	58 ml	52
Penicillin and streptomycin (PS: Nacalai tesque, 26252-94) (*see* **Note 2**)	5.8 ml	53 54
L-Glutamine (L-Gln; Nacalai tesque, 16948-04) (*see* **Note 2**)	5.8 ml	55

6. 2× Freeze solution. 56

EF medium	28 ml	57
DMSO (Sigma, D2650)	10 ml	58
FBS (Hyclone)	2 ml	59

7. Mitomycin C solution. 60
   Dissolve mitomycin C (2 mg, Sigma, M4287) in 2 ml PBS. 61
8. Mitomycin C-containing medium. 62

EF medium	200 ml	63
Mitomycin C solution (Final concentration: 10 μg per ml)	2 ml	64

9. Human ESCs medium. 65

Reproff (ReproCELL, RCHEMD004)	500 ml	66
PS (Nacalai Tesque, 26252-94) (*see* **Note 2**)	5 ml	67

10. Supplements for human ESCs medium. 68
    bFGF (Peprotech, 100-18B-2). 69

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Stock solution at 5 μg/ml in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μl and store at −80 °C. Once thawed, keep at 4 °C. Add to human ESCs medium at a final concentration of 5 ng/ml. 70 71 72

11. Y27632 (Wako, 253-00513) (*see* **Note 6**). 73
12. Matrigel (BD, 354234) (*see* **Note 7**). 74
13. Endoderm Differentiation basal Medium 1 (store at 4 °C). 75

RPMI 1640 medium (Invitrogen, 11875-093)	500 ml	76
PS (Nacalai tesque, 26252-94) (*see* **Note 2**)	5 ml	77
L-Glutamine (Nacalai tesque, 16948-04) (*see* **Note 2**)	5 ml	78
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5 ml	79
0.1 M ME (*see* **Note 3**)	500 μl	80

14. Endoderm Differentiation Basal Medium 2 (store at 4 °C). 81

Methionine-deprived medium (Ajinomoto, provided upon requests) (or Cell Science & Technology Institute, a custom-made medium)	500 ml	82 83 84
PS (Nacalai tesque, 26252-94) (*see* **Note 2**)	5 ml	85
L-Gln (Nacalai tesque, 16948-04) (*see* **Note 2**)	5 ml	86
NEAA (Invitrogen, 11140-050) (*see* **Note 2**)	5 ml	87
0.1 M ME (*see* **Note 3**)	500 μl	88

15. Supplements for endoderm differentiation Medium (store at 4 °C). 89 90
Activin (R&D, 338-AC). 91
Stock solution at 100 μg/ml in 0.1 % (w/v) BSA/PBS. Aliquot into 100 μl and store at −80 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 100 ng/ml. 92 93 94 95
B27 supplement (Invitrogen, 17504-044). 96
Stock solution at 100 % (50×). Aliquot into 500 μl and store at −20 °C. Once thawed, keep at 4 °C. Add to endoderm differentiation medium at a final concentration of 2 % (v/v, 1×). 97 98 99

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3 Methods 100

3.1 Preparation of Mitomycin C-Treated M15 Cells (MMC-M15 Cells)

3.1.1 Thawing M15 Cells

1. Put a vial of frozen M15 stock into 37 °C water bath until most cells are thawed. 101 102
2. Transfer cell suspension to 15 ml tube pre-added with 4 ml EF medium. 103 104
3. Spin down at 180 × *g* for 5 min. 105
4. Resuspend the pellets with 10 ml EF medium 106

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5. Transfer cell suspension into 90-mm dish 107
6. Incubate at 37 °C under 5 % CO_2. 108

3.1.2 Passage of M15 Cells

1. At full confluence, remove the medium. 109
2. Rinse with PBS. 111
3. Add 0.05 % trypsin/EDTA (1 ml per 90-mm dish, 3 ml per 150-mm dish) and incubate for 5 min at 37 °C under 5 % CO_2. 112 113
4. Add EF medium into the M15 cells dish (4 ml per 90-mm dish, 6 ml per 150-mm dish), suspend cells by gently pipetting, and transfer cell suspension into 15 ml tube or 50 ml tube. 114 115 116
5. Spin down at 180 $\times g$ for 5 min. 117
6. Resuspend the pellets with an appropriate amount of EF medium. 118 119
7. Seed the cells at 1.5 $\times 10^6$ cells per 150-mm dish 120
8. Incubate at 37 °C under 5 % CO_2 until they reach confluence. 121

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3.1.3 Mitomycin C-Inactivation of M15 Cells

1. Remove the medium. 123
2. Add mitomycin C-containing medium, and incubate for 2 h at 37 °C under 5 % CO_2. 124 125
3. Remove the mitomycin C-containing medium. 126
4. Rinse with PBS twice 127
5. Add 3 ml of 0.05 % trypsin/EDTA and incubate for 5 min at 37 °C under 5 % CO_2. 128 129
6. Add 5 ml EF medium, suspend cells by P1000 pipetting, and transfer cell suspension into 50-ml tubes. 130 131
7. Spin down at 180 $\times g$ for 5 min. 132
8. Resuspend the pellets with EF medium to the concentration at 2 $\times 10^7$ cells/ml. 133 134
9. Add equal volume 2$\times$ freeze solution and mix gently. 135
10. Transfer 1 ml of cell suspension into cryovials. 136
11. Put cryovials into Nalgene controlled-rate freezer box and then put the box into −80 °C freezer. 137 138
12. On the next day, transfer the vials of frozen MMC-M15 cells into −150 °C freezer for long-term storage. 139 140

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3.2 Preparation of Gelatin-Coat Plates

1. Add 50 μl of 0.1 % gelatin solution into 96-wells plate. 142
2. Incubate at 37 °C for 2 h (*see* **Note 4**). 143
3. Remove gelatin solution. 144
4. Add 100 μl EF medium into 96-well gelatin-coated plate. 145

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