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Assessing Human Germ-Cell Mutagenesis in the Postgenome Era: A Celebration of the Legacy of William Lawson (Bill) Russell

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Abstract

Birth defects, de novo genetic diseases, and chromosomal abnormality syndromes occur in ~5% of all live births, and affected children suffer from a broad range of lifelong health consequences. Despite the social and medical impact of these defects, and the 8 decades of research in animal systems that have identified numerous germ-cell mutagens, no human germ-cell mutagen has been confirmed to date. There is now a growing consensus that the inability to detect human germ-cell mutagens is due to technological limitations in the detection of random mutations rather than biological differences between animal and human susceptibility. A multidisciplinary workshop responding to this challenge convened at The Jackson Laboratory in Bar Harbor, Maine. The purpose of the workshop was to assess the applicability of an emerging repertoire of genomic technologies to studies of human germ-cell mutagenesis. Workshop participants recommended large-scale human germ-cell mutation studies be conducted using samples from donors with high-dose exposures, such as cancer survivors. Within this high-risk cohort, parents and children could be evaluated for heritable changes in (a) DNA sequence and chromosomal structure, (b) repeat sequences and minisatellites, and (c) global gene expression profiles and pathways. Participants also advocated the establishment of a bio-bank of human tissue samples from donors with well-characterized exposure, including medical and reproductive histories. This mutational resource could support large-scale, multiple-endpoint studies. Additional studies could involve the examination of transgenerational effects

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associated with changes in imprinting and methylation patterns, nucleotide repeats, and mitochondrial DNA mutations. The further development of animal models and the integration of these with human studies are necessary to provide molecular insights into the mechanisms of germ-cell mutations and to identify prevention strategies. Furthermore, scientific specialty groups should be convened to review and prioritize the evidence for germ-cell mutagenicity from common environmental, occupational, medical, and lifestyle exposures. Workshop attendees agreed on the need for a full-scale assault to address key fundamental questions in human germ-cell environmental mutagenesis. These include, but are not limited to, the following: Do human germ-cell mutagens exist? What are the risks to future generations? Are some parents at higher risk than others for acquiring and transmitting germ-cell mutations? Obtaining answers to these, and other critical questions, will require strong support from relevant funding agencies, in addition to the engagement of scientists outside the fields of genomics and germ-cell mutagenesis.

Keywords

inherited disease; mutation detection; molecular genetic analysis; human genetic risk

INTRODUCTION

The genetic heritage of the human species resides in our germ cells (egg and sperm), and it is our collective stewardship responsibility to protect the genomic integrity of germ cells to assure that today's societal actions do not increase the burden of abnormal pregnancies and genetic diseases for future generations. However, we are faced with a fundamental mystery – to date, no chemical or radiation has been confirmed as a human germinal mutagen. Decades of animal research have shown over 30 chemicals and ionizing radiation to be potent germ-cell mutagens in mice [Russell et al., 1981,1998;Shelby et al., 1993]. Recent studies have shown clearly that certain medical exposures can induce chromosomal mutations in human sperm [Marchetti and Wyrobek, 2005]. Data from these studies have created a new consensus that this long-standing mystery is due to limitations of the old technologies in detecting random de novo human germ-cell mutations rather than a special human biological resistance to environmentally induced germ-cell mutations.

The biology of gamete development differs substantially from somatic tissue development, negating the ability to effectively extrapolate effects induced by environmental exposures from somatic to germ cells. There is a wealth of data on mutation induction in somatic cells of rodents and humans [Hsie et al., 1981;Li et al., 1988;Albertini et al., 1993;Albertini, 1994;Seifried et al., 2006]; however, these data cannot readily be used to assess mutational risk of human germ cells due to the unique biological characteristics of human germ cells relative to those of other mammals or to somatic cells of either humans or other mammals [Bishop and Witt, 1995;Witt et al., 2003]. Thus, to accurately assess the impact of environmental mutagens on the genomic integrity of human germ cells, there is growing consensus that studies must be conducted directly in germ cells, and preferably in human populations [Adler, 1996].

Eighty years ago, Herman Muller [1927] discovered ionizing radiation as the first germ-cell mutagen in an animal species (*Drosophila*). Subsequent pioneering studies in rodent model systems (primarily male mice) have found numerous physical and chemical mutagens that can induce heritable mutations, i.e., mutations in the germ cells that are transmitted to offspring [Russell et al., 1981,1998;Shelby et al., 1993;Bishop and Witt, 1995;Witt and Bishop, 1996;Bishop, 2003]. Despite the similarities in many aspects of germ-cell biology among humans and mammalian model systems, four separate studies of human radiation or chemical exposures have failed to prove the occurrence of environmentally induced germ-cell mutations in humans. Beyond the unlikely possibility that there are no human germ-cell mutagens, several

explanations for this disparity between human and animal results have been suggested. These include insufficient numbers of human subjects; insensitive methods to detect mutations; multiplicity of DNA and chromosomal defects induced by individual mutagens; inadequate length, intensity, or type of exposure; inadequate dosimetry; and inadequate technology for detecting random de novo mutations in the human genome.

Biology is undergoing a major technological revolution as a consequence of the Human Genome Project (HGP) and its animal corollaries. Recent advances in genomic analysis technologies provide a new set of sensitive tools for finding human germ-cell mutations and for assessing the risks of environmental exposures. With this in mind, a workshop entitled “Assessing Human Germ-Cell Mutagenesis in the Postgenome Era” was organized at The Jackson Laboratory in Bar Harbor, Maine on September 28–30, 2004 to foster a dialogue among scientists in the fields of germ-cell mutagenesis and genomics, and to identify methods to be used in collaborative efforts to detect human germ-cell mutagens. While planning this conference, one of the pioneers in the field of mammalian germ-cell mutagenesis, Dr. William L. Russell, passed away, and we dedicated this workshop to his memory and the legacy of his research in this field.

This report summarizes the presentations and discussions of a multidisciplinary group of scientists with expertise in germ-cell biology, on the evidence for environmentally induced de novo human germ-cell mutations, and on the new technologies for detecting genomic changes. At the end of this report, we summarize the workgroup recommendations for research strategies and technological needs for detecting de novo germ-cell mutations in humans and for assessing the heritable risks associated with exposure to environmental mutagens.

This workshop identified the importance of large-study collaborations among scientists in the fields of genomics, biotechnology, bioinformatics, reproductive biology, and germ-cell mutagenesis. Realizing that it has been 80 years since the discovery of the first germ-cell mutagen in *Drosophila*, the participants voiced the urgent need to move ahead with collaborative efforts of human cohorts with well characterized mutagen exposures. Furthermore, it is important to gain support of both scientists and funding agencies for this effort so that society will not have to wait another 80 years to answer these questions: Are there any human germ-cell mutagens, what risks do they pose to future generations, and are some parents more susceptible than others for acquiring and transmitting germ-cell mutations?

KEYNOTE ADDRESS

Liane B. Russell, Oak Ridge National Laboratory

In the opening address, Liane Russell gave an overview of the pioneering work of her late husband, Bill Russell [Russell, 2004]. Bill Russell held an early fascination with phenotypic variation within inbred strains of mice, which led to his development of the technique of ovarian transplantation to study the influence of the prenatal environment on phenotypic variability among genetically uniform mice. He also developed the mouse specific-locus test, which allowed quick and objective detection of visible recessive mutations among any of seven gene loci. Russell utilized the specific-locus test to characterize the effects of different qualities of radiation, total dose, dose protraction, and dose fractionation. His results with dose protraction provided early evidence that certain germ cells could repair premutational damage [Russell and Russell, 1992]. Russell and coworkers explored the roles of sex, parity, age, and (most importantly) germ-cell stage on mutation rate and type [Russell, 2004]. Later in his career, Russell pioneered the application of the specific-locus test to study the induction of germ-cell mutations by chemicals. These studies led to what is perhaps one of the most important legacies of his work in germ-cell mutagenesis—his discovery that ethylnitrosourea (ENU) is a highly potent germ-cell mutagen in the mouse [Russell, et al., 1979]. Because ENU induces primarily

single base-pair changes, it has become a highly valuable tool for gene discovery in laboratories around the world [Michaud et al., 2005].

By analyzing the nature of mutations detected in the specific-locus test, the Russells found that, although each chemical had its own pattern for frequency of mutations induced in the different germ-cell stages, all chemicals were similar in yielding large lesions (e.g., deletions, inversions) from exposure of postspematogonial stages but generally not from exposures of either differentiating or stem cell spermatogonia. For ENU, which induces primarily single base-pair changes in spermatogonia, the locus spectrum roughly parallels the length of the various coding regions. Locus-spectra of spontaneous mutations, many of which were shown to originate during the perigametic interval, differ from treatment-induced spectra of mutations [Russell, 2004]. Liane Russell concluded with a discussion of future prospects in germ-cell mutagenesis research, expressing the hope that new genomics-based molecular tools would further the understanding of germ-cell biology and mutagenesis in mice and in humans, thereby enhancing the approaches used for the estimation of germ-cell risk.

SESSION 1: HISTORICAL PERSPECTIVES AND IMPACT OF GENOME PROJECT

Session Chair: John S. Wassom, YAHSGS LLC and Oak Ridge National Laboratory

John Wassom focused his presentation on the central question in the field of germ-cell mutagenesis: Do environmental agents induce heritable mutations in humans? He summarized the scientific evidence published on this topic from 1960 to the present [Wassom, 1989; Brusick, 1990; Neel and Lewis, 1990; Hoffmann, 2004; Malling, 2004]. The contrast between the strong positive evidence from animal germ cells and the indefinite evidence for humans [Shelby, 1994], he said, was a primary motivation for the workshop. Wassom discussed the recent evidence implicating particulate air pollution as a cause of germ-cell mutations in mice [Samet et al., 2004; Somers, 2006] and referenced the finding of germline mutations at minisatellite loci in children born to parents living in heavily polluted areas of the Mogilev district of Belarus after the Chernobyl accident [Dubrova et al., 1996, 2002]. Wassom echoed Liane Russell in calling for the development and application of new strategies based on sensitive genomic analyses to identify and characterize environmental agents with potential for inducing heritable mutations in humans. He emphasized that even though it has not been proven conclusively that an agent can induce heritable changes in the genetic material of human germ cells, the time and resources are now at hand to initiate intense study on this issue as witnessed by the work cited previously on minisatellite mutations and work showing increased frequencies of chromosomal aberrations in human sperm following exposures to radiation or chemotherapeutic agents [Marchetti and Wyrobek, 2005]. The advances from the HGP (1990–2003) upon which new technologies will be based have, he said, already had a major impact on research in other areas in the life sciences. In closing, Wassom noted that many of the new technologies were, in fact, on the agenda for the current workshop. Organizers hoped that the information presented would stimulate new ideas for experimentation and collaborations and set the stage for a new era in germ-cell mutagenesis research.

The Human Perspective

John J. Mulvihill, University of Oklahoma Health Sciences Center—John Mulvihill described heritable mutagenesis as a process that has both a background component that is inherent to the individual and an induced component that results from environmental exposures. He emphasized that an as-yet-undefined fraction of hereditary human disease can be attributable to the latter, reaffirming the lack of definitive evidence for environmentally induced germ-cell mutations in humans. He proposed that cohort study designs monitoring sentinel phenotypes and other endpoints would be more advantageous than case-control study

designs to identify human germ-cell mutagens and to characterize their roles in human disease. Sentinel phenotypes are easily recognizable human defects that result from highly penetrant mutations in dominant (or X-linked) genes, and they have a high mutational component. Alternatively, molecular methods could be used to identify genomic mutations in exposed human populations [Foster and Sharp, 2005]. As described later in this report, molecular approaches have been developed in animal models and for somatic cells that allow for direct measurement of aneuploidy, chromosome aberrations, inversions, deletions, copy number changes, and point mutations; some of these may be immediately applicable for human germ-cell mutagenicity studies [Bendure and Mulvihill, 2006; Wyrobek et al., 2006].

Mulvihill described two human cohorts that have been investigated for germ-cell mutations: (1) Japanese atomic bomb survivors and their offspring and (2) cancer survivors and their offspring. To date, no evidence of a statistically significant increase in adverse pregnancy outcomes or heritable genetic disease has been observed in the offspring of atomic bomb survivors [Neel and Lewis, 1990]. Studies of cancer survivors, which are ongoing, are particularly advantageous for germ-cell mutagenesis studies because cancer survivors are increasingly common due to improved therapy regimens, and most importantly, because the timing and dose of their exposure to radiation and/or mutagenic chemicals has been accurately documented in their medical records. Initial analyses of the frequency of birth defects in offspring of cancer survivors have found no significant increases in the prevalence of major congenital malformations compared with the offspring of non-cancer populations [Mulvihill et al., 1987, 2001; Byrne et al., 1998; Fossa et al., 2005]. These data suggest that the agents and doses to which these individuals have been exposed do not induce transmissible mutations in human spermatogonial stem cells or resting oocytes at a frequency high enough to be detected over the background of spontaneous mutations. However, these studies were considered preliminary, and larger human studies are needed that have better coordination with studies in animal models, as described in later sections of this report.

History of Germ-Cell Mutagenesis

R. Julian Preston, US Environmental Protection Agency—Julian Preston reviewed the history of research in germ-cell mutagenesis and the current paradigm for human risk assessment. Early data on germ-cell mutagenesis generated using the mouse specific-locus tests conducted in the 1950s and 1960s demonstrated how the fundamental aspects of male and female germ-cell biology impact our understanding of germ-cell mutagenesis. These studies established that dose, dose-rate, sex, germ-cell stage, and radiation quality influence experimental outcomes [Russell, 2004] and that the results were species-specific and difficult to extrapolate in a quantitative manner from one sex and one species to another [Neel and Lewis, 1990].

One approach to the identification of germ-cell mutagens is by direct analysis of gene mutations and chromosome aberrations in human and animal sperm [Wyrobek et al., 2005a]. Studies of sperm samples from cancer patients who received radiation and/or chemotherapy were analyzed and showed that sperm-cell stage, the time of exposure, and the dose of radiation all influence the frequency of aneuploidy and/or chromosome aberrations in the sperm that are subsequently produced [Wyrobek et al., 2005b]. Corollary animal models for chromosomal abnormalities also have been developed for mouse and rat sperm [Wyrobek et al., 2005a]. In general, there are fewer studies of chemical-induced germ cell mutations in humans and experimental animals than of radiation-induced mutations.

Recent studies have reported increased minisatellite instability in human and animal germ cells exposed to ionizing radiation [Dubrova, 2005; Bouffler et al., 2006]. The significance of these data for heritable disease is not yet clear, and more studies are needed to assess their implications for human risk assessment [Preston, 2004]. Finally, Preston emphasized that better

genomic technologies are needed to determine which environmental exposures increase the frequency of germ-cell mutations in human populations. He also emphasized that the effects of radiation or chemical exposure are likely to depend on the interaction between DNA replication and DNA repair pathways in germ cells.

Preston reiterated that germ-cell stage at the time of exposure is a critical determinant of the frequency of genetically defective gametes that will be produced and the proportions of pregnancies and offspring carrying genetic defects. Preston described how germ-cell stage determines specific sensitivities for various mutagenic outcomes. Because the exact timing of germ-cell maturation is species-specific, the relative sensitivity of the germ cells to radiation at different life cycle stages varies significantly from one species to another and, therefore, must be determined empirically.

How Genome Sequence Impacts Germ-Cell Mutation and Health

Robert K. Moyzis, University of California, Irvine—Robert Moyzis gave a historical overview of the landmark HGP and its impact on the field of human germ-cell mutagenesis. Referring to the 1984 Alta Conference, the 1986 Santa Fe meeting, and the 1988 Cold Spring Harbor meeting, Moyzis emphasized that the HGP initially arose out of concern for the potential impact of environmental mutagens on the integrity of the human genome and received initial funding from the Department of Energy. As the HGP developed and involved other agencies, it changed its focus to concentrate on large-scale DNA sequencing technology, the development of model organisms for genomics studies and comparative genomics, and the ethical implications of human genomics research.

Although there may be only one major organizational structure of the human genome, there are likely to be up to ~6 billion minor sequence variations of this genome, one for each unique individual on the planet. This diversity of DNA sequence among individuals is thought to be the basis for differences in susceptibility to disease. The completion of the sequencing of the human genome and the subsequent resequencing of genomic segments among large numbers of individuals has led to rapid progress in understanding DNA sequence changes that underlie the rare, single-gene Mendelian disorders (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>). However, common polygenic diseases have presented a greater challenge, and pathway approaches have been applied to study such diseases. Moyzis illustrated the pathway approach using his research on attention deficit hyperactivity disorder (ADHD), a behavioral syndrome that involves altered neurological function. Moyzis selected a handful of genes that encode neurotransmitter receptors to screen for disease-associated variants. One of the three most common alleles coding for dopamine receptor D4, the 7R allele, was enriched ~2-fold in individuals with ADHD [Ding et al., 2002]. The 7R allele is not distributed randomly worldwide, being more common in individuals from North and South America. The association of 7R with ADHD may reflect its interaction with genetic or environmental factors that were absent when the allele became prevalent in the population. Moyzis suggested that further study of variants of the human genome will yield significant insight into the evolutionary history of humans and the environmental forces that may cause germ-cell mutation [Wang et al., 2006].

SESSION 2: GERM-CELL BIOLOGY AND MUTAGENESIS

Session Co-Chairs: Susan E. Lewis, Consultant; John B. Mailhes, LSU Health Sciences Center

Germ-Cell Biology

Animal Models vs. Humans, Male vs. Female Mary Ann Handel, Jackson Laboratory
Mechanisms of Germ-Cell Stage Susceptibilities Jack B. Bishop, National Institute of Environmental Health Sciences
 Mary Ann Handel and Jack Bishop reviewed male and

female germ-cell biology and the susceptibility of germ cells to environmental mutagens. The major topics presented were sexual dimorphism, the unique characteristics and susceptibilities of male and female germ-cell stages, differences in the time line for the production and maturation of germ cells in both sexes, sex-specificity of checkpoints during germ-cell maturation, and the sex- and stage-specificity of DNA repair capacity [Inselman et al., 2003; Lynn et al., 2004, 2005]. The major differences in the competence of the various germ-cell stages, both for checkpoint control of the cell cycle and for DNA repair, are related to the ability of environmental agents to induce mutations in the various germ-cell stages. Male germ cells appear to have a more efficient meiotic checkpoint than female germ cells; however, male germ cells are DNA repair-deficient in postmeiotic stages when the sperm chromatin condenses. In contrast, postmeiotic female germ cells using stored mRNAs retain the capacity for DNA repair until after fertilization.

Figure 1 compares the stage specificities of more than 30 chemical mutagens that have been tested by the National Toxicology Program [Witt and Bishop, 1996] and in studies of the effects of various chemotherapeutic agents [Marchetti and Wyrobek, 2005]. However, these results are based on the analyses of a very small number of genotypes, and it is well known that genetic variation among strains of mice affects their susceptibility to toxicants. Thus, genotype differences are expected to affect the susceptibility to germ-cell mutagenicity, as was recently reported for the model germ-cell mutagen acrylamide [Ghanayem et al., 2005; Manson et al., 2005]. Most germ-cell mutagens in rodents induce mutations in postmeiotic stages of spermatogenesis but not in premeiotic stages of spermatogenesis or in oocytes. As illustrated in Figure 1, very few agents induce chromosomal or gene defects in stem cells, which is of high relevance for designing studies of offspring analysis of cancer survivors. Bishop also stressed that, when designing experiments to assess the impact of environmental agents on germ cells, it is important to know both the germ-cell stage(s) at which the exposure occurs and when the gametes are sampled relative to that exposure.

Handel described a large-scale joint initiative involving The Jackson Laboratory and Cornell University for the discovery of genes required for meiosis and fertility in mice [Yang et al., 2005; Handel et al., 2006]. Gene mutations were generated by treating male mice with ENU and screening the progeny of G2 backcrosses for infertility. Among 11,000 mice tested to date, 36 mutations were identified, of which 25 were male but not female infertile, 8 caused infertility in both sexes, and 3 resulted in female infertility only. The trend for sex bias might indicate a higher level of complexity in male vs. female gametogenesis or greater pleiotropy among the genes that control female reproduction. Molecular studies indicate that many of these mutants affect mitotic proliferation of primordial germ cells or meiotic mechanisms of chromatid cohesion and recombination [Qin et al., 2004]. Future studies will test the epistatic relationship of these new mutants to previously characterized meiotic mutants. Additional information about this project can be found at <http://reprogenomics.jax.org/>.

Egg Repair of DNA Damage

Francesco Marchetti, Lawrence Berkeley Laboratory Francesco Marchetti reviewed the role of DNA repair in mammalian germ cells and during early embryogenesis. DNA repair capacity varies dramatically during germ-cell development; thus, the relative timing of exposure during gametogenesis has a major impact on the magnitude and type of DNA and chromatin damage that will be present in the germ cell at fertilization (Fig. 2). There are variable effects on embryo viability depending on which germ-cell stage is exposed [Marchetti and Wyrobek, 2005; Marchetti et al., 2006].

Oocytes have a high DNA repair capacity that they retain during and after fertilization, using stored maternal protein and mRNA, including abundant DNA repair proteins and transcripts that encode DNA repair proteins. However, the protein and mRNA complement, as well as the

transcriptional profile of the fertilized oocyte, change significantly during the first few mitotic divisions (through the 4-cell stage); zygotic transcription is initiated near the beginning of the 2-cell stage. Thus, there is a narrow window of time in which sperm DNA damage can be repaired by maternal factors in the early embryo. This is particularly important for the male genome, which has not undergone DNA repair for several weeks prior to fertilization because of the poor repair capacity of postmeiotic late-step spermatids. Experimental evidence shows that the maternal genotype affects the efficiency with which oocyte enzymes can repair DNA damage in the paternal genome, suggesting that the repair capacity of oocytes may vary across individuals of different genetic backgrounds [Generoso, 1980; Marchetti and Wyrobek, 2005].

Marchetti described his research on the role of specific DNA repair pathways in repairing radiation-induced DNA lesions during early embryogenesis. He mated irradiated male mice with unirradiated female mice that were deficient for genes involved in homologous recombination-dependent double-strand DNA break repair (HR) or non-homologous end-joining (NHEJ) repair, and measured chromosome damage using fluorescence in situ hybridization (FISH) whole-chromosome painting probes. These experiments revealed that both HR and NHEJ pathways are active in the early mouse embryo and play important roles in preventing the formation of chromatid-type and chromosome-type aberrations resulting from DNA damage in the fertilizing sperm. Similar experiments conducted by Shimura et al. [2002] and Toyoshima et al. [2005] demonstrated that a p53-dependent S-phase checkpoint also is active during early mouse embryogenesis. In conclusion, the available evidence from mouse studies demonstrates the existence of maternal factors that result in differential conversion of sperm DNA lesions into paternally transmitted chromosome damage, and suggest that quantitative and qualitative limitations in maternal DNA repair can have profound effects on modifying the risks for abnormal reproductive outcomes of paternal origin [Marchetti and Wyrobek, 2005].

Mosaicism and Germ-Cell Mutagenesis

Harvey W. Mohrenweiser, Oregon Health Sciences University Harvey Mohrenweiser reviewed the effects of mosaicism on phenotypic and genotypic variation in mammals [Jones et al., 2005]. Although mosaicism may arise from a number of processes, Mohrenweiser focused on genetic heterogeneity caused by mutations that arise after fertilization during embryogenesis. Some mosaicism occurs as the result of normal biological processes, including genomic imprinting, X-inactivation, and DNA methylation. Aberrant mosaicism can be associated with pathological conditions or environmental exposures. For example, mutations that occur during embryogenesis can be spontaneous or can result from exposure to mutagens; they also may arise from chromosome aberrations occurring during mitotic cell divisions. The fraction of embryonic tissue affected by such mutations (i.e., mosaicism) depends on the stochastic distribution of cells during embryonic development. When the fraction of affected cells increases, the potential for adverse outcome also increases.

Mohrenweiser estimated that as many as 10–20% of so called *de novo* mutations in a gene can be attributed to events occurring during early embryogenesis; the remaining 80% of new mutations are inherited via the parental germline or arise postnatally. When more than one offspring of the same parent is affected by the same disease, the parent is likely to have germline mosaicism. Germline mosaicism is not uncommon, but the frequency is gene-specific and varies dramatically across different regions of the human genome. Recent studies indicate that mosaicism occurs at a higher frequency in human embryos produced in vitro using assisted reproductive technology (ART). For example, ~75% of day 2 embryos produced by ART show mosaicism, and 100% of ART embryos in more advanced stages have some mosaic characteristics. These frequencies are likely to reflect the abnormal conditions in which ART

embryos are formed and manipulated. However, they suggest that the rate and potential impact of mosaicism under biologically relevant conditions may be underestimated; somatic and germ-line mosaicism could make a significant contribution to sporadic and inherited genetic disease.

SESSIONS 3/4: NEW APPROACHES FOR DETECTING HERITABLE MUTATIONS

Session Chairs: David M. DeMarini, US Environmental Protection Agency; Carole L. Yauk, Health Canada

Single-Sperm PCR Genotyping

Detecting Chromatin, DNA, and Chromosomal Abnormalities in Sperm

Andrew J. Wyrobek, Lawrence Berkeley Laboratory Norman Arnheim and Andrew Wyrobek reviewed recent advances in the molecular detection of gene mutations, DNA damage, chromatin damage, and chromosome defects in human sperm as summarized in Table I [Marchetti and Wyrobek, 2005; Wyrobek et al., 2006]. A major advantage of analyzing sperm directly vs. analyzing groups of offspring is that a much larger number of gametes per individual can be assessed for genomic defects compared with the relatively small numbers of affected offspring typically available for epidemiological studies of male-mediated effects. The statistical advantage of sperm studies was illustrated by estimating the number of families with offspring that would need to be screened to detect a doubling of affected offspring after a hypothetical parental exposure: ~900 families for birth defects, ~300 for spontaneous abortions, and ~250,000 for childhood leukemia. In contrast, sperm studies usually require ≤ 10 men per group to detect ~2-fold effects, depending on the specific assay used. More research will be needed to elucidate the cellular and biochemical events that occur after sperm release that may modify the probability of a genetically defective sperm producing a child with a heritable defect of paternal origin, such as the role of selection pressures for or against defective sperm during fertilization, maternal DNA repair of sperm DNA lesions in the zygote, and epigenetic modifications of sperm DNA. Nevertheless, sperm assays are a promising approach for screening for potentially hazardous compounds and prioritizing medical, occupational, and lifestyle factors that may induce heritable disease due to gene mutations and chromosomal alterations in male germ cells. They also provide a unique opportunity to prescreen cancer survivors to identify chemotherapeutic regimens that induce gene mutations or chromosomal aberrations in spermatogenic stem cells, which represents only a subset of chemicals tested in mice (Fig. 1).

Arnheim described several PCR and direct sequencing assays that measure mutation frequency in sperm at specific loci in the fibroblast growth factor receptor genes *FGFR2* or *FGFR3*, which are associated with dominantly inherited Apert syndrome and achondroplasia, respectively [Goriely et al., 2005]. These loci have relatively high mutation frequencies, and mutations in these two genes occur predominantly in the paternal genome. Quantitative, allele-specific PCR is used in these assays to amplify specific mutant alleles with a sensitivity of approximately 10^{-5} ; a DNA sample of ~5 μ g is required. However, more DNA (~5 mg) is needed to detect a more typical mutation at 10^{-8} , which is not yet feasible in all cases. To improve the current sensitivity and specificity of single-base mutation detection methods, Arnheim developed a modified assay that uses a dideoxy-blocked, mutant-specific oligonucleotide as the PCR primer. Using a DNA polymerase that carries out pyrophosphorolysis, the primer annealed to mutant DNA becomes unblocked, and the rare mutant template is preferentially amplified. The assay background is decreased dramatically, and the sensitivity of the assay improves by about three orders of magnitude compared to standard allele-specific PCR.

Arnheim also briefly described methods to enhance assay sensitivity and detect low-frequency mutations in human sperm DNA. These methods can enrich for a mutant allele, deplete the wild-type allele, or selectively tag and sort mutant and wild-type alleles (i.e., selective restriction digestion of the wild-type allele, sequestering the mutant allele via specific binding probes, followed by PCR with beads-emulsion-amplification-magnetics or “BEAMing”).

Wyrobek reviewed the molecular techniques for analyzing DNA damage, chromatin damage, and cytogenetic defects directly in human sperm (Table I): sperm comet assay, sperm chromatin structure assay (SCSA), and sperm fluorescence in situ hybridization (sperm FISH) [Wyrobek et al., 2005a]. The sperm comet assay is a single-cell gel electrophoretic method typically applied to ~100 sperm nuclei per sample to quantitate single-strand or double-strand DNA breaks by using different pH conditions. SCSA is a flow cytometric method that employs acridine orange to measure the relative proportions of single- and double-stranded DNA in several thousand sperm. SCSA results represent the degree of DNA fragmentation per specimen and the proportions of sperm with immature chromatin (i.e., incomplete chromatin condensation).

Several FISH methods have been developed since 1990 to detect aneuploidy and structural chromosomal alterations directly in human sperm, including breaks as well as partial chromosomal duplications and deletions. Multicolor sperm FISH has been applied to detect aneuploidies for several chromosomes simultaneously in ~10,000 sperm per specimen; DNA probe combinations can detect sperm associated with increased risks for aneuploidy syndromes including Down, Edwards, Turner, Klinefelter, XXX, and XYY, as well as other types of aneuploidies that lead to embryo death during pregnancy. Most of these human sperm assays already have been adapted for studies in animal models [Wyrobek et al., 2005a].

Wyrobek summarized the human studies that have applied sperm FISH technologies to assess the effects of chemotherapy [Wyrobek et al., 2005b], occupational exposures, and lifestyle factors [Robbins et al., 2005] on the incidence of sperm aneuploidy. He observed a 5-fold variation in baseline frequencies of sperm aneuploidy among healthy donors; however, he also noted that aneuploidy frequencies may remain stably elevated for years within the same donor. There is also limited evidence that the frequency of aneuploidy in sperm is correlated with the frequency of aneuploidy in lymphocytes, suggesting that there may be constitutive mechanisms leading to aneuploidy that affect both germ cells and somatic cells [Rubes et al., 2002]. Recently, Kauppi et al. [2005] showed that localized breakdown in linkage disequilibrium does not always predict sperm crossover hot spots in humans. Wyrobek summarized the results of a recent study that found a strong association between advancing male age and the levels of physiological and genetic damage in sperm, including detrimental effects on motility, DNA fragmentation, chromosomal aberrations, DNA damage, and gene mutations, but not aneuploidy frequency [Wyrobek et al., 2006].

Wyrobek called for investigations of the predicative values of sperm biomarkers for abnormal reproductive outcomes, including developmental defects and heritable genetic disease. Sperm biomarkers of genomic damage would serve to identify medical, occupational, and environmental exposures as well as human lifestyle factors that may be detrimental to the genetic integrity of the male germline. They would also be useful in comparing the relative sensitivities of human somatic and human germ cells to these kinds of genotoxic exposures, in assessing the relative risks of somatic genetic diseases vs. heritable diseases, and for linking the rodent model and human data.

Oocyte Microenvironment and Transmitted Chromosomal Abnormalities

Ursula Eichenlaub-Ritter, University of Bielefeld, Germany Ursula Eichenlaub-Ritter described several techniques of genomic analyses of human oocytes based on FISH, spectral

karyotyping (SKY), and comparative genomic hybridization (CGH), primarily with unfertilized human oocytes generated by ART. Using these methods, she found that 20–52% of human oocytes were aneuploid. In contrast, studies using conventional chromosome analysis methods have estimated a lower frequency of 11%, which may be attributable to variations in maternal age because it is well known that total aneuploidy and specific types of chromosome abnormalities increase with advancing age of female donors. Eichenlaub-Ritter proposed that age-related differences in gene expression during oogenesis are important factors that determine the susceptibility to meiotic error. In support of this model, mouse studies suggest that an age-dependent decrease in the efficiency of the Mad (Mitotic Arrest Deficiency) 2-dependent cell cycle checkpoint in oocytes can predispose to induction of aneuploidy, a mechanism that is likely similar in human oocytes [Eichenlaub-Ritter, 2005].

Eichenlaub-Ritter also reviewed the evidence for chemically induced errors in chromosome distribution in mammalian oocytes, using enhanced polarizing microscopy, a noninvasive technique that reveals changes in spindle formation that predispose to nondisjunction [Eichenlaub-Ritter et al., 2002]. This method can be combined with more conventional methods to analyze the impact of hormones, life style, age, or environmental exposure on the frequency of chromosomal abnormalities in human oocytes, including those that are fertilized by intracytoplasmic sperm injection in assisted reproduction. Such noninvasive methods may help to identify intrinsic factors detrimental to female fertility, such as a reduced follicle pool, altered hormonal homeostasis or maternal age, and length of the meiotic arrest, as well as extrinsic factors like chronic or acute exposures to aneugens in induction of chromosomal aberrations in human oocytes and embryos. She also described an in vitro mouse ovarian follicle culture method for modeling the growth, maturation, and ovulation of oocytes. This technique may facilitate the assessment of risk to mammalian oocytes within follicles from acute, subchronic, or chronic exposures to meiotic aneugens. In addition, this model may aid in the analysis of direct and indirect mechanisms of aneuploidy induction in oocytes and in the identification of targets of drug action in both somatic and germ cells. Eichenlaub-Ritter pointed out that such models also might be useful to detect risks for exposure-induced epigenetic changes in mammalian oocytes.

Haplotype Analysis and Human Genetic Disease

Jack A. Taylor, National Institute of Environmental Health Sciences Jack Taylor gave an overview of human genetic diversity associated with single nucleotide polymorphisms (SNPs). He reviewed the Environmental Genome Project (EGP), a systematic human genome resequencing project sponsored by the National Institute of Environmental Health Sciences (<http://www.niehs.nih.gov/envgenom/home.htm>). The goal of the EGP is to assess genetic diversity in a subset of human genes that are predicted to influence susceptibility to environmentally induced disease. Initial studies focused on the resequencing of 100 genes involved in cell cycle control and DNA repair among ~90 ethnically diverse individuals. Approximately 9,000 total SNPs were identified, including ~2,000 SNPs with a frequency $\geq 5\%$ (<http://www.niehs.nih.gov/envgenom/home.htm>). There were on average 20 common SNPs per gene and SNPs associated with one another into linked groups called haplotypes. On average, there were 3–4 common haplotypes per gene. Many haplotypes were shared across ethnic groups, suggesting that they arose before human ethnic divergence, i.e., before ~100,000 years ago [Taylor et al., 2006]. Taylor suggested that selected subsets of SNPs, each representing a different haplotype, might be used to examine whether the common variants of a gene are associated with disease.

However, new germline mutations present a more difficult problem because they can occur anywhere on the complex background of preexisting haplotypes. Unless recombination has occurred, a rare mutation will continue to persist on the haplotype background on which it

arose. SNPs that identify the haplotype in which the new mutation has occurred also may serve as a surrogate for that mutation and further aid in the discovery of the genetic variants that modulate disease susceptibility. Further studies will be needed to evaluate the utility of SNP analyses for environmentally induced de novo gene mutations that are expected to occur in random genomic locations.

Inherited Microdeletions, Translocations, and Rearrangements

Jane Fridlyand, Cancer Research Institute/UCSF Comprehensive Cancer Center Jane Fridlyand overviewed several methods for conducting whole-genome scans for genomic alterations, including FISH, SKY, end-sequence profiling (ESP) [Volik et al., 2003], and array-based CGH [Willenbrock and Fridlyand, 2005]. Fridlyand indicated that an ideal method for a whole-genome scan to detect abnormal cells should be inexpensive and readily automated for high throughput, provide high resolution and high sensitivity, and be able to detect both balanced and unbalanced DNA rearrangements, i.e., translocation or copy number changes. No currently available method fulfills all of these requirements. For example, the resolution of SKY is 1–10 Mb, making it unsuitable for detecting gene-dosage alterations. ESP, on the other hand, can be used to detect all types of chromosomal alterations, including changes in the number of copies of a gene, but it is currently too expensive for routine use. ESP is discussed further in a later section. Although chromosome CGH has relatively low resolution, the resolution of array-based CGH can be high because the sensitivity is determined by the size and number of hybridization probes on the array. Current technology allows for maximal resolution approaching 100 kb using tiled arrays of Bacterial Artificial Chromosome (BAC) clones. BAC arrays providing complete coverage of the human genome are now becoming available from academic centers. In addition, commercial manufacturers are working to develop oligonucleotide-based arrays for DNA copy number analysis.

Fridlyand described the sensitivity of array CGH using examples from several experiments in which array CGH was used to analyze gene dosage for human and mouse genes. Fridlyand used a 2,500-element BAC array with ~1.4 Mb resolution to analyze DNA samples from human peripheral blood and somatic tissue. Whole-genome scans with this array can detect a deletion or amplification involving DNA sequences within a single BAC array element. In a study of 44 patients, 22 of whom having a deletion affecting a single array element, no false positives or negatives were observed. Fridlyand also presented array CGH data that identified copy number variation in Kringle repeat elements in the human apolipoprotein gene and indicated that this is just one example of the frequent “normal” polymorphisms in the human genome that result in DNA dosage variation. She also presented results that showed that changes in copy number detected by a mouse BAC array are specific to individual inbred strains and that these data can be used to cluster strains of mice and accurately distinguish among individuals of different strains. These data suggest that rare deletions or amplifications can be detected with relatively high accuracy using array CGH of appropriate resolution. However, Fridlyand cautioned that it remains a challenge to differentiate between technical noise, i.e., false positives or negatives, normal genetic variation, and novel variants due to copy number events in the target genome.

Several of these methods have been adapted to the study of gametes. Sperm and zygote FISH methods have been developed for detecting environmental agents that induce chromosomal damage in human, mouse, and rat male germ cells [Marchetti et al., 1996; Wyrobek et al., 2000, 2005a], and SKY has been applied to study of oocytes [Eichenlaub-Ritter, 2005]. However, ESP and CGH are not readily adaptable to gamete analyses. In addition, array-based CGH can detect chromosome aberrations at 50–100 kb resolution and link changes in an offspring’s clinical phenotype and function with specific chromosomal duplications and deletions. These attributes have been used successfully in human prenatal testing [Sahoo et al.,

2006], but the applications of CGH to the analyses of environmentally induced heritable effects remains to be established.

Comprehensive Genome Structure and Transcriptome Analysis for Mutation Detection

Thomas J. Vasicek, Medtronic, Inc Thomas Vasicek gave an overview of applications in genome analysis using Massively Parallel Signal Sequencing (MPSS), which is a method for evaluating genome structure [Jongeneel et al., 2005] or gene expression by counting mRNA molecules present in a sample [Crawford et al., 2006]. Individual mRNAs are identified by sequencing 17–20 nucleotides at a unique site in each mRNA. Typically, 2 million molecules are analyzed from a single sample. This technology can be used for comprehensive genome-wide analyses of the cellular transcriptome. MPSS traditionally has been used for comprehensive, quantitative gene expression profiling. Because MPSS determines transcript abundance by a transcript counting method, it measures absolute transcript abundance and is, therefore, more quantitative than technologies based on hybridization. MPSS also is capable of detecting low-abundance transcripts (<100 transcripts per million) that are not detectable using microarray technology. For example, MPSS analysis of the transcriptome of human monocytes and immature dendritic cells revealed that ~90% of all transcripts in these cells are represented by less than 100 transcripts per million and, therefore, would not be detected in a typical microarray study.

Vasicek also reviewed several new applications for MPSS, some of which could be used to map chromosome rearrangements and other mutational events [Jongeneel et al., 2005]. These applications include comprehensive analyses of protein binding sites, DNase I hypersensitivity, DNA methylation, chromosome breakpoints, and SNPs. Vasicek emphasized that the MPSS methodology is well established, accurate, and reliable; however, for many specific applications of MPSS, the method of sample preparation requires development and improvement.

MPSS was adapted to carry out BAC end-sequencing for mapping large-scale polymorphisms. This version of the assay uses the same principle as ESP: i.e., a short stretch of DNA sequence is determined at both ends of all clones in a BAC library of the target genome. BAC insert lengths are estimated, and the sequence tags are mapped against the normal genomic sequence. This permits identification of all amplifications, rearrangements, insertions, and deletions. Vasicek also described a modified ESP approach in which genomic restriction fragments are sequenced directly, and the BAC cloning step is omitted. These sequences can be used to identify novel SNPs. However, MPSS and its variants have not yet been evaluated for the detection of de novo heritable mutations.

Bioinformatics Tools in Germ-Cell Analyses

Michael Primig, Biozentrum, and Swiss Institute of Bioinformatics Michael Primig described GermOnline, a bioinformatic knowledge base for genes and functions relevant to mitosis, meiosis, germline development, gametogenesis, and fertility in yeast and higher eukaryotes [Primig et al., 2003; Wiederkehr et al., 2004a,b] (see <http://www.germonline.org>). GermOnline was developed at the Biozentrum in Basel, Switzerland and the Swiss Institute of Bioinformatics, and has mirror sites in Europe, Asia, and the US to ensure continuous access worldwide.

GermOnline is unique in that the data and annotations are provided, curated, and updated by members of the scientific community. The mechanism of community-based curation was adopted to increase the number of available curators and, therefore, increase the efficiency of database management. This approach solves the problem of the small ratio of curators to data

that threatens to overwhelm the information technology (IT)/bioinformatics communities. Several mechanisms are employed to maintain the quality and integrity of the knowledgebase: oversight by an international board of qualified scientists and use of harmonized language and terms, i.e., Gene Ontology (GO) terms. Thus, in most areas of GermOnline, data entry is restricted to GO keywords and terms.

GermOnline is a cross-species knowledge base that provides access to data curated in other molecule- and species-specific databases. It is unique among integrated databases because it is focused on specific biological components and processes that play roles in germ-cell biology. It also provides access to microarray and image data relevant to the knowledge base [Schlecht et al., 2004; Zhou et al., 2005; Hermida et al., 2006]. Future goals for GermOnline include adding capacity to curate data on protein-DNA interactions and SNPs, and adding video and other types of image data.

SESSION 5: OPEN DISCUSSION1; SESSION 6: POSTERS; SESSION 7: LINGERING PUZZLES IN MUTAGENESIS AND INHERITANCE

Session Chair: Heinrich V. Mallng, National Institute of Environmental Health Sciences

Something Curious about Paternal Age Effects and Other Questions in Germ-Cell Mutagenesis

James F. Crow, University of Wisconsin, Madison James Crow presented an overview of the effects of paternal age on human germ-line mutations—the so-called “paternal age effect” [Crow, 2003], as exemplified by several human diseases with well-characterized paternal age effects such as Apert syndrome, achondroplasia, X-linked hemophilia, retinoblastoma, and neurofibromatosis. Clinical observations of these diseases suggest that the number of affected offspring increases more quickly than predicted with increasing paternal age [Crow, 2003]. For these syndromes, the germline mutation rate increases with paternal age but not with maternal age, and the mutation rate is higher in human males than in females.

Molecular analyses of human and mouse germ cells have confirmed some of these clinical observations. Three classes of mutations have been identified that contribute differentially to the paternal age effect: hot spots, insertions and deletions (indels), and base substitutions. When present, as in the genes linked to Apert syndrome and achondroplasia, hot spot mutations tend to occur in males only, and in some cases they increase dramatically with paternal age [Crow, 2003]. However, hot spot mutations are gene- and sequence-specific and are, therefore, relevant only to paternal age effects in a subset of genes. Approximately two-thirds of the documented new mutations in the human genome are base substitutions, with the remaining one-third of mutational events being mostly small and large indels that show no increase with paternal age [Crow, 2003]. Crow argued that base substitutions show a slight paternal bias and a smaller, but significant, paternal age effect than hot spot mutations. The total paternal age effect in a specific gene reflects the relative contributions of base substitutions, indels, and hot spot mutations. Thus, the magnitude of the paternal age effect should vary significantly from one gene to another.

Radiation and Germline Mutation at Repeat Sequences: Are We in the Middle of a Paradigm Shift?

Bryn A. Bridges, University of Sussex Bryn Bridges reviewed several hypotheses proposed to explain the estimated 10-fold excess of childhood cancer that occurred among inhabitants of Seascale, UK between 1950 and 1990 [Bouffler et al., 2006]. Seascale is near the Sellafield nuclear power plant, and its inhabitants have been exposed to higher than background doses of radiation, in some cases exceeding 100 mSv. Although initial studies correlated paternal

preconception dose with the number of affected children, this observation has not been reproduced in studies at other international sites for groups of fathers with similar occupational exposures to ionizing radiation. In addition, it was concluded that the radiation dose received by Seascale residents and other individuals living close to nuclear power plants could not alone induce enough mutations through direct mechanisms to account for the excess cancer cases in Seascale.

Subsequent analyses of Seascale data suggest that two additional factors may contribute to the increased cancer rates in this exposed population [Bouffler et al., 2006]. The first factor is population mixing, as a surrogate for an, as yet unknown, infectious agent that may increase susceptibility to radiation-induced mutation. The second factor is an, as yet unidentified, epigenetic mechanism that may amplify the mutagenicity of a given radiation dose and cause nontargeted mutations distal from sites of radiation-induced DNA damage, possibly at unstable repeat sequences.

However, none of the explanations for the excess cancers in Seascale have been confirmed. Bridges also suggested that prior studies of germinal mutations in offspring of radiation-exposed human populations have been inconclusive because they did not investigate sufficiently comprehensive endpoints in offspring, such as subtle birth defects that manifest later in childhood.

Tandem Repeat DNA Germ-Cell Mutagenesis in Chernobyl, Japanese, and Animal Studies

Yuri E. Dubrova, University of Leicester Yuri Dubrova reviewed recent findings of increased mutation rates in tandem repeat DNA sequences in radiation-exposed human populations. The human genome contains three types of repeated DNA sequences: minisatellites, microsatellites, and extended simple tandem repeats (ESTRs). The microsatellite and ESTR loci account for up to 15% of the gamete genome, and the spontaneous mutation rate at these loci is several orders of magnitude higher than in the rest of the human genome [Dubrova, 2003]. Minisatellites appear to have a high mutation rate in both human somatic cells and germ cells, and they may be ideal for studying induced mutations in the human germline. Most mutations in ESTRs are gains or losses of repeats, suggesting that they arise via replication slippage. Importantly, because of the high mutation rate in ESTRs and minisatellite sequences, samples from fewer individuals are needed to detect exposure-induced mutations in these sequences than in single-copy genes. Dubrova estimated that minimum sample sizes of 240, 2,400, and 240,000 individuals are sufficient to detect statistically significant inductions of mutations in human minisatellites or mouse ESTR, microsatellite, and single-copy genes, respectively.

Initial studies compared the radiation dose-response curve for ESTRs with that of the specific-locus test in mice [Dubrova, 2005; Singer et al., 2006]. The linear portion of the dose-response curve was lower for ESTRs, but both data sets appear to fall on the same dose-response curve, suggesting that ESTR sequences are more sensitive to X-rays than are single-copy genes, and that both endpoints may reflect similar biological responses to radiation-induced DNA lesions. Similar results were obtained for ENU mutagenesis in mice. These data suggest that ESTR loci can be used to monitor the mutagenic response to low-dose exposures using a relatively small number of exposed animals or humans.

Several studies have analyzed mutations in human repeat sequences in exposed human populations [Dubrova, 2003]. Dubrova summarized the results of four studies that investigated mutations in (1) 6 repeat loci among 64 children of Hiroshima bomb survivors, (2) 8 repeat loci among 367 children of Chernobyl survivors, (3) 8 repeat loci among 232 children of residents near the Semipalatinsk nuclear test site, and (4) 8 repeat loci among 338 offspring of

Chernobyl cleanup workers [Barber and Dubrova, 2006]. Studies 1 and 4 were negative, and Studies 2 and 3 showed significant associations between paternal (but not maternal) exposure and increased mutation rates.

Dubrova discussed three possible explanations for the negative result in atomic bomb survivors: (1) this population generally received smaller radiation doses than the other exposed populations; (2) the number of individuals studied may have been too small for detecting significant differences; and (3) this study did not distinguish subjects according to paternal or maternal exposure. Dubrova also suggested that the negative result in Study 4 (Chernobyl cleanup workers) may be due to fractionated radiation exposures.

Dubrova summarized that the initial findings with repeat sequences in the human germline are encouraging and that epidemiological studies with relatively small exposed populations are expected to have sufficient power to detect radiation-induced mutations. Dubrova's laboratory currently focuses on the comparative effects of radiation on minisatellite mutation rates in several exposed cohorts. Dubrova also recommended additional studies to understand the relative effects of chemical or radiation exposures on repeat-sequence variability in the human germline and to examine the correlation between the mutation rate in repeat sequences and the rate of functional mutations in single-copy genes in the human genome [Singer et al., 2006].

Heritable Mitochondrial Mutagenesis

Eric A. Shoubridge, McGill University Eric Shoubridge gave an overview of mouse and human mitochondrial genetics and the impact of mutations in the mitochondrial genome on fertility and disease. The mitochondrial genome is small, with 13 protein-coding genes, most of which play essential roles in oxidative phosphorylation [Wallace, 2005]. Generally, mitochondrial DNA (mtDNA) is homoplasmic, but in some cases, more than one mtDNA variant co-exists within a single mitochondrion (heteroplasmy). The replication of mtDNA is not under cell cycle control, but the number of copies of mtDNA per mitochondrion is regulated and maintained at 2–10. mtDNA is transmitted to embryos exclusively from the cytoplasm of the maternal germline. Thus, its inheritance is non-Mendelian. Mitochondria have the capacity to repair DNA lesions, but the mutation rate in mtDNA is ~10-fold higher than in genomic DNA [Wallace, 2005]. A number of human diseases and conditions are associated with mtDNA variants that arise through spontaneous or induced mutations in the mtDNA in somatic or germ cells. Variant mtDNAs can be present as a variable fraction of the total mtDNA, and they cause multiple complex phenotypes that complicate diagnosis.

Shoubridge used a mouse model to analyze the segregation of mtDNA variants during oocyte development and early embryogenesis [Battersby et al., 2005]. His findings suggest that mtDNA rapidly segregates by a stochastic process that does not ensure equal distribution of variant mtDNA genomes. Through detailed analysis of the segregation of mtDNA variants at different stages in oogenesis, Shoubridge concluded that all mtDNA segregation occurs prior to formation of the primary oocyte. He also observed that the number of mtDNA molecules per primordial germ cell is low, and he proposed that this creates a bottleneck for mtDNA distribution during female gametogenesis. There also appeared to be no selection against mutant mitochondrial genomes; defective mtDNA genomes do not appear to reduce female fertility.

SESSION 8: RESEARCH CHALLENGES IN MUTAGENESIS AND INHERITANCE

Session Chair: Steve S. Sommer, City of Hope Medical Center

Germ-Cell Methylation in Mutagenesis

Jaquetta M. Trasler, Montreal Children's Hospital Research Institute Jaquetta Trasler described the mechanisms for establishing and maintaining patterns of DNA methylation in mammalian genomes [Trasler, 2006]. DNA methylation is one of the best-characterized epigenetic mechanisms for modulating gene function. DNA methylation is required for X-inactivation and gene silencing, and recent studies show that DNA methylation is critical for gene imprinting. Defective imprinting is associated with human diseases such as Prader-Willi, Angelman, and Beckwith-Wiedemann syndromes, and aberrant DNA methylation also has been linked to some human cancers. Some studies also suggest that aberrant DNA methylation may occur in human embryos produced by ART, and if confirmed, this could have implications for the susceptibility to imprinting-associated diseases in children conceived by ART. In addition, Barton et al. [2005] have shown that epigenetic programming in the preimplantation rat embryo is disrupted by chronic paternal cyclophosphamide exposure.

The mechanisms by which DNA methylation patterns are inherited are complex. Most methylation is referred to as maintenance methylation, which occurs postreplicatively on hemimethylated DNA. However, the genome is reprogrammed twice, once during gametogenesis and once during embryogenesis. The DNA of both male and female primordial germ cells initially undergoes demethylation at about the time the cells enter the embryonic gonads. Remethylation occurs according to different time lines in male and female gametes [Trasler, 2006]. Male germ cells begin to be methylated before birth and continue to be methylated at some sites after birth. In contrast, the DNA of female germ cells is remethylated only after birth during the oocyte growth phase. Following fertilization, there is a second wave of demethylation that takes place during preimplantation development, although some sites are spared, such as imprinted genes and repeat sequences that maintain their gamete-derived methylation patterns. In the somatic tissue of the mouse embryo, the genome undergoes a second period of remethylation during Days 5–7 of gestation. However, the exact timing of methylation in embryonic cells varies in a gene-specific manner.

Several murine DNA methyltransferase (*DNMT*) genes that play roles in de novo and maintenance DNA methylation have been cloned and characterized [Trasler, 2006]. *DNMT1* is involved in maintenance methylation of hemimethylated DNA, and *DNMT3A* and *DNMT3B* are involved in de novo methylation during DNA reprogramming. Knockout mice lacking an oocyte-specific form of DNMT1, DNMT1o, show a lack of maintenance of methylation patterns on imprinted genes at the 8-cell stage of preimplantation development, consistent with its role in maintenance methylation. Knockout mice for DNMT3A show impaired germ-cell development in both males and females, with complete infertility in males. This result confirms the role of DNMT3A in de novo methylation in the mouse germ-line. DNMT3B knockouts have no apparent defect in gametogenesis. Although DNMT3L has no detectable DNA methyltransferase activity, it works together with DNMT3A in methylating DNA; deficiency of DNMT3L interferes with maternal imprinting and causes male sterility.

Trasler pointed out that little is known about how environmental or mutagenic agents affect DNA methylation in somatic or germ cells. However, molecular approaches have been developed for detailed analysis of DNA methylation in specific genes or on a genome-wide basis. These methods, including bisulfite sequencing, restriction landmark genome scanning, and methylation profiling, can be used to address this question and to study the consequences of defects in DNA methylation in animal model systems.

Epigenetic Transgenerational Actions of Endocrine Disruptors on the Male Germline and Fertility

Michael K. Skinner, Washington State University Michael Skinner described recent novel findings of environmentally induced transgenerational changes in methylation in male germ cells [Anway et al., 2005]. Skinner discovered this phenomenon while testing the effects of the endocrine disruptor, vinclozolin, on testis development and germ-cell differentiation during male rat embryogenesis [Skinner and Anway, 2005]. When rat embryos were exposed to vinclozolin in utero from embryonic day (ED) 7 to ED15, male offspring demonstrated decreased seminiferous cord formation and increased rates of apoptosis in spermatogonial cells. Decreases in sperm motility and sperm number were noted in male offspring only when embryos were exposed during ED13–ED15, which is the most critical period for testis development in the rat embryo. Approximately 10–15% of the exposed male pups were completely infertile. A number of the in utero-exposed, sperm-defective but fertile males were inadvertently mated, which led to the discovery that the exposure-induced effects on spermatogenesis were transmitted to the offspring of the in utero-exposed males for at least four generations without further exposures to vinclozolin.

Skinner tested the hypothesis that vinclozolin exposure disrupted normal methylation patterns in the male germ-line during embryonic development [Anway et al., 2005]. Characterization of methylation patterns in the genomic DNA of affected males identified 25 known genes and 21 unknown genes whose methylation patterns differed in exposed and unexposed animals. Similar results were observed when gene-specific DNA methylation was examined in male germ cells. The affected genes included STAT-like transcription factors. These results led Skinner to propose that transient exposure to vinclozolin during stages ED13–ED15 caused a permanent epigenetic reprogramming of specific genes in male germ cells that led to an exposure-induced transgenerational phenotype, including adverse effects on male fertility. One possible mechanism for the genetic reprogramming might be vinclozolin-induced changes in transcription during stages ED13–ED15. Additional characterization of the health of in utero-exposed males revealed significant adverse effects when they reached older ages [Anway et al., 2005; Skinner and Anway, 2005]. Late-onset phenotypes included male breast tumors, premature aging, prostate degeneration, increased prevalence of severe and/or complete male infertility, and a pre-eclampsia-like phenotype in late-stage pregnancy in females.

In summary, these findings suggest that in utero exposures to environmental agents can cause transgenerational effects by an epigenetic mechanism, and point to the existence of gender-specific windows of susceptibility during embryogenesis in which germ cells and/or germline progenitor cells are susceptible to environmentally induced adverse effects.

Transgenerational Effects of Low-Dose Mutagenesis

Diana Anderson, University of Bradford Diana Anderson reviewed the rodent and human evidence that low-dose environmental exposures of male and female parents to radiation and chemicals can induce adverse heritable effects. The evidence for such effects in humans is very limited, e.g., based on observations of increased cancer prevalence in the vicinity of nuclear power plants and increased cancer incidence among children of male smokers [Anderson, 2005]. The majority of studies of exposed humans have failed to provide convincing evidence of exposure-related adverse heritable effects, including a study of the rate of birth defects among the offspring of ~18,000 cancer survivors, who presumably received high doses of mutagenic agents [Anderson, 2005].

Anderson emphasized the value of rodent models for detecting the effects of low- or high-dose exposure by monitoring adverse birth outcomes or induction of chromosomal aberrations among offspring. Substantial data are available to support high-dose exposure effects on

heritable mutations in rodents. However, this association is more ambiguous for low-dose exposures. Anderson reviewed studies of adverse effects in animals and humans exposed to environmental chemicals, including cyclophosphamide, 1,3-butadiene, and urethane. Using an animal model of male-mediated teratogenicity, offspring of male rodents acutely exposed to cyclophosphamide had numerous birth defects as well as chromosome abnormalities and increased tumor incidences [Anderson, 2005].

Anderson also summarized several low-dose studies [Anderson, 2005]. Chronic low-dose exposure to 1,3-butadiene increased the frequency of adverse pregnancy outcomes, which is consistent with recent epidemiological data that support the possibility that occupational exposure to 1,3-butadiene induces adverse reproductive effects in humans. Low-dose urethane exposures increased the rate of liver tumors in exposed males but did not cause birth defects.

In closing, Anderson emphasized the potential usefulness of animal models in identifying chemicals that may be hazardous to germ cells and in establishing the affected germ-cell stages and the exposure doses of greatest concern. She further recommended additional use of these models for characterization of potential adverse health impacts resulting from environmental exposures to male germ cells in humans.

SESSION 9: CHALLENGES IN LINKING HUMAN EXPOSURE TO INCREASED MUTATIONS AND HEALTH CONSEQUENCES IN OFFSPRING

Session Chair: Mort Mendelsohn, Lawrence Liver-more National Laboratory

Inherited DNA Repair Defects and Human Disease

Philip C. Hanawalt, Stanford University Philip Hanawalt reviewed the major cellular pathways for repairing damaged DNA with examples of several human hereditary diseases resulting from defects in DNA damage processing. The genome is continuously assaulted by many exogenous and endogenous agents, e.g., reactive oxygen species, which induce lethal or mutagenic DNA lesions. The recognition of lesions, or their inhibitory effects on the processes of replication or transcription, triggers cellular responses that may arrest the cell cycle, induce apoptosis, and/or upregulate expression of DNA repair enzymes to repair DNA damage. Variation in DNA repair capacity may alter the susceptibility of individual parents to germ-cell mutagenesis after exposure to environmental mutagens.

There are three major DNA excision repair pathways: nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) targeted to different types of DNA damage, but with a considerable degree of overlap in substrate specificity. Additionally, recombinational mechanisms are utilized to deal with double-strand breaks, interstrand crosslinks, closely spaced lesions on the respective DNA strands, and some types of replication fork arrest. Mutations in genes encoding DNA repair proteins cause phenotypic effects on growth, cell-cycle progression, susceptibility to DNA damage, meiosis, and other biological processes in model systems, such as yeast. Although defects in NER and MMR may result in genomic instability, BER appears to be essential in mammalian species because it has not been possible to isolate viable BER-deficient mutants in mice.

NER is a complex process involving the concerted action of ~30 proteins that recognize the lesion, remove a short segment of the damaged strand, replace it using the complementary DNA strand as template for repair replication, and seal the “repair patch” by ligation to the contiguous parental strand [Hanawalt et al., 2003]. NER targets primarily bulky helix-distorting lesions, and there are two NER repair subpathways: global-genome repair (GG-NER) and targeted repair, which is also called transcription-coupled repair (TCR). Human hereditary defects in NER cause several distinct diseases, including xeroderma pigmentosum

(XP), Cockayne syndrome (CS), UV-sensitive syndrome (UVSS), and trichothiodystrophy (TTD).

Hanawalt noted that there are important differences in the efficiency of GG-NER in rodent cells compared to human cells—rodent cells lack expression of the *XPE* gene, which codes for a component of a DNA damage-binding activity. The reduced GG-NER efficiency in rodents may account for the higher rates of mutagenesis and carcinogenesis compared to humans following exposure to carcinogens such as UV. This difference in GG-NER capability in somatic cells establishes that rodents may be imperfect surrogates for humans in certain mechanisms of genetic toxicology.

Hanawalt also summarized recent progress in understanding the role of NER in murine spermatogonial cells. Overall, the results of these studies suggest that NER activity is cell-type specific [Xu et al., 2005]. Although all cell types were proficient in TCR, A- and B-type spermatogonia displayed low-to-moderate ability to repair the test lesion (UV-induced cyclobutane pyrimidine dimers) on both strands of expressed genes. GG-NER was more efficient in mouse primary keratinocytes than in the spermatogenic cell types. Also, GG-NER activity appeared to decrease with age in postmeiotic cells. Variation in DNA repair capacity may alter the susceptibility of individual parents to germ-cell mutagenesis after exposure to environmental mutagens.

Clinical and Economic Aspects of Mutation Detection

Robert P. Erickson, University of Arizona Health Science Center Robert Erickson reviewed current issues in implementing molecular genetic assays in clinical medicine. High-throughput molecular genetics has changed the face of medical research dramatically, but it has perhaps had less of an impact on clinical medicine than one might expect [Tischfield et al., 2005]. For example, it is technically feasible to use gene-chip methodology to screen for any of several hundred possible mutations that cause cystic fibrosis [Schrijver et al., 2005]. However, the logistics of gene-chip development are difficult. The cost per chip is high because of the large number of patents on DNA sequences and DNA-based technologies. Furthermore, there appears to be significant resistance among clinical professionals to increased use of molecular diagnostics. Erickson predicted that efficient gene-chip technology will eventually emerge and be widely used to rapidly screen for genetic defects, but this implementation will occur only after many existing DNA patents have expired and chip costs drop significantly.

Another factor that limits the use of molecular diagnostics in clinical settings is the fact that insurance companies are reluctant to pay for these tests. This attitude may change as therapeutic intervention options increase because insurance companies prefer to pay for tests whose results will influence the selection of therapy and, thereby, possibly improve prognosis. Erickson encouraged wider future attention to developing tests for genetic mosaicism in somatic tissue because mosaicism often goes undetected and is known to be potentially important in determining the tissue distribution and severity of human genetic disease.

Feasibility of Epidemiologic Studies

John D. Boice, Vanderbilt University Medical Center John Boice reviewed epidemiological studies of heritable disease phenotypes in exposed human populations, including atomic bomb survivors, radiation workers, individuals exposed to diagnostic X-rays, individuals exposed to high levels of environmental radiation, and cancer survivors [Boice et al., 2003; Winther et al., 2004; Rahu et al., 2006]. These studies looked at many health indicators, e.g., adverse pregnancy outcome, sex ratio, childhood cancer, death of offspring, cytogenetic abnormalities, and minisatellite mutation rate. Approximately 80,000 individual offspring were included in the study of atomic bomb survivors alone. On the whole, the results were negative. The 2001

United Nations UNSCEAR panel concluded that “no radiation-induced genetic diseases have so far been demonstrated in humans ...[therefore] estimates of risk have to be based on mouse experiments” [United Nations, 2001]. Boice argued that doses below 0.2 Gy (20 rad) are unlikely to double the risk of an adverse pregnancy outcome, and UNSCEAR estimated the genetic risk of heritable disease to be ~0.2% per Gy and the doubling dose ~1 Gy (100 rad).

In contrast to the absence of evidence for radiation-induced heritable disease in humans, there is evidence of radiation-induced chromosome aberrations in somatic cells of exposed persons. For example, although offspring of cancer survivor cohorts do not show increased cancer rates, cancer survivors themselves show strong evidence of exposure-induced secondary cancers [Curwen et al., 2005]. One explanation for the differences seen between studies of heritable mutations vs. somatic mutations is that studies assessing heritable disease phenotypes in exposed human populations may have lacked sufficient power and dose range, or failed to measure appropriate outcomes. In addition, affected offspring may elude detection because they may be eliminated by natural biological processes such as early miscarriage.

Epidemiologists are continuing to look for evidence of effects on the rate of heritable genetic disease in offspring of human cancer survivors [Tawn et al., 2005]. A large-scale international collaboration is currently conducting a demonstration project focused primarily on adverse pregnancy outcomes. Analysis of chromosome abnormalities will be carried out in offspring, and selected trios will be studied using molecular methods, including minisatellite characterization. Variation in radiation response will be examined using in vitro cytogenetic assays in conjunction with gene profiling. The studies are designed to quantify the dose of chemotherapy administered and to calculate precisely the gonadal dose received by each exposed individual. This study is expected to provide definitive answers to questions about the integrity of the germline in human populations exposed to mutagenic chemotherapies.

Risk Assessment

K. Sankaranarayanan, Leiden University Medical Center K. Sankaranarayanan discussed the approaches for assessing human genetic risk after exposure to ionizing radiation. He pointed out that the existing paradigm is based on the assumption that adverse effects of radiation will be manifested in the progeny of exposed individuals as genetic diseases distributed similarly to those that occur naturally in the population. Because human data are extremely limited, risk is generally estimated using three components: the doubling dose for radiation-induced germ-cell mutations in mice, the background rate of “spontaneous” genetic disease in humans, and population genetics theory. Recent estimates suggest that the genetic risk associated with chronic, low-dose irradiation is ~4,000 affected cases per million births per Gy. This rate represents 0.4–0.6% of the baseline frequency of affected births (738,000 cases per million births). The baseline estimate includes chronic multifactorial diseases in the population (650,000 per million; mostly of adult onset); congenital abnormalities (60,000 per million); Mendelian diseases (24,000 per million); and chromosomal diseases (4,000 per million).

Recent studies in experimental systems demonstrate that most radiation-induced mutations are large DNA deletions encompassing multiple genes. Thus, it is reasonable to assume that many radiation-induced deletions cannot be recovered and characterized in offspring because they are lethal during the early stages of development. Further, large DNA deletions that are viable are likely to cause developmental abnormalities in multiple organs/systems.

Sankaranarayanan described a recent approach for predicting the rate at which nonlethal radiation-induced multi-gene deletions should occur in the mouse or human genome [Sankaranarayanan and Wassom, 2005]. This analysis is based on a molecular understanding of the mechanisms by which such deletions occur and the distribution of DNA repetitive

sequences, e.g., segmental duplications or low-copy repeats (LCRs) in the mouse and human genomes.

It is thought that a large fraction of the biological impact of radiation at the cellular level is due to misrepair or lack of repair of radiation-induced double-strand breaks (DSBs). DSBs are repaired by three pathways in mammalian cells: homologous recombination repair (HRR); NHEJ; and single-strand annealing (SSA), which is an error-prone variant of HRR. Another error-prone form of HRR, namely, nonallelic homologous recombination (NAHR), which occurs between misaligned LCRs in germ cells during meiosis, underlies the origin of large deletions in human genetic diseases. Sankaranarayanan proposed that germ cells favor NAHR-dependent DSB repair, that LCRs are hotspots for radiation-induced deletions, and that detailed analysis of genome architecture should allow one to predict the sites where a multigene deletion mediated by a pair of LCRs will not be lethal. It may be possible to test these ideas in experimental studies with mice and through molecular analysis of human fetuses or neonates. Additional analysis of the distribution of LCRs in the human and mouse genome also is needed. Sankaranarayanan suggested that these data and available bioinformatics tools could be used to predict the expected rates of NAHR-mediated radiation-induced deletions that can be recovered in human live births.

OPEN DISCUSSION SESSION 1

Integrating New Technologies in Germ-Cell Mutagenesis

Discussion leaders: Joe Gray, Lawrence Berkeley Laboratory; Sally D. Perreault; US Environmental Protection Agency; and Colin H. Collins, University of California, San Francisco In this session, the Workshop participants discussed ways in which new molecular technologies, currently being used to address the detection of mutations in somatic and other cell types, could be applied to the field of germ-cell mutagenesis. Gray presented a list of technologies for assessing DNA and genomic defects, and related them in a matrix to the biological endpoints that could potentially be measured or analyzed with those technologies, and their detection capabilities (Table II). The technologies evaluated included PCR-based sequencing, PCR-based conformation analysis, sequencing by hybridization, ESP [Volik et al., 2003,2006], high-throughput sequencing, primer extension, FISH, CGH, high-throughput LOH, optical mapping, genome subtraction, expression arrays [Wang et al., 2005], Serial Analysis Gene Expression (SAGE, both RNA, and DNA), chromatin immunoprecipitation assays, protein lysate arrays, 1D and 2D gel electrophoresis, mass spectrometry, and computational biology. Many of the advantages and limitations of these technologies were presented by earlier speakers.

Colin Collins (UC, San Francisco) noted that ESP is a relatively new technology that can clone and map many kinds of chromosomal defects, e.g., deletions, insertions, translocations, inversions, and copy number changes, in a single step [Volik et al., 2006]. The resolution of ESP is relatively high (10 kb), and this powerful technology could be useful for identifying and characterizing environmentally induced germ-cell mutations. The two main disadvantages of ESP are its high cost and the expertise required for BAC library construction. The biological parameters that might be analyzed by ESP included genome-wide mutation rate, chromosome aberrations, multigenic disorders, locus-specific mutation rate, DNA methylation, genetic mapping, gene function, and gene expression profiling. On the other hand, the cost of high-throughput DNA sequencing, a rapidly advancing technology, has dropped dramatically (now ~US \$1/1,000 bp), making it particularly promising for investigations of genome-wide DNA sequence alterations in the children of parents with induced germ-cell mutations.

An animated discussion on the advantages and limitations of the methods listed in Table II concluded this Session, and the two primary recommendations were:

1. A large-scale collaborative project needs to be initiated that applies new genomic analysis technologies to the measurement of induced heritable mutations in the offspring of human populations who have received a well-characterized germ-cell exposure from either radiation or chemicals.
2. A bio-bank should be established to serve as a central resource for tissue samples from exposed populations for use by the germ-cell mutagenesis research community.

In conjunction with the recommendation for immediate initiation of a large-scale collaborative study and establishment of a bio-bank to support such a study, the participants also raised several other important issues for consideration.

1. A well-characterized exposure regimen should be selected as a focus of the large-scale collaborative project; radiation and mutagenic cancer chemotherapeutic exposures were suggested for prime consideration.
2. A battery of mutagen-detection systems should be used to test for the broad spectrum of genomic abnormalities that are likely to be associated with exposure-induced germ-cell mutations. This recommendation is based on extensive animal studies showing that multiple types of genomic damage can result from single-agent exposures. Depending on the exposure agent selected, the test battery should include methods to detect random genomic damage of the following types:
 - DNA sequence mutations (base-pair substitution in coding and noncoding regions and various repeat sequence mutations). The average spontaneous base-pair mutation rate in the human genome is $\sim 1 \times 10^{-7}$ per cell per generation. Thus, technologies for detecting induced germ-cell mutations must be able to measure changes at a rate of 1×10^{-7} bp.
 - Chromosome aberrations including partial chromosomal deletions, duplications, and rearrangements;
 - Whole chromosomal aneuploidies;
 - Changes in chromatin modifications; and
 - Changes in gene expression.
3. Improved database resources are needed to provide researchers ready access to data and information. These resources could be used to make results obtained by various mutation-detection technologies available for comparison and contrast by researchers from different laboratories using samples from the same central bio-bank.
4. A complementary mouse model needs to be employed to mimic and manipulate experimental conditions to overcome the relative difficulties of determining exposures, controlling variables, and obtaining samples of human subjects.
5. Only a subset of agents induce mutations in spermatogonial stem cells (as shown in Fig. 1). Thus, sperm analysis might be useful to prescreen candidate chemotherapy exposure regimens for those that induce stem-cell mutations (e.g., gene mutation, chromosomal rearrangements; see Table I). However, results from sperm analyses cannot be extrapolated to oocytes because of the unique biologies of spermatogenesis and oogenesis, and separate methods would be needed to study effects of environmental exposures on oocytes.

Meeting participants discussed the use of laboratory rodent and transgenic models in germ-cell mutagenesis and the assessment of heritable risks. Transgenic models using reporter genes have the relative advantage of measuring gene mutation directly in sperm; however, such

models permit only gene (and not chromosomal) mutations to be measured; the reporter gene is not expressed [Lambert et al., 2005].

OPEN DISCUSSION SESSION 2

Research Recommendations for Assessing Mutations in the Postgenome Era

Leader: John J. Mulvihill, University of Oklahoma Health Sciences Center This session was devoted to developing proposals for future directions in germ-cell mutagenesis research. In his opening comments, Victor A. McKusick (Johns Hopkins University) provided a historical perspective, relating the current task of understanding germ-cell mutagenesis to his personal experience working on the HGP and the Online Mendelian Inheritance of Man database [Hamosh et al., 2005] (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>). The draft human genome sequence and the catalogue of human genetic diseases and associated mutations have proven to be valuable tools for understanding the mutability of the human genome.

John Mulvihill reviewed the list of recommendations for germ-cell mutagenesis research that had been developed at the 1984 meeting in Alta, Utah that presaged the HGP (listed below, http://www.ornl.gov/sci/techresources/Human_Genome/project/alta.shtml) and discussed how they were still relevant today:

- Assured continued funding for human mutation research and related studies.
- Designation of a lead federal agency for tracking progress, facilitating interchange of information, and encouraging coordinated interdisciplinary studies.
- Creation of a repository of biological materials (a bio-bank) from high-risk individuals, their spouses, and children for studies of somatic and heritable mutations; improved specimen-storage technologies.
- Continued studies in animals to define mechanisms of mutagenesis and to elucidate the relationship between mutagenic potency in animals and humans.

Mulvihill pointed out that the new genomic technologies introduced at this workshop provide new avenues for detecting and characterizing induced germ-cell mutations in a manner not previously possible.

Richard P. Woychik (The Jackson Laboratory) discussed the need to understand cell type-, species-, and exposure-dependent differences in mutagenic outcome. Woychik argued that this complexity dictates an integration of different approaches and strategies for understanding and analyzing human germ-cell mutagenesis. Woychik also emphasized the importance of distinguishing the proposed program from other genome-based programs with implications for human mutagenesis [Austin et al., 2004]. Woychik proposed generating a white paper on the mutability of the human germline, whose *raison d'être* would be to increase awareness of issues and concerns in this field. Extensive discussions focused on funding initiatives, resources, and technologies of value to future research efforts, as well as experimental designs for studying human germ-cell mutagenesis.

Several specific research approaches were proposed toward identifying human germ-cell mutagens. Jack A. Taylor (NIEHS) proposed a SNP analyses and resequencing project of a target population of ~100 offspring of childhood cancer survivors plus each of their parents, i.e., 100 trios. He suggested that ~100 genes be resequenced in each of the offspring, and all SNPs recorded and analyzed. If putative novel SNPs were found, then the parental (somatic) genomes would be resequenced to determine if the polymorphic sites were rare variants, pre-

existing in the parental DNA, or if some of them could be attributed to new mutations in parental germ cells.

Harvey W. Mohrenheiser (Oregon Health Sciences University) also strongly advocated using childhood cancer survivors as a cohort for extensive study of genetic changes and heritable genetic disease. By analyzing this exposed population, he argued, the human genetic risk from exposure to mutagens becomes a tangible, real-world problem, and not a theoretical question lacking public health relevance. Mulvihill emphasized that one of the benefits of studying childhood cancer survivors and their offspring is that the levels and conditions of exposure are precisely known from patient medical records. This information greatly increases the potential value of analyzing such an exposed population relative to most other exposed human populations.

Bryn A. Bridges (University of Sussex) proposed a project to investigate whether the minisatellite system is a valid indicator for human germ-cell mutations. He proposed the analysis of minisatellite sequences in exposed human populations, including childhood cancer survivors, as a promising experimental strategy because there is already evidence that increased mutation rate in minisatellites is associated with human exposure to ionizing radiation. He proposed that a system for rapidly and efficiently detecting minisatellite mutations should be developed, automated, and applied to other exposed human populations. The link between exposure and hereditary disease would then be explored by correlating minisatellite mutations and functional coding-sequence mutations in the same exposed individuals. Bridges emphasized that studies will be needed that employ across several dose ranges and conditions, from acute high-dose exposure to chronic low-dose exposure.

George R. Douglas (Health Canada) emphasized the need for parallel studies of mutational mechanisms in mouse models, and he presented data from ENU-treated *lacZ* transgenic mice (Muta mouse) that illustrated the utility of transgenic rodent models for studying the etiology and mechanisms of induced gene mutation in male germ cells [Douglas et al., 1995]. Although the *lacZ* model does not permit the detection of gene mutations in the ovum per se, Douglas described a method to detect *lacZ* mutations in granulosa cells, which provide the immediate environment for the ovum in female mice [Yauk et al., 2005]. Lambert et al. [2005] have reviewed the use of transgenic rodent assays, including their use in germ-cell studies.

George R. Hoffman (Holy Cross College) re-emphasized that the study of germ-cell mutagenesis needs to remain broad-based, encompassing diverse effects such as point mutations, chromosome aberrations, aneuploidy, complex traits, epigenetic effects, and minisatellite variation. A multifaceted strategy is needed on the molecular level, and a similarly broad approach is needed to assess phenotypic effects of germ-cell mutagens.

The following points were raised in subsequent discussions:

- The human germ-cell mutation project must be designed to differentiate hereditary effects from somatic-cell variation and/or mosaicism.
- A comprehensive understanding of human germ-cell biology will be essential for understanding the mutability of human germ cells in males and females, including the effects of age at exposure. This endeavor should include understanding germ-cell, stage-dependent, or sex-specific variation in the genetic risk associated with environmental exposure.
- The mouse remains a valuable model for the study of genetic susceptibility to germ-cell mutations. The Complex Trait Consortium (www.complextait.org) is generating 1,000 recombinant inbred mouse strains from 8 parental inbred strains, which might be a useful resource for studies of susceptibility in germ-cell mutagenesis.

- Animal models also are essential for developing a mechanistic understanding of germ-cell mutagenesis. Thus, it will be important to understand the differences and similarities in the effects of mutagen exposure on mouse and human germ cells, especially with regards to mechanisms of induction, persistence, and transmission to offspring.

WORKSHOP SUMMARY AND RECOMMENDATIONS

Pregnancies and children affected by birth defects and genomic abnormalities stand to suffer from lifelong mild to severe health consequences associated with high familial and societal costs. This workshop addressed the challenges to understanding the causes of these seemingly “random” genomic defects and to identifying the relevant risk factors so that they might be minimized. Affected children often carry de novo mutations (i.e., those not present in somatic cells of either parent), and thus these genomic changes are likely to have arisen in germ cells of one parent or during early development. In stark contrast to cancer, where associations with environmental exposures have been identified and where differences in genetic susceptibility can dramatically alter an individual’s risks, the causes or individual susceptibilities for human birth defects and heritable diseases are largely unknown. Indeed, the status of the research is such that there is still no direct scientific evidence for the existence of transmissible, environmentally induced, human germ-cell mutations, although the indirect evidence from human and animal studies indicates that they should exist. Recent major advances in genome analysis technologies may provide the tools to obtain such evidence, and these rapidly developing technologies were a major motivation for holding this workshop.

Research into the detection of human germ-cell mutagens and the prevention of associated developmental defects and heritable genetic diseases faces three major challenges:

- Understanding the special biology of male and female gametogenesis as it pertains to mutation susceptibility and the risk of damage transmission;
- Developing effective technologies for detecting the broad spectrum of mutations known or predicted to be associated with germ-cell mutagens, developmental defects, and heritable diseases; and
- Initiating research strategies to investigate the induction of germ-cell mutations in exposed human populations.

Male and female germ cells each have a unique biology that influences their susceptibilities to germ-cell mutagens, and these susceptibilities change dramatically throughout the course of germ-cell development, maturation, and fertilization (Fig. 1). In animal studies, the types of mutations seen in offspring depend on the agent and the exact timing of exposure during germ-cell development. Similar specificities have been noted in sperm studies with patients receiving mutagenic chemotherapy. This critical relationship between agent, dose, timing, and outcome was considered by the workshop participants to be of paramount importance for identifying appropriate exposed human populations for germ-cell mutagenesis studies.

The detection of germ-cell mutagens is complicated by the broad spectrum of chromosomal defects and gene mutations known to be associated with birth defects and heritable diseases. Rodent studies have shown that even exposures limited to one germ-cell mutagen often induce a spectrum of transmissible damage and that mutagens can differ dramatically in the types of transmissible damage they induce. The types of transmissible damage include base-pair alterations, repeat-sequence changes, and a variety of chromosomal abnormalities, e.g., duplications, deletions, rearrangements, and aneuploidies. In addition, recent animal studies have shown that altered imprinting patterns, not associated with mutations, can lead to heritable multigenerational defects. Therefore, Workshop attendees emphasized that approaches to

studying germ-cell mutagenesis in humans must remain broad-based, and investigations should include the full spectrum of detectable genetic and chromosomal endpoints.

This workshop highlighted the impressive technological advances for investigating DNA sequence and chromosomal alterations that were developed in conjunction with the HGP (Table II). These technological innovations include major advances in high-throughput DNA sequencing; detection of chromosomal duplications, amplifications, and deletions; gene-transcript profiling; and proteomics. The advantages and limitations of these technologies were discussed in detail.

One of the major issues facing the field of germ-cell mutagenesis is to identify candidate germ-cell mutagens for intensive human study, and several strategies for identifying these were discussed during the workshop. These included animal breeding screens, animal and human gamete analyses (especially defects in sperm genomes, Table I), and epidemiological pilot data. Consistent with the special biology of germ cells, it was emphasized that data from somatic-cell mutagenicity studies cannot be extrapolated directly to germ-cell risk and that the determination of human germ-cell risk requires direct studies of exposed germ cells.

There was consensus on the importance of mounting coordinated animal and human germ-cell mutagenesis studies to explore the impact of important societal concerns, such as exposure to cancer therapy in childhood cancer survivors. It was recommended that such studies be initiated as soon as possible, both in humans and in parallel animal models, using some of the genomic tools currently available. Cancer survivors represent a unique cohort with well-defined exposures and genetic alterations, including base-pair changes, chromosomal alterations, repeat-sequence and minisatellite mutations, and gene-expression profiles. Other types of genomic alterations can be measured in their offspring, using as references both the parent without cancer as well as the parental cancer survivor.

The need to create a bio-bank of human tissue samples, e.g., from cancer patients and their children, also was advocated by attendees. Such bio-banks will be critical in conducting multi-endpoint, comprehensive, collaborative international efforts aimed at detecting exposure-induced heritable alterations in the human genome.

There was also strong support for using animal models of human germ-cell mutagenesis in parallel with studies in humans to provide insights into the biology and biochemical mechanisms of germ-cell mutagenesis.

In contrast to the germ-cell mutagenicity data from animal studies, the following questions regarding human germ-cell mutagens stand unanswered and remain a challenge for the research community:

1. What environmental, occupational, or medical agents increase the risk for human germ-cell mutations, and do they act via direct mechanisms (point mutations, chromosomal alterations, etc.) or indirect, epigenetic mechanisms (methylation, etc.)?
2. For both direct and indirect mechanisms, what is the potential for transmissibility of genomic damage from the affected gamete to a zygote, and what is the effect on the live-born child?
3. Does individual susceptibility affect parental risk to germ-cell mutations, and can we identify individuals with increased susceptibility?
4. What is the differential risk to male vs. female germ cells for the same exposure, and do these risks change with age, diet, and other physiological factors?

The workshop attendees were in strong agreement regarding the need to initiate large-scale collaborative human and animal exposure studies to identify and define the environmental factors that contribute to adverse human pregnancy outcomes and genetic diseases among children. This need is compelling and timely because there is strong animal evidence that exposure to environmental agents can induce germ-cell mutations and heritable genetic disease. There are growing human health concerns from exposures to an increasing complexity of environmental chemicals, and we have, for the first time, new and sensitive tools for genome analyses. We must initiate these studies now rather than wait another 80 years to answer the questions: Are there any human germ-cell mutagens, what risks do they pose to future generations, and are some parents at higher risk for germ-cell mutations than others?

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Fig 1.

Germ-cell stage-specificity for 28 chemicals tested for heritable mutations and dominant lethality in male and female mice. Gametogenic stages are shown in which positive results have been reported in dominant lethal (top solid bar), heritable translocation (middle /// bar), and specific-locus (bottom \\\ bar) tests. A white bar indicates a mild (<25%) response, a gray bar indicates a moderate (25–50%) response, and a black bar indicates a high (>50%) mutation response. Compiled and adapted from Figures in Witt and Bishop [1996] and Marchetti and Wyrobek [2005].

Fig 2.

Comparison of male and female germ-cell differentiation. The occurrence and duration of various cell cycles (e.g., mitosis/meiosis) and cell types (e.g., spermatocytes, oogonia) in gametogenesis for males (spermatogenesis) and females (oogenesis) during developmental stages (e.g., embryo/fetus) are shown. Adapted from Witt and Bishop [1996].

TABLE I

Molecular Technologies for Assessing DNA Damage, Gene Mutations, Chromosomal Aberrations, and Aneuploidies in Human and Animal Gametes

Technology		Major Use	Applications	
Major type	Subtype		Gametes	Species
FISH	Spot probes, multicolor, multiprobe	Aneuploidy and diploidy	Sperm and oocytes	Human, rodent, others
	Spot probes, multicolor, multiprobe	Partial chromosomal duplications and deletions	Sperm	Human (ACM assay), Mouse (CT8 assay)
	Painting, SKY	Chromosomal aberrations	Oocytes	Human, rodent models, other
Single-cell electrophoresis (comet)	Alkaline assay	Single-stranded DNA damage	Sperm	Human, rodent models, others
	Neutral assay	Double-stranded DNA damage	Sperm	Human, rodent models, others
Sperm chromatin structure (SCSA)	DNA fragmentation index (DFI)	DNA strand damage	Sperm	Human, rodent models, others
	High-DNA staining cells (HDS)	Immature sperm chromatin	Sperm	Human, rodent models, others
Allele-specific PCR	<i>FGFR3</i> mutation, achondroplasia	Specific-locus mutation frequency	Sperm	Human
	<i>FGFR2</i> mutation, Apert syndrome	Specific-locus mutation frequency	Sperm	Human
Adducts	DNA and protein adducts	Germ-cell DNA exposure to mutagenic agent	Sperm ^b	Rodent models ^a

^a Analyses with biological accelerator mass spectrometry (bio-AMS) may be feasible for human sperm exposed to certain agents.

^b Possibly also oocytes.

TABLE II

Molecular Technologies for Assessing DNA and Genomic Defects, Using DNA From Mutagenized Parents and Their Children

Technology	Major use	Applicable to single-cell analyses	Genomic resolution
PCR-based assays (sequencing or conformation) ^{a,b}	Single-gene assessment, methylation	Yes	Single base
Sequencing by hybridization	Allelotyping, mutation detection	Yes	Single base
End-sequence profiling (DNA and transcripts)	Structural rearrangements, large-scale resequencing, genome immortalization	No	Single base
Chromatin immunoprecipitation	Antibody-based multiprotein interrogation	No ^a	Single protein
FISH ^b	Numerical and structural chromosomal aberrations	Yes	Megabase
CGH	Copy number gains and losses, allele-specific, methylation-specific	Possibly	10–100 Kbp
High throughput LOH	LOH mapping, gene localization	Possibly	10 Kbp
Optical mapping	Numerical and structural chromosomal aberrations	Yes	100 bp
Genome subtraction	Mapping and cloning of lost or gained regions	Possibly	Megabase
Expression arrays	Comprehensive, semiquantitative expression, splicing	Yes	Single exon
SAGE (RNA and DNA)	Comprehensive, semiquantitative expression, splicing	No	Single gene
ChIP	DNA binding proteins, chromatin structure	No	Single gene
Protein lysate arrays	Antibody-based multiprotein interrogation	No ^a	Single protein
1D and 2D gel electrophoresis	Mutant protein detection	No	Single protein
Mass spectrometry	Qualitative protein composition	No	Single protein

^a ~100 cells are needed.

^b Technologies adapted to the analyses of genomic defects in human and animal sperm.