

Meeting Report

Assessing Human Germ-Cell Mutagenesis in the Post-Genome Era:

A Celebration of the Legacy of William Lawson (Bill) Russell

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ABSTRACT

Although numerous germ-cell mutagens have been identified in animal model systems, to date, no human germ-cell mutagens have been confirmed. Because the genomic integrity of our germ cells is essential for the continuation of the human species, a resolution of this enduring conundrum is needed. To facilitate such a resolution, we organized a workshop at The Jackson Laboratory in Bar Harbor, Maine on September 28-30, 2004. This interactive workshop brought together scientists from a wide range of disciplines to assess the applicability of emerging molecular methods for genomic analysis to the field of human germ-cell mutagenesis. Participants recommended that focused, coordinated human germ-cell mutation studies be conducted in relation to important societal exposures. Because cancer survivors represent a unique cohort with well-defined exposures, there was a consensus that studies should be designed to assess the mutational impact on children born to parents who had received certain types of mutagenic cancer chemotherapy prior to conceiving their children. Within this high-risk cohort, parents and children could be evaluated for inherited changes in (a) gene sequences and chromosomal structure, (b) repeat sequences and minisatellite regions, and (c) global gene expression and chromatin. Participants also recommended studies to examine trans-generational effects in humans involving mechanisms such as changes in imprinting and methylation patterns, expansion of nucleotide repeats, or induction of mitochondrial DNA mutations. Workshop participants advocated establishment of a bio-bank of human tissue samples that could be used to conduct a multiple-endpoint, comprehensive, and collaborative effort to detect exposure-induced heritable alterations in the human genome. Appropriate animal models of human germ-cell mutagenesis should be used in parallel with human studies to provide insights into the mechanisms of mammalian germ-cell mutagenesis. Finally, participants recommended that

scientific specialty groups be convened to address specific questions regarding the potential germ-cell mutagenicity of environmental, occupational, and lifestyle exposures. Strong support from relevant funding agencies and engagement of scientists outside the fields of genomics and germ-cell mutagenesis will be required to launch a full-scale assault on some of the most pressing and enduring questions in environmental mutagenesis: Do human germ-cell mutagens exist, what risk do they pose to future generations, and are some parents at higher risk than others for acquiring and transmitting germ-cell mutations?

INTRODUCTION

Because the future of the human species resides in our germ cells (egg and sperm), it is vitally important that we determine if environmental factors are adversely impacting the genomic integrity of our germ cells. There is a wealth of data on mutation induction in somatic cells of rodents and humans; however, these data cannot 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. Thus, to accurately assess the impact of environmental mutagens on the genomic integrity of human germ cells, studies must be conducted directly in human populations.

Starting 80 years ago with Herman Muller's discovery in 1927 of the first germ-cell mutagen (ionizing radiation) in an animal (), studies in various animal model systems have shown that certain physical and chemical mutagens can induce heritable mutations, i.e., mutations in the germ cells that are transmitted to offspring. However, despite the similarities in many aspects of germ cell biology among humans and mammalian model systems, studies during the past 80 years have failed to find clear and convincing evidence of environmentally induced heritable mutations in humans.

Beyond the unlikely possibility that there are no human germ-cell mutagens, the reasons for this disparity between human and animal studies are many: insufficient numbers of human subjects; insensitive methods to detect mutations; the multiplicity of DNA and chromosomal defects induced by individual mutagens; inadequate length, intensity, or type of exposure; and inadequate dosimetry. Nonetheless, to date, there are still no confirmed, universally agreed-upon mutagens that produce transmissible human germ-cell mutations—not one.

However, recent advances in genomic biology and biotechnology now offer the unprecedented opportunity to apply new technologies and molecular methods to the critically important questions regarding the existence of human germ-cell mutagens and the heritable risks they might pose for the next generation. With this in mind, we organized a workshop titled “Assessing Human Germ-Cell Mutagenesis in the Post-Genome Era” at The Jackson Laboratory in Bar Harbor, Maine on September 28-30, 2004. Our intent was to foster a dialogue among scientists in the fields of germ-cell mutagenesis and genomics, with the goal of encouraging 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 B. Russell, died. We dedicated this workshop to his memory and legacy of research in this field.

The workshop was attended by 98 scientists, and their presentations and discussions reviewed the special biology of germ cells as well as the epidemiological and sperm-based evidence for environmentally induced de novo human germ-cell mutations. Participants also provided an update and evaluation of the new genomic technologies available for measuring random DNA sequence mutations, chromosomal alterations, and changes in gene expression across the human genome. Participants made suggestions for research strategies and technical developments that could aid in addressing the existence of and risk associated with exposure to human germ-cell mutagens.

We anticipate that the topics and issues discussed in this workshop report will help to spark more intense collaborations among scientists in the fields of genomics and germ-cell mutagenesis. We trust that the urgency for such collaborative efforts will be recognized by scientists and funding agencies alike, such that we will not have to 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 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. 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 (from inbreds into their F1 hybrids) to study the influence of the prenatal environment on phenotypic variability among genetically uniform mice. He then developed the mouse specific-locus test, which allowed quick and objective detection of visible recessive mutations among any of seven loci. Russell utilized the specific-locus test to study 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 pre-mutational damage. Russell and coworkers explored the roles of sex, parity, age, and (most importantly) germ-cell stage on mutation rate and type. 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 the most important legacy of his work in germ-cell mutagenesis—his discovery that ethylnitrosourea (ENU) is a potent germ-cell mutagen in the mouse. ENU is still used extensively in a wide variety of mutagenesis studies because of its propensity for generating point mutations and not insertions/deletions or chromosomal rearrangements.

Key discoveries by the Russells regarding the nature of mutations detected in mouse spermatogonial cells following specific chemical or radiation exposures revealed that each chemical had its own characteristic germ-cell stage-sensitivity pattern, and all chemicals, as well as radiations, produced primarily large lesions, e.g., deletions and inversions, in post-spermatogonial stages but not in pre-meiotic or meiotic stages. Furthermore, the Russells

discovered that the locus-spectra of spontaneous mutations identified in mouse sperm, many of which were shown to originate during the perigametic interval, differed from the ENU-induced spectra.

Liane Russell concluded with a discussion of future prospects in germ-cell mutagenesis research. She expressed the hope that new high-throughput, genomics-based molecular tools would lead to rapid progress in understanding unresolved questions about 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, Oak Ridge National Laboratory

John S. Wassom posed one of the central questions in the field of germ-cell mutagenesis: "Do environmental agents induce heritable mutations in humans?" and summarized published and unpublished evidence from 1960 to the present. The lack of a definitive answer to this question was a significant motivation for this workshop. He echoed Liane Russell in emphasizing the need for developing new strategies employing recently developed genomic biotechnologies to identify and characterize environmental agents that have the potential of inducing heritable mutations in humans.

The Human Perspective

John J. Mulvihill, University of Oklahoma Health Sciences Center

John J. 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 argued that an as-yet-undefined fraction of hereditary human disease is attributable to the latter. He acknowledged the lack of definitive evidence for environmentally induced germ-cell mutations in humans, and he proposed that cohort studies that monitor sentinel phenotypes and other endpoints would be more advantageous than case-control studies to understand human germ-cell mutagenesis and its role in human disease. Sentinel phenotypes 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. Molecular approaches are currently available in animal models that allow for direct measurement of aneuploidy, chromosome aberrations, inversions, deletions, copy number changes, and point mutations; some of these are immediately applicable for human studies.

Mulvihill described two available cohorts and their advantages for future molecular studies: (1) 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 has been observed in the offspring of atomic bomb survivors. Studies of cancer survivors are ongoing. Cancer survivors are particularly advantageous because they are numerous and, most importantly, because the timing and dose of their exposure to radiation and/or mutagenic chemicals is accurately documented. Initial studies of the frequency of birth defects in offspring of cancer survivors have shown no significant differences from the average frequency in the US population. 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 and resting oocytes at a frequency high enough to be detected over the background of spontaneous mutations, but larger studies are needed.

History of Germ-Cell Mutagenesis

R. Julian Preston, US Environmental Protection Agency

R. Julian Preston reviewed the history of research on germ-cell mutagenesis and the current paradigm for human risk assessment. Early data on germ-cell mutagenesis generated in mouse specific-locus tests conducted in the 1950s and 1960s demonstrated the impact of fundamental aspects of germ-cell biology on germ-cell mutagenesis. These studies established that dose, dose-rate, sex, germ-cell stage and radiation quality influence experimental outcomes, and the results were species-specific and difficult to extrapolate in a quantitative manner from one species to another. Preston emphasized the role of germ-cell stage in determining specific sensitivities for various mutagenic outcomes. Because the exact timing of germ-cell maturation is in itself 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.

One approach to the identification of germ-cell mutagens is by direct analysis of chromosome aberrations in human and animal sperm. 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. In general, there are significantly fewer data on chemically induced compared to radiation-induced germ-cell mutagenesis in both humans and experimental animals.

Recent studies have reported increased minisatellite instability in human and animal germ cells exposed to ionizing radiation. The significance of these data is not yet clear, and more studies are needed to assess their implication for human risk assessment. Finally, Preston emphasized that more studies 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. Because the mechanisms underlying these processes vary during gametogenesis, germ-cell stage is a critical determinant of the effects of exposure on the frequency of offspring with heritable defects.

How Genome Sequence Impacts Germ-Cell Mutation and Health

Robert K. Moyzis, University of California, Irvine

Robert K. Moyzis gave a historical overview of the landmark Human Genome Project (HGP) and its relationship to the field of human germ-cell mutagenesis. Referring to deliberations at the 1984 Alta Conference, the 1986 Sante Fe meeting, and the 1988 Cold Spring Harbor meeting, Moyzis explained that the HGP initially arose out of concern for the potential impact of environmental mutagens on the integrity of the human genome. However, with time, the HGP changed its focus to concentrate on large-scale comparative genomics and DNA sequencing technology, the development of model organisms for genomics studies, and the ethical implications of human genomics research.

Although there is one organizational structure of the human genome, there are ~6 billion unique sequence variations of this genome. This diversity among individuals contributes to differences in susceptibility to disease. The completion of the human genome map has led to rapid progress in understanding the DNA sequence changes underlying rare, single-gene Mendelian disorders. Common polygenic diseases have presented a greater challenge, but technological advances now enable scientists to use a pathway approach to study such diseases. Moyzis illustrated the pathway approach using attention deficit hyperactivity disorder (ADHD).

Because this behavioral syndrome involves altered neurological function, Moyzis selected a handful of genes that encode neurotransmitter receptors to screen for disease-associated variants. He discovered that one of the three most common alleles of dopamine receptor D4 (DRD4), the 7R allele, is enriched ~2-fold in individuals with ADHD. This allele carries a 7-mer VNTR (variable number of tandem repeats) in exon 3, unlike the most common variant, which has a 4-mer VNTR repeat, or the third most common variant, which has a dimer VNTR repeat. The 7R allele is not distributed randomly worldwide, being more common in individuals from North and South America. Haplotype analysis of the 7R and other alleles showed uncommonly high linkage disequilibrium for the 7R allele. This led Moyzis to propose that the 7R allele arose relatively recently in evolutionary history (50,000 years ago) and was driven to high population frequency by positive selection. 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.

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 versus Human, Male versus 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 B. Bishop presented broad overviews of germ-cell biology and its relevance to the susceptibility of germ cells to environmental mutagens. Common themes discussed in both talks were sexual dimorphism and the unique characteristics and susceptibilities of distinct germ-cell stages. The time line for the production and maturation of germ cells is dramatically different in female and male mammals. For all mammals, female germ cells complete meiotic prophase I during fetal growth and remain arrested in diplotene until the onset of puberty. In contrast, male germ cells undergo mitosis prior to birth but do not undergo a meiotic division until puberty. In human females, all germline mitotic divisions are completed within days during embryonic development; completion of meiosis I requires years and does not begin until puberty; and completion of meiosis II occurs within hours after fertilization. There is limited mitotic proliferation in females, and the number of female germ cells is limited. In human males, mitotic proliferation begins near the time of puberty and continues throughout life well into senescence. Spermatogonial stem cells undergo asymmetric division, generating a stem cell and germ cells that undergo numerous mitotic divisions over several weeks, followed by two meiotic divisions that occur within a few hours of each other.

Handel emphasized the sex-specificity of checkpoints during germ-cell maturation and Bishop emphasized the sex- and stage-specificity of DNA repair capacity. The relative competence of the various germ-cell stages, both for checkpoint control of the cell cycle and for DNA repair, has a clear impact on 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 post-meiotic stages, while post-meiotic female germ cells retain the capacity for DNA repair. Bishop stressed

the importance of being aware of which germ cell stage(s) are exposed and which are sampled when designing experiments to assess the impact of environmental agents on germ cells.

Handel reported on a large-scale joint initiative between investigators at the Jackson Laboratory and Cornell University to identify genes required for meiosis and fertility in mice. Mice with meiotic gene mutations were generated by treating males with ENU. The progeny of G2 backcrosses were then screened for infertility. Approximately 11,000 mice have been tested for fertility defects to date. Thirty-six mutations have been identified: 25 of these resulted in male infertility, 8 caused infertility in both sexes, and 3 resulted in female infertility. This sex bias might indicate a higher level of complexity in male gametogenesis than in female gametogenesis, or it could suggest greater pleiotropy in the action of genes required for female reproduction. Molecular studies indicate that many of these mutants have defects that affect either mitotic proliferation of primordial germ cells or meiotic mechanisms of chromatid cohesion and recombination. Future studies will include testing the epistatic relationship of these mutants to mice with previously characterized defects in meiosis. 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 discussed DNA repair capacity and mechanisms in mammalian germ cells and during the early stages of embryogenesis. DNA repair capacity varies with developmental stage; thus, the relative timing of the induction of DNA damage, DNA replication, and DNA repair strongly influence the potential impact of DNA damage on germ-cell and embryo viability.

Oocytes have a high DNA repair capacity, and they retain maternal protein and mRNA, including abundant DNA repair proteins and transcripts encoding DNA repair proteins. However, the protein and mRNA complement, as well as the transcriptional profile of the fertilized oocyte, changes significantly during the earliest 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 in the early embryo. This is particularly important for the male genome, which has not undergone DNA repair for several weeks prior to fertilization. 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.

Marchetti examined the role of specific DNA repair pathways in repairing radiation-induced DNA lesions during early embryogenesis by mating irradiated male mice with unirradiated female mice that were deficient for genes involved in homologous recombination-dependent double-strand DNA break repair (HR-DSBR) or non-homologous end-joining (NHEJ) repair. His experiments revealed that both 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 were conducted to demonstrate that a p53-dependent S-phase checkpoint 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.

Mosaicism and Germ-Cell Mutagenesis

Harvey W. Mohrenweiser, Oregon Health Sciences University

Harvey W. Mohrenweiser discussed the potential impact of mosaicism (genetic heterogeneity caused by mutations that arise after fertilization during embryogenesis) on phenotypic and genotypic variation in mammals. Some mosaicism is the result of normal biological processes, including genomic imprinting, X-inactivation, and DNA methylation. Some aberrant processes 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 may also arise from chromosome aberrations occurring during mitotic cell divisions. The fraction of embryonic tissue affected by such mutations depends on the stochastic distribution of cells during embryonic development. The greater the fraction is of affected cells, the greater the potential for an adverse outcome.

Mohrenweiser estimated that as many as 10-20% of mutations in a given gene can be attributed to events occurring during early embryogenesis; the remaining 80% of new mutations are inherited from the parental germline or arise postnatally. When more than one offspring of the same parent is affected by the same disease, and neither parent carries the mutant allele in their somatic cells, then 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 high frequency in human embryos produced using assisted reproductive technology (ART). For

example, ~75% of ART embryos on day 2 show mosaicism and 100% of ART embryos in more advanced stages have some mosaic character. These frequencies likely 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 germline 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

Norman Arnheim, University of Southern California

Detecting Chromatin, DNA and Chromosomal Abnormalities in Sperm

Andrew J. Wyrobek, Lawrence Berkeley Laboratory

Norman Arnheim and Andrew J. Wyrobek described recent advances in molecular detection of gene mutations, DNA damage, and chromosome defects in human sperm. A major advantage of analyzing sperm directly versus groups of offspring is that a much larger number of gametes per individual can be assessed for genomic defects compared with the 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. The main limitation of sperm assays is that they are restricted to paternal exposures. In addition, 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.

Arnheim described several PCR- and direct sequencing-based assays that measure mutant frequency at a specific nucleotide in the fibroblast growth factor receptor genes *FGFR3* or *FGFR4*, which are associated with dominantly inherited Apert syndrome and achondroplasia, respectively. The two specific nucleotides have a mutation frequency that is much higher than the average, and mutations in these two genes occur predominantly in the paternal genome at the same nucleotide. Quantitative, allele-specific PCR is used in these assays to amplify specific mutant alleles with a sensitivity of approximately 10^{-5} . To use this method successfully to detect Apert syndrome mutations, a DNA sample of ~5 μg is required; however, to detect a more typical mutation at 10^{-8} , ~5 mg of DNA would be needed, which is not 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 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 (BEAMing).

Wyrobek described three molecular techniques for analyzing DNA damage and cytogenetic defects directly in human sperm: sperm comet assay, sperm chromatin structure assay (SCSA), and sperm fluorescence in situ hybridization (FISH). 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. 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 are thought to represent the degree of DNA fragmentation per specimen and the proportions of sperm with immature (no protamines) chromatin.

Several human sperm FISH methods have been developed to detect aneuploidy and structural chromosomal alterations, including breaks as well as partial chromosomal duplications and deletions. Multi-color FISH has been applied to detect aneuploidies for multiple chromosomes 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 YYY, as well as other autosomal trisomies. Most of these human sperm assays have already been adapted for studies in animal models.

Wyrobek summarized studies that have applied sperm FISH technologies to assess the effects of cancer chemotherapy, occupational exposures, and lifestyle factors 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. 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. Wyrobek summarized the results of a large study of the association between advancing male age and the levels of physiological and genetic damage in sperm. Results showed that advancing male age was strongly associated with detrimental effects on motility, DNA fragmentation, chromosomal aberrations, DNA damage, and gene mutations in sperm, but not with aneuploidy frequency.

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 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 exposures, in assessing the relative risks of somatic versus 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 studies of the human oocyte genome using FISH, spectral karyotyping (SKY) and comparative genomic hybridization (CGH). The majority of these studies were carried out with unfertilized human oocytes generated by assisted reproductive technology (ART). FISH and CGH analyses indicate aneuploidy rates from 20 to 52% in human oocytes. In contrast, studies using conventional chromosome analysis methods estimated a lower frequency of 11%. These differences may be associated with maternal age-effects. The extent of total aneuploidy and specific types of chromosome abnormalities strongly increased with age of the female donor. Eichenlaub-Ritter proposed that age-related differences in gene expression during oogenesis are important factors in susceptibility to meiotic error. For instance, 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 in human oocytes.

Eichenlaub-Ritter also described studies of chemically induced errors in chromosome distribution in mammalian oocytes, using enhanced polarizing microscopy, a non-invasive technique that reveals changes in spindle formation that predispose to nondisjunction. This method can be combined with other more conventional methods to analyze the impact of hormones, life style, age, or environmental exposure on the frequency of chromosomal abnormalities in human oocytes that are fertilized by intra-cytoplasmic sperm injection (ICSI) in assisted reproduction. Such retrospective or prospective non-invasive 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. Furthermore, an in vitro mouse ovarian follicle culture method was described for modeling the growth, maturation, and ovulation of oocytes. This technique may facilitate the assessment of risk to mammalian oocytes within follicles from acute, sub-chronic, 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 identification of targets of drug action in the somatic compartment or in the germ cell itself. Eichenlaub-Ritter pointed out that such models might also 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 A. Taylor discussed studies of human genetic diversity associated with single nucleotide polymorphisms (SNPs). In particular, he described the Environmental Genome Project (EGP), a systematic human genome resequencing project sponsored by the National Institute of Environmental Health Sciences. 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 100 cell cycle and DNA repair genes. These genes were resequenced in 90 ethnically diverse individuals. Approximately 9000 total SNPs were identified, including ~2000 SNPs with a frequency $\geq 5\%$. There were on average 20 common SNPs per gene. Not all possible combinations of SNPs were observed. Instead, SNPs associate with one another into linked groups called haplotypes. On average, there are 3-4 common haplotypes per gene. Many haplotypes are shared across ethnic groups, suggesting that they arose before human ethnic divergence ~100,000 years ago. Taylor suggested that a few SNPs,

each representing a different haplotype, can be used to examine whether the common variants of a gene are associated with disease.

New germline mutations occur on the background of an existing haplotype. Unless there has been recombination, a rare mutation will continue to persist on the haplotype background on which it arose. The common haplotypes of genes may thus also act as a surrogate for the rare mutation and further aid in the discovery of the genetic variant that increases disease susceptibility.

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) and array-based cellular genomic hybridization (CGH). Fridlyand indicated that an ideal method for a whole-genome scan 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 to 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. 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 of oligonucleotide-based arrays are working to develop these for DNA copy number analysis.

Fridlyand described several experiments in which array CGH was used to analyze gene dosage for human and mouse genes. Fridlyand used a 2500-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 had 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 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 the 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 an 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.

In summary, Fridlyand indicated that array-based CGH can be used effectively to detect heritable chromosome aberrations at 50-100 kb resolution, to link a clinical phenotype with a functional change in copy number, and to detect segmental duplications and associated variations

in copy number. These attributes are used successfully in human prenatal testing and can also be used for rapid genotyping of inter-specific backcross mice.

Comprehensive Genome Structure and Transcriptome Analysis for Mutation Detection

Thomas J. Vasicek, Medtronic, Inc.

Thomas J. Vasicek described applications in genome structure analysis using Massively Parallel Signal Sequencing (MPSS), which is a method for evaluating gene expression by counting mRNA molecules in a sample. Individual mRNAs are identified by sequencing 17-20 nucleotides at a unique site in each mRNA. Typically, two million molecules are analyzed from a single sample. This technology can be used for comprehensive genome-wide analyses of the transcriptome.

MPSS has traditionally 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 is also 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 briefly reviewed several new applications for MPSS, some of which could be used to map chromosome rearrangements and other mutational events. 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 end-sequence profiling (ESP): 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. The estimated false-positive frequency for SNPs identified twice in these sequences is 6 per million base pairs (bp).

Bioinformatics Tools in Germ-Cell Analyses

Michael Primig, Biozentrum and Swiss Institute of Bioinformatics

Michael Primig described GermOnline, a bioinformatic knowledge base focused on genes and annotation relevant to mitosis, meiosis, germline development, gametogenesis, and fertility in yeast and higher eukaryotes (see <http://www.germonline.org>). The concept of GermOnline is unique in that the data and annotation are provided, curated, and updated by members of the scientific community. GermOnline was developed at the Biozentrum in Basel, Switzerland and the Swiss Institute of Bioinformatics. It has mirror sites in Europe, Asia, and the US to ensure continuous access worldwide.

GermOnline adopted the mechanism of community-based curation to increase the number of available curators and, therefore, increase the efficiency of database management.

This approach was chosen to solve the problem of the small ratio of curators to data that threatens to overwhelm the IT/bioinformatics community. Several mechanisms are employed to maintain the quality and integrity of the knowledgebase, one of which is oversight by an international board of qualified scientists. Another mechanism to maintain database quality is to enforce 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 integrated knowledge base that provides access to data curated in other molecule- and species-specific databases. GermOnline is unique among integrated databases because it is focused on specific biological components and processes that play roles in germ-cell biology. GermOnline also provides access to microarray and image data relevant to the knowledge base. Future goals for GermOnline include increasing funding, adding capacity to curate data on protein-DNA interactions and SNPs, and adding video and other types of image data.

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 F. Crow presented an overview of past and current studies on the effect of paternal age on human germline mutations—the so-called "paternal age effect." These studies have focused on several well-characterized human disease syndromes such as Apert, achondroplasia, X-linked hemophilia, retinoblastoma and neurofibromatosis. Clinical observations suggest that,

for these diseases, the number of affected offspring increases more quickly than predicted with increasing paternal age. This association suggests that the germline mutation rate increases with paternal age but not with maternal age and that the mutation rate is higher in human males than in females.

Molecular analysis of human or mouse germ cells has confirmed some of these clinical observations. Further, three classes of mutations have been identified that contribute differentially to the paternal age effect: hotspots, insertions/deletions (indels), and base substitutions. When present, as in the genes linked to Apert syndrome and achondroplasia, hotspot mutations tend to occur in males only, and in some cases they increase dramatically with paternal age. However, hotspot 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 argued that base substitutions show a slight paternal bias and a smaller, but significant, paternal age effect than hotspot mutations. The total paternal age effect in a specific gene reflects the relative contributions of base substitutions, indels, and hotspot 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 A. Bridges described several hypotheses that have been proposed to explain the estimated 10-fold excess of childhood cancer that occurred among inhabitants of Seascale, UK between 1950 and 1990. Seascale is near the Sellafield nuclear power plant, and inhabitants of Seascale are known to have been exposed to substantial 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 of other 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 produce enough mutations to account for the excess cancer cases in Seascale.

Subsequent analyses of Seascale data have proposed that two additional factors may have played a role in increasing human cancer rates in the exposed population. The first factor is population mixing, as a surrogate for an infectious agent that increases susceptibility to radiation-induced mutation. The second factor is a possible epigenetic factor that could amplify the mutagenicity of a given radiation dose and cause non-targeted mutations distal from sites of radiation-induced DNA damage, possibly at unstable repeat sequences.

Bridges indicated that no viable explanation for the excess cancer in Seascale has yet been established. Bridges also indicated that other studies of the offspring of exposed human populations remain inconclusive regarding the possible mutagenic effects of radiation exposure. However, Bridges pointed out that these studies might not have been sufficiently comprehensive because they did not evaluate 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 E. Dubrova described studies of the mutation rate in tandem repeat DNA sequences in exposed human populations. The human genome has three types of repeated DNA sequences: minisatellites, microsatellites and extended simple tandem repeats (ESTRs). The spontaneous mutation rate at microsatellite and ESTR loci is several orders of magnitude higher than in the rest of the human genome, and these types of loci account for up to 15% of all gamete genomes. Minisatellites appear to have a high mutation rate in both human somatic cells and germ cells, so 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, fewer samples are needed to detect exposure-induced mutations in these sequences than in single-copy genes. Thus, Dubrova estimated that minimum sample sizes of 240, 2,400 or 240,000 individuals are sufficient to measure induced 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. The linear range of effect 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 single-copy genes, but that they both reflect the same biological response 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.

A small number of studies have analyzed mutations in human repeat sequences in exposed human populations. Dubrova summarized the results of four of these studies: (1) 6 repeat loci in 64 children of Hiroshima bomb survivors, (2) 8 repeat loci in 367 children of Chernobyl survivors, (3) 8 repeat loci in 232 children of residents near the Semipalatinsk nuclear test site, and (4) 8 repeat loci in 338 offspring of Chernobyl cleanup workers. Studies 1 and 4 were negative, and studies 2 and 3 showed significant correlations between paternal but not maternal exposure and mutation rate. Dubrova proposed three factors to explain the negative result in atomic bomb survivors: first, this population generally received smaller radiation doses than the other exposed populations; second, the number of individuals studied may have been too small for detecting significant differences; and third, this study did not distinguish subjects according to paternal or maternal exposure. Dubrova indicated that the negative result in study 4 was expected because Chernobyl cleanup workers were exposed to fractionated radiation doses.

In closing, Dubrova indicated that more studies are needed to determine the effect of chemical or radiation exposure on repeat-sequence variability in the human germline. The initial results reported here are encouraging, suggesting that epidemiological studies with relatively small exposed populations have sufficient power to detect induced mutations in repeat sequences in the human germline. Additional studies will be needed to determine the relationship between the mutation rate in repeat sequences in the human genome and the rate of functional mutations in single-copy human genes. Dubrova's laboratory currently focuses on the comparative effects of radiation on minisatellite mutation rates in a number of exposed cohorts.

Heritable Mitochondrial Mutagenesis

Eric A. Shoubridge, McGill University

Eric A. Shoubridge discussed 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. In most cases, mtDNA is homoplasmic, but in some cases, more than one mtDNA variant co-exists in a single organelle (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.

Many human diseases are associated with mtDNA variants that arise through spontaneous or induced mutations in the mtDNA in somatic or germ cells. The variant mtDNAs can be present as a variable fraction of the total mtDNA, and they cause multiple complex phenotypes that are poorly understood.

Shoubridge used a mouse model to analyze the segregation of mtDNA variants during oocyte development and early embryogenesis. Most mice within a strain are homoplasmic for a single mtDNA variant, but the mitochondrial genome in NZB mice differs at ~100 sites from the mtDNA of BALB/C. Shoubridge generated cytoplasmic hybrid mouse embryos carrying NZB and BALB/C mtDNA and then bred a resulting founder heteroplasmic female, in which ~4% of the total mtDNA was the NZB variant, to a male from a different strain. The pups from this female carried from 0 to 25% NZB mtDNA, but the mean NZB mtDNA frequency was equal to the frequency in the mother. This finding suggests 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; however, these defective 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 M. Trasler described mechanisms for establishing and maintaining patterns of DNA methylation in mammalian genomes. 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 has also 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.

The mechanisms by which DNA methylation patterns are inherited are complex. Most methylation is maintenance methylation, which occurs post-replicatively on hemi-methylated DNA. However, the genome is "reprogrammed" twice, once during gametogenesis and once during embryogenesis. Reprogramming occurs according to different time lines in male and

female gametes. Male progenitor germ cells begin to be methylated before birth and continue to be methylated at some sites after birth. In contrast, female germ cells are reprogrammed primarily after birth. In the somatic tissue of the mouse embryo, reprogramming takes place during days 15-17 of gestation. However, the exact timing of methylation in embryonic cells varies in a gene-specific manner.

Several murine DNA methyltransferases (DNMT) that play roles in and maintenance DNA methylation have been cloned and characterized. DNMT1 is involved in maintenance methylation of hemi-methylated DNA and DNMTs 3A and 3B are involved in methylation during DNA reprogramming. Knockout mice lacking DNMT1 show a lack of imprinting at the 8-cell embryo stage, 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 females. This result confirms the role of DNMT3A in methylation in the mouse germline. DNMT3B knockouts have no defect in gametogenesis, and DNMT3L has no detectable methyl transferase activity; nevertheless, deficiency in this enzyme interferes with maternal imprinting and causes male sterility. The mechanism of this effect is not understood.

Trasler pointed out that little is known about how environmental 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 begin to address this question and to study the consequences of defects in DNA methylation in animal model systems.

Epigenetic Trans-generational Actions of Endocrine Disruptors on the Male Germline and Fertility

Michael K. Skinner, Washington State University

Michael K. Skinner described environmentally induced trans-generational changes in methylation in male germ cells in the rat. Skinner discovered this phenomenon while testing whether the endocrine disruptor, vinclozolin, had adverse effects on testis development and germ-cell differentiation in male rat embryos. When rats were exposed to vinclozolin from embryonic day (ED) 7 to ED15, male pups demonstrated decreased seminiferous cord formation and increased rates of apoptosis in spermatogonial cells. Decreases in sperm motility and sperm number were also 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. When a number of the in utero exposed, sperm-defective but fertile males were inadvertently mated, Skinner discovered that the exposure-induced symptoms were transmitted to the offspring of the affected males. His laboratory demonstrated that these exposed animals acquired a heritable phenotype that was transmitted through at least four generations.

Skinner tested the hypothesis that vinclozolin exposure had disrupted methylation patterns in the male germline of exposed rats. 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 to ED15 caused a permanent epigenetic

reprogramming of specific genes in male germ cells that led to an exposure-induced trans-generational 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.

Subsequent characterization of affected males revealed significant adverse effects in older animals. The late phenotype 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. This study suggests that environmental agents might cause trans-generational effects by an epigenetic mechanism. It also confirms the existence of gender-specific windows of susceptibility in which germ cells and/or germline progenitor cells are susceptible to environmentally induced adverse effects.

Trans-generational Effects of Low-Dose Mutagenesis

Diana Anderson, University of Bradford

Diana Anderson reviewed the evidence that low-dose environmental exposures to radiation and chemicals can induce adverse heritable effects in humans and animals. The evidence for such effects in humans is limited and includes observations of increased cancer prevalence in the vicinity of nuclear power plants or among children of male smokers. Numerous studies of exposed humans have failed to provide evidence of adverse effects, including a study of the rate of birth defects among the offspring of ~18,000 cancer survivors.

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

mutations in rodents. Nevertheless, this association is more ambiguous for low-dose than for high-dose exposures, which cause abundant birth defects in rodents.

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, Anderson showed that the offspring of male rodents acutely exposed to cyclophosphamide had numerous birth defects as well as chromosome abnormalities and increased tumor incidences. In addition, she discussed the results of several low-dose exposure studies. A chronic low dose of 1,3-butadiene increased the frequency of adverse pregnancy outcomes. Recent epidemiological data support the possibility that occupational exposure to 1,3-butadiene induces adverse reproductive effects in humans. In contrast, 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 Livermore National Laboratory

Inherited DNA Repair Defects and Human Disease

Philip C. Hanawalt, Stanford University

Philip C. Hanawalt outlined the multiple cellular pathways for repairing damaged DNA, and he reviewed a number of 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 up-regulate expression of DNA repair enzymes to repair DNA damage. There are three 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 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 which 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. NER targets primarily bulky helix-distorting lesions, and there are two NER repair sub-pathways: global-genome repair (GG-NER) and targeted repair (TC-NER or TCR). Human hereditary defects in NER cause several distinct diseases, including xeroderma pigmentosum (XP), Cockayne syndrome (CS), UV-sensitive syndrome

(UVSS), and trichothiodystrophy (TTD). These syndromes are all characterized by marked sun sensitivity and, usually, a striking clinical and genetic heterogeneity with pleiotropic features, including developmental and neurological abnormalities and variable signs of premature aging. XP patients, defective in GG-NER, have a 1000-fold increase in skin cancer predisposition, whereas CS, UVSS, and TTD patients, defective in TCR, do not have increased cancer risks. In the case of UVSS, sensitivity to sunlight is the only clinical feature, whereas in CS the hallmarks are the neurological and developmental problems. The basis for the difference is unclear because both diseases are characterized by the TCR defect. A current working hypothesis is that UVSS patients retain the ability to carry out some TCR, whereas patients with CS are totally deficient in TCR.

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 *xpc* gene, which codes for a component of a DNA damage-binding activity. The reduced global repair 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 are imperfect surrogates for humans in genetic toxicology studies.

Hanawalt briefly described studies by others on NER in murine spermatogonial cells. Overall, the results suggested cell-type specific NER activity. 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.

Other cancer-prone human diseases caused by DNA repair defects include ataxia telangiectasia, Bloom syndrome, and Werner syndrome. The defects in these human diseases lead to aberrant DNA damage signaling and/or improper processing of DNA repair intermediates. Bloom syndrome and Werner syndrome patients have defects in multifunctional RecQ family proteins with DNA helicase activity. Finally, it was noted that defects in human mismatch repair genes are linked to a hereditary form of colon cancer and to sporadic colon and endometrial cancer. 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 P. 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. For example, it is technically feasible to use gene-chip methodology to screen for any of 1000 possible mutations that cause cystic fibrosis. However, the logistics of gene-chip development are difficult, and 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 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 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. He felt that this advance could have significant impact because genetic mosaicism may currently go undetected as the cause of a significant fraction of human disease.

Feasibility of Epidemiologic Studies

John D. Boice, Vanderbilt University Medical Center

John D. 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. 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." Boice argued that doses below 0.2 Gy (20 rad) are unlikely to double the risk of an adverse pregnancy outcome, and UNSCEAR estimated genetic risk as ~0.2% per Gy and the doubling dose ~1 Gy (100 rad).

In contrast, there is evidence of radiation-induced chromosome aberrations in exposed persons. Although offspring of cancer survivor cohorts do not show increased cancer rates, cancer survivors themselves show strong evidence of exposure-induced secondary cancers. One explanation for these results is that epidemiological studies of exposed human populations to date 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 such natural biological processes 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. 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. Efforts have been made to calculate precisely the gonadal dose received by each exposed individual. Administered chemotherapy will also be quantified. Results of this study may provide definitive answers to questions about the integrity of the germline in exposed human populations.

Risk Assessment

K. Sankaranarayanan, Leiden University Medical Centre

K. Sankaranarayanan discussed several novel concepts that could be used to develop a new or modified paradigm for assessing human genetic risk from 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 progeny of exposed individuals as genetic diseases similarly distributed 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 to 0.6% of the baseline frequency of affected births (738,000 cases per million births). The baseline estimate includes chronic multi-factorial 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). Therefore, among diseases recognized at birth, chronic low-dose radiation may account for 0.5%, which is nearly equivalent to the incidence of chromosomal abnormalities recognized at birth.

Recent studies in experimental systems have demonstrated 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 early stages of development. Further, large DNA deletions that are viable are likely to cause developmental abnormalities in multiple organs/systems.

Sankaranarayanan presented an analysis that can be used to predict the rate at which non-lethal radiation-induced multigene deletions should occur in the mouse or human genome. This analysis is based on molecular understanding of the mechanisms by which such deletions occur and the distribution of non-segmental duplications in the mouse and human genomes. It is thought that a large fraction of the biological impact of radiation on the cellular level is due to misrepair or lack of repair of radiation-induced double-strand breaks (DSBs). DSBs are repaired

by three major pathways in mammalian cells: non-allelic homologous recombination (NAHR), homologous recombination (HR), and non-homologous end-joining (NHEJ). Sankaranarayanan proposed that germ cells favor NAHR-dependent DSB repair and that low-copy repeats (LCRs) are hotspots for radiation-induced deletions. Thus, Sankaranarayanan argued 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 in mice and through molecular analysis of human fetuses or neonates. Additional analysis of the distribution of LCRs in the human and mouse genome is also 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 National Laboratory; Sally Perrault-Darney; US Environmental Protection Agency; and Colin H. Collins, University of California, San Francisco

The goal of this session was to explore how current molecular technologies could be applied to answer key questions in the field of germ-cell mutagenesis. Gray presented a matrix of technologies for assessing DNA and genomic defects and related them to the biological endpoints that could potentially be measured or analyzed with those technologies (Table 1). The most promising technologies included PCR-based sequencing, PCR-based conformation analysis, sequencing by hybridization, end-sequence profiling (ESP), primer extension, FISH,

CGH, optical mapping, genome subtraction, expression arrays, Serial Analysis Gene Expression (SAGE, both RNA and DNA), Chromatin Immuno Precipitation (ChIP) assays, protein lysate arrays, 1- & 2-D gel electrophoresis, mass spectrometry, and computational biology. ESP is a relatively new technology that can clone and map many kinds of chromosomal defects, e.g. deletions, insertions, translocations, inversions, copy number changes, in a single step. 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.

The ensuing discussion explored this matrix in detail, and several important points were raised during the discussion:

- There was a consensus in support of a collaborative project to use these new genomic technologies to measure induced mutation rates in germ cells in a human population with a well-characterized germ-cell exposure. A complementary mouse model system would be essential because of the relative ease of manipulating experimental conditions in mice, in contrast to the relative difficulty of determining exposure and controlling variables in human subjects, and the relative difficulty of obtaining human samples. Although no specific technology was identified, it was suggested that different technologies could be evaluated by expert laboratories using a central source of samples, i.e, a bio-bank. Discussants recommended that a single mutagenic agent be selected as a focus of this project. Because of the extensive knowledge base in animals and humans, radiation exposure was suggested as

the focus for the new project. Offspring of childhood cancer survivors were also suggested as an ideal study population.

- The average spontaneous base-pair mutation rate in the human genome is approximately 1×10^{-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, with error rates below that level.
- Potential germ-cell mutagens should be assessed for ability to act through both genetic and epigenetic mechanisms.
- Molecular techniques should identify various types of mutations, including single-base substitutions, chromosome aberrations, and aneuploidy. Alterations induced in noncoding regions would be more difficult to assess.
- There are advantages to measuring mutation rates directly in sperm DNA, and methods are already available for the analysis of human and animal sperm. Results from experiments with sperm cannot be extrapolated to oocytes, and separate methods would be needed to study effects of environmental exposures on oocytes.
- Meeting participants urged establishment of a bio-bank as a resource for the germ-cell mutagenesis community. Additional and improved database resources were also recommended.
- Meeting participants discussed the relative advantages and limitations of the use of transgenic mouse models in germ-cell mutagenesis and assessment of heritable risks.

OPEN DISCUSSION SESSION 2

Research Recommendations for Assessing Mutations in the Post-Genome Era

Leader: John J. Mulvihill, University of Oklahoma Health Sciences Center

This session was devoted to developing recommendations and proposals for future activities in germ-cell mutagenesis research. In his opening comments, Victor A. McKusick (Johns Hopkins University) provided some historical perspective, relating the current task of understanding germ-cell mutagenesis to his personal experience working on the Human Genome Project and the Online Mendelian Inheritance of Man database. These projects, which generated a draft human genome sequence and a catalogue of human genetic diseases and associated mutations, respectively, generated tools that will be useful in understanding the mutability of the human genome. 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.

John J. Mulvihill noted that many of these recommendations for germ-cell mutagenesis research were first proposed at the 1984 meeting in Alta, Utah that presaged the Human Genome Project (listed below) and were still relevant today.

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Mulvihill pointed out that the new genomic technologies introduced at this meeting 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. Woychik proposed generating a white paper on the mutability of the human germline, whose *raison d'etre* would be to increase awareness of issues and concerns in this field.

Several specific experimental approaches were proposed that could increase our understanding of human germ-cell mutagenesis. Jack A. Taylor (NIEHS) proposed that 100 offspring of childhood cancer survivors and their parents, i.e., 100 trios be used as a target population for SNP analysis. He also suggested that ~100 genes would be resequenced in each of the offspring, and all SNPs would be recorded and analyzed. If putative novel SNPs were found, then the parental (somatic) genomes would be re-sequenced 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) suggested that analysis of minisatellite sequences in exposed human populations, including childhood cancer survivors, is a promising experimental strategy because there is already evidence that increased mutation rate in minisatellites is associated with human exposure to ionizing radiation. Thus, 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. The objective in this experimental strategy is to ascertain whether the minisatellite system is a valid indicator for human germ-cell mutagenesis. Bridges emphasized that studies will be needed across several dose ranges and conditions, from acute high-dose exposure to chronic low-dose exposure.

George R. Hoffman (Holy Cross College) suggested that the study of germ-cell mutagenesis needs to remain broad-based, encompassing such diverse effects 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 also raised in this discussion session:

- Care should be taken to differentiate hereditary effects from somatic-cell variation and/or mosaicism.

- The Complex Trait Consortium (www.complextait.org) is generating 1000 recombinant inbred mouse strains from 8 parental inbred strains. This might be a useful resource for studies of germ-cell mutagenesis in the mouse.
- Animal models will continue to be essential for developing a mechanistic understanding of germ-cell mutagenesis. Thus, it will continue to be important to understand the differences and similarities in the effects of mutagen exposure on mouse and human germ cells.
- A comprehensive understanding of human germ-cell biology will be essential for understanding the mutability of human germ cells. This endeavor should include understanding of germ cell stage-dependent or sex-specific variation in the genetic risk associated with environmental exposure.

WORKSHOP SUMMARY AND RECOMMENDATIONS

Birth defects, genetic diseases, and chromosomal abnormality syndromes occur in approximately 5% of all live births, and affected children suffer from a broad range of lifelong health consequences. Many of these consequences are 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 mitigated. Because affected children often carry de novo mutations, i.e., those not present in either somatic cells of either parent, these genomic changes are likely to have arisen in germ cells of one parent or during early development. In stark contrast to cancer, where the majority of causes are associated with environmental exposures and where differences in genetic susceptibility can dramatically alter an individual’s risks, there is no comparable understanding of the causes or individual susceptibilities for human birth defects and heritable diseases.

Indeed, the status of the research is such that there is still no direct scientific evidence for the existence of a transmissible, environmentally induced, human germ-cell mutation, although the indirect evidence from human and animal studies is increasingly strong. Recent major advances in genome analysis technologies have provided 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: (1) understanding the special biology of male and female gametogenesis as it pertains to mutation susceptibility and risk of damage transmission; (2) 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 (3) initiating research strategies to investigate 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. In animal studies, the types of mutations seen in offspring depend strongly 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 highly 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. 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 Human Genome Project (Table 1). These technological innovations include 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 by the workshop participants. For example, the cost of high-throughput DNA sequencing was noted to have dropped dramatically (now ~US\$1/1000 bp), making it particularly promising for investigations of genome-wide DNA sequence alterations in the children of parents with induced germ-cell mutations.

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), 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 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 exposures, such as the effects of advancing age or 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 reference 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, was also advocated by attendees. Such a bio-bank is critical to conducting a multi-endpoint, comprehensive, collaborative assault aimed at detecting exposure-induced heritable alterations in the human genome. There was also strong support for using appropriate 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 conclusion, based on the wealth of data from animal studies, the following questions regarding the risks for inducing human germ-cell mutations stand to be addressed by the research community:

1. What environmental, occupational, or medical agents increase the risk for germ-cell mutations through classical, direct mechanisms, i.e., point mutations, chromosomal alterations?

2. What environmental, occupational, or medical agents increase the risk for germ-cell alterations through indirect or epigenetic mechanisms?
3. For both direct and indirect mechanisms, what is the potential for transmissibility to a zygote and to a live born child?
4. To what degree does individual susceptibility affect risk, and can we identify individuals with increased susceptibility?
5. What is the differential risk to male germ cells compared with female germ cells for the same exposure?

Because of the relatively high incidence of adverse reproductive outcomes and genetic diseases that characterize the human species, there is a pressing need to identify, define, and control the environmental factors that contribute to these adverse outcomes and genetic diseases. Given the potential magnitude of overall risk resulting from our exposure to the complex mixture of environmental chemicals, it is essential that human studies and parallel animal studies be designed and initiated to provide an accurate estimate for risk of transmissible mutations and to develop approaches to reduce that risk. The technologies are now available to allow us to investigate and address this urgent issue, which has an enormous potential public health impact.

As noted in the Introduction, it has been 80 years since the discovery of the first germ-cell mutagen. Unless we initiate immediately the studies proposed here, we may find ourselves asking the same questions 80 years from now: 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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TABLE I. Technologies for Assessing DNA and Genomic Defects

Genome analysis technology	Major utility	Applicable to single-cell analyses	Genomic resolution
PCR-based assays (sequencing or conformation) ^a	Single-gene assessment, methylation	Yes	Single base
Sequencing by hybridization	Allelotyping, mutation detection	Yes	Single base
End-Sequence Profiling (DNA & transcripts)	Structural rearrangements, large- scale resequencing, genome immortalization	No	
Primer extension	Antibody based multi-protein 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	10-100Kbp
High throughput LOH	LOH mapping, gene localization		100 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, semi-quantitative expression, splicing	Yes	Single exon
SAGE (RNA & DNA)	Comprehensive, quantitative expression, splicing	No	Single gene
ChIP	DNA binding proteins, chromatin structure	No	Single gene
Protein lysate arrays	Antibody based multi-protein interrogation	No ^a	Single protein
1D & 2D gel electrophoresis	Mutant protein detection		Single protein
Mass spectrometry	Qualitative protein composition		Single protein

^a~100 cells are needed.

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

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