Workshop Summary

Workshop on Assessing Human Germ Cell Mutagenesis in the Post-Genome Era
September 28-30, 2004

The Jackson Laboratory and the Environmental Mutagen Society recently organized and sponsored a workshop on Assessing Human Germ Cell Mutagenesis in the Post-Genome Era: A Celebration of the Legacy of William Lawson (Bill) Russell.** The workshop was held at The Jackson Laboratory in Bar Harbor, Maine from September 28-30, 2004 and was attended by a diverse group of clinicians and scientists from academia, government, and industry. These scientists provided expertise in many disciplines from genomics and toxicology to epidemiology and reproductive biology. The overall objectives of the workshop were to assess the current state of the field of germ cell mutagenesis and to develop a strategy for future research. Another workshop goal was to evaluate available molecular and epidemiological techniques for their ability to identify human germ cell mutagens and their effects in exposed human populations and in animal model systems.

The workshop included a keynote presentation by Liane Russell, one poster session and 8 plenary sessions. Topics for presentations and discussions were: Impact of the Human Genome Project, Germ Cell Biology and Mutagenesis, New Approaches for Detecting Inherited DNA and Chromosomal Mutations, Lingering Puzzles in Mutagenesis, and Challenges in Linking Human Exposure to Health Consequences in Offspring.

Two-hour open discussion sessions were also held at the end of each workshop day. These discussion sessions were highly productive, had wide participation from workshop attendees and produced recommendations and guidelines for future research (see below). This summary recapitulates the main outcomes of the workshop.

Introduction and Keynote Address
by Liane B. Russell
Oak Ridge National Laboratory

In an opening address to the workshop participants, Liane Russell gave an overview of the pioneering work of her husband, Bill Russell, in whose honor this workshop was held. Before he began his well known work on mutagenesis using inbred mouse strains, Russell was already keenly interested in phenotypic variation in inbred mouse strains and the potential effects of radiation fallout on animals used in laboratory research. Russell developed the technique of ovarian transplantation in mice, using hybrid strains to avoid immunologically-based transplant rejection, and used it to study the role of the prenatal environment on phenotypic outcome in newborn mice. Later, Russell advocated strongly for developing the mouse instead of relying on Drosophila as a primary model for studying mammalian mutagenesis. Russell took a key step towards developing a useful mouse model for mutagenesis when he devised the seven marker mutagenesis test for recessive visible traits in mice. This test was
heavily used in the following years to develop qualitative understanding of mammalian mutagenesis. Seminal experiments with the seven locus test demonstrated that germ cell stage is critical in determining sensitivity to mutagenic agents. Russell and others investigated how sex, age, dose, dose-fractionation and dose-protraction modulate the effects of radiation exposure on germ cell mutagenesis in mice. However, the most important legacy of Bill Russell's work on mutagenesis may be the discovery late in his career of the potent germ cell mutagen ethynitrosourea (ENU). The discovery of ENU came after extensive screening of many chemical agents, many of which did not have the desired mutagenic potency. A key characteristic of ENU mutagenesis, still heavily used in mutagenesis screens today, is its tendency to generate point mutations instead of strand breaks, insertions/deletions or chromosomal rearrangements.

In her keynote address presented later in the meeting, Liane Russell examined future prospects in germ cell mutagenesis research. She expressed excitement about newly developed high throughput, genomics-based molecular tools that are now available, but were not available when she and Bill Russell worked together at Jackson Laboratory or Oak Ridge National Laboratory. These technologies, if applied appropriately, could lead to rapid progress in understanding long-standing unresolved questions about germ cell biology and mutagenesis in mice and humans.

**Session 1: Historical Perspective and Impact of Genome Project**  
**Session Chair:** John Wassom, Oak Ridge National Laboratory

Session Chair John Wassom set the stage for the workshop by directly posing one of the central questions in the field of germ cell mutagenesis to workshop participants: "Do environmental agents induce inherited mutations in humans?" Wassom quoted several published and unpublished statements from 1960 to the present that attempt to address this question. These statements expressed uncertain and conflicting opinions, and they support the notion that the answer to this question is still not known. This realization was largely the motivation for this workshop, whose goal was to develop a strategy for using recently developed molecular techniques in future research to identify and characterize human germ cell mutagens.

**The Human Perspective – Why Me? Why Us?**  
John Mulvihill, University of Oklahoma Health Sciences Center

John Mulvihill described mutagenesis as a process that has a background (or baseline) component and an induced component, where the latter reflects mutagenic effects of environmental agents. Mulvihill argued that some fraction of hereditary human disease may be attributable to the induced fraction of mutagenesis in human germ cells. However, there is no definitive evidence that proves this hypothesis, and additional research, as described below and in other workshop presentations, is needed to address this issue.

Referring to Sobel's parallogram, Mulvihill indicated that there is very little data on human germ cell mutagenesis, while there is a large amount of data on human somatic mutagenesis, mouse somatic and mouse germ cell mutagenesis. Recent genetic studies of human cancer provide relatively strong evidence that somatic mutation is associated with cancer pathology in humans. To better understand human germ cell mutagenesis and its role in human disease, Mulvihill argued that cohort studies that monitor sentinel phenotypes would be more advantageous than case control studies. Sentinel phenotypes are caused by highly penetrant mutations in
dominant (or X-linked) genes and they have a very high mutational component. Alternatively, molecular methods could be used to identify genomic mutations in exposed human populations. Molecular approaches allow for direct measurement of aneuploidy, chromosomal aberration, inversions, deletions, copy number changes and point mutations. Technical details regarding the use of these methods to study germ cell mutagenesis are discussed in later workshop sessions.

Mulvihill mentioned two possible cohorts that could be useful for future studies: atomic bomb survivors and their offspring and cancer survivors and their offspring. To date, no evidence of a statistically significant increase in adverse pregnancy outcomes have been observed in studies of atomic bomb survivors. Studies of cancer survivors are ongoing. Cancer survivors are a particularly useful cohort because they are numerous, and most importantly because the exact nature of their exposure to radiation and/or mutagenic chemicals is known with a high degree of accuracy and precision. Initial studies of the rate of birth defects in cancer survivors show no significant difference from the average rate in the US population. These data suggest that the agents and doses to which these individuals have been exposed have not induced enough mutations in human germ cells to be detected over the background of spontaneous mutations.

History of Germ Cell Mutagenesis
R. Julian Preston, US Environmental Protection Agency

Julian Preston reviewed the history of research on germ cell mutagenesis with an emphasis on its role in the current paradigm for human risk assessment. Early data on germ cell mutagenesis relied primarily on the 7 locus and 6 locus tests for radiation-induced germ cell mutations in male mice. These early studies, performed in the 1950s and 1960s, demonstrated the impact of fundamental aspects of germ cell biology on germ cell mutagenesis. In particular, these studies established that dose, dose-rate, sex, germ cell stage and radiation quality influence experimental outcome in the 7 or 6 locus tests. While these principles may be conserved across species, it was also established early in these studies that the results were species-specific and difficult to extrapolate in a quantitative manner from one species to another. Preston emphasized the role of stage specificity in this effect; 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.

Another approach used in early studies of germ cell mutagenesis was direct analysis of chromosome aberrations in sperm. In some of these studies, sperm samples from cancer patients who received radiation and/or chemotherapy were analyzed. These studies showed that sperm cell stage and radiation dose influence the frequency of aneuploidy and/or chromosome aberrations in irradiated human sperm. In general, there is significantly less data on chemically-induced germ cell mutagenesis than on radiation-induced germ cell mutagenesis in humans and experimental animals.
Very recent studies report increased minisatellite instability in human and animal germ cells exposed to radiation. The significance of these data are not yet clear and more studies are needed to assess their implication for human risk assessment.

In closing, Preston emphasized that more studies are needed to determine if environmental exposures cause germ cell mutations in human populations. He also emphasized that the outcome of exposure to radiation or chemicals is likely to depend on competition between DNA replication and DNA repair pathways in germ cells. Thus germ cell stage is a critical determinant of exposure outcome.

**How Genome Sequence Impacts Germ Cell Mutation and Health**

Robert K. Moyzis, University of California, Irvine

Robert Moyzis gave a historical review of the Human Genome Project (HGP) and its relationship to the field of human germ cell mutagenesis. Referring to deliberations at the Alta Conference in 1984, the Sante Fe meeting in 1986 and the Cold Spring Harbor meeting in 1988, Moyzis indicated that the HGP grew out of concern over the potential impact of environmental mutagens on the integrity of the human genome. Ultimately, however, the focus of the HGP became large scale comparative genomics as well as DNA sequencing technology, developing model organisms in the field of genomics, and the ethical implications of human genomics research.

Although there is one human genome sequence, there are approximately 6 million unique human genomes, and the diversity in genomes among individuals contributes to differences in their susceptibility to disease. The availability of the human genome sequence has led to rapid progress in understanding rare single gene Mendelian disorders. Progress has been less rapid in understanding common multigenic diseases, but it is now possible to use a pathway approach to facilitate such studies.

Moyzis described an example of using the pathway approach from his research on the common behavioral syndrome, Attention Deficit Hyperactivity Disorder (ADHD). Assuming that this behavioral syndrome is likely to involve altered neurological function, a handful of genes encoding neurotransmitter receptors were selected to be screened for disease-associated variants. Moyzis discovered that one of the three most common alleles of dopamine receptor D4 (DRD4), the 7R allele, is enriched approximately 2-fold in individuals with ADHD. This allele carries a 7mer tandem VNTR repeat in exon 3, unlike the most common variant, which has a 4mer VNTR repeat or the third most common variant, which has a dimer VNTR repeat. Moyzis also observed that the 7R allele is non-randomly distributed in worldwide populations, 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 first arose and became prevalent in the population. Moyzis noted that these observations suggest that study of the
Human genome sequence and its variants can lead to significant insight about the evolutionary history of the human population and the human genome.

**Session 2: Germ Cell Biology and Mutagenesis**

**Session Co-Chairs:** Susan Lewis, Consultant and John Mailhes, LSU Health Sciences Center

**Germ Cell Biology: Animal Models vs Human, Male vs Female**
Mary Ann Handel, The Jackson Laboratory

**Mechanisms of Germ Cell Stage Susceptibilities**
Jack Bishop, National Institute of Environmental Health Sciences

Mary Ann Handel and Jack 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. For example, the time line of germ cell maturation 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 after birth and arrest transiently at meiotic prophase I at puberty. In human females, all germ line mitotic divisions are completed in days, completion of meiosis I requires years and completion of meiosis II occurs post-fertilization in hours. There is limited mitotic proliferation in females and the number of female germ cells is limited. In human males, there is extensive mitotic proliferation and germline mitotic divisions occur over years. Spermatogonial stem cells undergo asymmetric division, generating germ cells that undergo 4 mitotic divisions over weeks and subsequent meiotic divisions in hours.

Handel emphasized the sex-specificity of checkpoints during germ cell maturation and Bishop emphasized the sex- and stage-specificity of DNA repair capacity. Competence for both checkpoints and DNA repair have a clear impact on mutant frequency. 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 have a large capacity for DNA repair.

Handel and her colleagues John Eppig (Jackson Laboratory) and John Schimenti (Cornell University) have begun a large scale initiative to identify genes that are required for meiosis in mice. Mutant mice with defects in meiotic genes were generated by large scale ENU mutagenesis of male mice. The progeny of F2 backcrosses are then screened for infertility. Approximately 9000 mouse lines have been tested for fertility defects to date. Thirty-two potential mutants have been identified: 24 of these mutants demonstrate male infertility, and one mutant demonstrates female infertility. Handel cautiously interpreted this sex bias as indicating a higher level of complexity in male gametogenesis than in female gametogenesis. Molecular studies indicate that these mutants have defects in 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 meiotic functions. For more information see reprogenomics.jax.org.

Egg Repair of DNA Damage  
Francesco Marchetti, Lawrence Livermore National Laboratory

Francesco Marchetti discussed DNA repair capacity and mechanisms in human gametes and the early stages of embryogenesis. Marchetti emphasized that DNA repair capacity differs at different developmental stages. Therefore, the relative timing 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 very high DNA repair capacity because they contain large amounts of maternal protein and maternal mRNA, including abundant DNA repair proteins and transcripts encoding DNA repair proteins. However, the protein and mRNA complement and the transcription profile of the oocyte changes significantly during the earliest mitotic divisions (i.e., zygote to 2-cell stage, 2-cell to 4-cell stage), with zygotic transcription initiating near the beginning of the 2-cell stage. Marchetti emphasized that there is a narrow window of time in which DNA damage can be repaired in the early embryo. Experimental evidence also shows that the genotype of the oocyte differentially affects the efficiency with which DNA damage in the paternal genome is repaired. This reflects the fact that mature sperm are DNA repair-deficient, and that the repair capacity of oocytes is determined at least in part by genetic factors.

Marchetti examined role of specific DNA repair pathways in repairing radiation-induced DNA lesions during early embryogenesis by mating irradiated male mice with repair-deficient female mice. Female knockout mice deficient in RAD54 or the SCID gene were used to selectively inactivate homologous recombination-dependent dsDNA break repair (DSBR) or non-homologous end-joining (NHEJ), respectively. These experiments revealed that both DSBR and NHEJ pathways are active in oocytes and play important roles in preventing chromotid-type and chromosomal aberrations caused by DNA damage in the paternal genome of the early mouse embryo. Similar experiments were used to demonstrate that a p53-dependent S phase checkpoint is active during early mouse embryogenesis.

Mosaicism and Germ-Cell Mutagenesis  
Harvey Mohrenweiser, University of California, Irvine

Harvey Mohrenweiser discussed the potential impact of mosaicism on phenotypic and genotypic variation in mammals. Mohrenheiser defined mosaicism as genetic heterogeneity caused by mutations that arise during embryogenesis. This excludes genetic variation present when chimeric embryos are first formed and genetic variation that accumulates post-nataally. Some normal biological processes cause mosaicism including genomic imprinting, X-inactivation and DNA methylation. In other cases, mosaicism results from aberrant processes that can be associated with pathological conditions. Mutations that occur during embryogenesis can result from spontaneous mutation, exposure to mutagenic compounds or
conditions, or chromosome aberrations arising during mitotic cell division. The fraction of embryonic tissue affected by such mutations depends on stochastic distribution of cells during embryonic development. The greater the fraction of affected cells in the embryo at term, the greater the potential for adverse outcome.

Mohrenheiser estimated that approximately 20% of mutations in a given gene can be attributed to mutagenic events during embryogenesis; the remaining 80% of mutations are inherited from the parental germ line or arise post-natally. When more than one offspring of the same parent are affected by the same disease, the parent is likely to have germ line mosaicism. Germ line mosaicism is not uncommon, but the rate of germ line mosaicism is gene-specific and varies dramatically in different regions of the human genome. Recent studies indicate that mosaicism occurs at a high rate in human embryos produced in vitro using assisted reproductive techniques (ART). For example, ≈75% of day 2 ART embryos show mosaicism and 100% of ART embryos in more advanced stages have some mosaic character. These rates likely reflect the abnormal conditions in which ART embryos are formed and manipulated. However, the rate and potential impact of mosaicism under biologically relevant conditions may be underestimated. Mohrenheiser suggested that somatic and germ line mosaicism could make a significant contribution to sporadic and inherited genetic disease in the human population.

**Sessions 3/4: New Approaches for Detecting Heritable Mutations**

**Session Chairs:** David DeMarini, US EPA and Carole 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 Livermore National Laboratory

Norman Arnheim and Andrew Wyrobek described molecular techniques for measuring mutations and/or chromosome aberrations directly in sperm DNA. One advantage of analyzing sperm directly is that a large number of gametes per individual can be analyzed, whereas a very small number of offspring can usually be analyzed per individual. Thus, assays of mutations in sperm DNA are more sensitive than phenotypic or genetic assays. This is very important because the incidence of germ line defects in the human population is low (i.e., birth defects ≈3%, childhood leukemia ≈0.0004%), and it is difficult to conduct epidemiological studies large enough to detect small changes in these rates. Direct analysis of mutations in sperm DNA also has a few disadvantages, including that selection during fertilization and DNA repair in the zygote are not assessed, poor ability to detect epigenetic DNA modifications, and neglect of sex-specific mutagenic outcomes. Nevertheless, these assays may be extremely useful for screening and prioritizing chemicals of concern.

Arnheim described several PCR- and direct sequencing-based assays that measure mutant frequency at a single nucleotide in FGFR2 or FGFR3, which cause APERT and achondroplasia, respectively. These nucleotides have a mutant frequency of $10^{-4}$ to $10^{-5}$,
which is much higher than the average mutant frequency in the human genome. Most mutations in these two genes occur in the paternal genome at the same nucleotide and the mutations are linked to dominantly-inherited diseases. Quantitative allele-specific PCR is used in these assays to specifically amplify mutant alleles with a sensitivity to approximately $10^{-5}$. To use this method successfully for APERT mutations, a DNA sample of ≈5 µg is required; however, to detect a more typical mutation at $10^{-8}$, ≈5 mg DNA is needed, which is not feasible in most cases. To overcome this problem, Arnheim developed a modified assay that uses a pyrophosphorylated allele-specific oligonucleotide as the PCR primer. When the pyrophosphorylated primer is used, the assay background decreases dramatically and the sensitivity of the assay improves to $10^{-7}$.

Arnheim also briefly described methods that enrich for a mutant allele, deplete the wild-type allele or selectively tag and sort mutant and wild-type (i.e., selective restriction digestion of wild-type allele, sequestering mutant allele via specific binding probes, PCR with beaming). These methods also enhance assay sensitivity and thus could potentially be used to detect low frequency mutations in human sperm DNA.

Wyrobek described three other molecular techniques for analyzing mutations in sperm DNA: sperm comet, sperm chromatin structure assay (SCSA) and sperm FISH. Sperm comet is optimized for quantifying ssDNA and dsDNA breaks in sperm DNA; SCSA detects small regions of ssDNA and DNA fragmentation; and sperm FISH can be adapted for detecting various types of aneuploidy or chromosome aberrations and rearrangements.

Wyrobek used sperm FISH assays to assess the extent of interindividual variation in aneuploidy in sperm from single male donors. He observed approximately 5-fold variation in baseline aneuploidy in 15 male sperm donors. In general, a high rate of aneuploidy did not necessarily correlate with a high rate of other types of chromosomal defects in these donors. Wyrobek also showed data suggesting that the frequency of aneuploidy and chromosome aberrations in germ cells may correlate with the frequency in lymphocytes. Other data indicate no strong tendency for increased frequency of DNA or chromosome abnormalities in sperm from healthy older males.

As stated by Wyrobek, the ultimate goal of these studies is to develop sperm biomarkers that have predictive value for reproductive outcome. Such biomarkers could then be used to evaluate the impact of environmental exposures as well as human lifestyle factors on the integrity of the male germ line. It would also be useful to compare the relative sensitivity of human somatic and human germ cells to these factors and to compare results in rodents and humans.

**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). Most of these studies were carried out with unfertilized oocytes generated by ART. FISH and CGH analyses indicate aneuploidy rates from 20 to 52%; in contrast, studies using conventional chromosome analysis methods estimated a lower rate of 11% total aneuploidy. The extent of total aneuploidy and specific types of chromosome abnormalities strongly increased with increasing age of the female donor. Eichenlaub-Ritter proposed that this may reflect age-dependent decrease in the efficiency of the Mad2-dependent cell cycle checkpoint in oocytes.
Eichenlaub-Ritter also described studies of dose-response to chemical exposures that cause errors in chromosome distribution in mammalian oocytes. These studies used enhanced polarizing microscopy (Polscope), a non-invasive technique that reveals changes in spindle formation that predispose to nondisjunction in mammalian oocytes. This method can be combined with other conventional methods to analyze the impact of hormones, life style, age, or environmental exposure on the frequency of chromosomal abnormalities in human oocytes.

**Haplotype Analysis and Human Genetic Disease**  
**Jack Taylor, National Institute of Environmental Health Sciences**

Jack Taylor discussed studies of human genetic diversity associated with single nucleotide polymorphism (SNPs). In particular, he described data from the Environmental Genome Project (EGP), a systematic human genome re-sequencing 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 re-sequenced in 90 ethnically diverse individuals. Approximately 9000 total SNPs were identified including \( \approx 2000 \) SNPs with a frequency \( \geq 5\% \). There were on average 20 SNPs per gene. All combinations of SNPs are not observed. Instead, SNPs are associated with one another into linked groups called haplotypes. On average, there are 3-4 haplotypes per gene. Haplotypes are shared across different ethnic groups, suggesting that they arose before human ethnic divergence \( \approx 100,000 \) years ago.

Spontaneous mutations occur on the background of the existing haplotypes of a particular gene. In general, these mutations are extremely rare, and is assumed that they arose relatively recently in the evolutionary history of the human genome. It is predicted that a new mutation will only be associated with a single haplotype, and recent studies validate this prediction. This facilitates epidemiological studies designed to test the association of a disease phenotype with rare SNPs in the human genome.

**Inherited Microdeletions, Translocations and Rearrangements**  
**Jane Fridlyand, Cancer Research Institute/UCSF Comprehensive Cancer Center**

Jane Fridlyand briefly described several methods for conducting whole genome scans for genomic aberrations including FISH, SKY, end sequence profiling (ESP) and array-based CGH. Fridlyand indicated that an ideal method for a whole genome scan should be inexpensive, 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 change). No currently available method fulfills all these requirements. For example, the resolution of SKY is 1 to 10 Mb, and it is a poor method for detecting dosage alterations. ESP is too expensive for widespread use, although it formally can be used to detect all types of chromosomal alterations including gene copy number changes. Although chromosome CGH has relatively low resolution, the resolution of array CGH can be very high, because it is determined by the size and number of hybridization probes on the array; current technology allows for maximal resolution near 100 kb using tiled BAC array elements.

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 \( \approx 1.4 \) Mb resolution to analyze human DNA samples from peripheral blood or somatic tissue. Whole genome scans with this array can detect a deletion or amplification involving DNA sequences within a single array element. In a study of 44 patients, 22 of which had a deletion affecting a single array element, no false positive or negative analyses were observed. Fridlyand also presented array CGH data that identified copy
number variation in kringle repeats in the human apolipoprotein gene, and results with a 2000 element BAC mouse genomic array that clustered inbred mouse strains by profiling genome wide copy number variation. These data suggest that rare deletions or amplifications can be detected with relatively high accuracy using array CGH of appropriate resolution. However, Fridlyand cautioned that it remains necessary to differentiate between technical noise (i.e., false positives or negatives), biological noise (signals due to normal genetic variation) and novel variants due to mutational events in the target genome.

In summary, Fridlyand indicated that array CGH can be used effectively to detect heritable chromosome aberrations at 50-100 kb resolution, to link a clinical phenotype with a functional copy number change, for prenatal testing, to detect segmental duplications and associated copy number variations, and for rapid genotyping of inter-specific backcross mice.

Comprehensive Genome Structure and Transcriptome Analysis for Mutation Detection
   Thomas Vasicek, Lynx Therapeutics, Inc.

Thomas Vasicek described applications in genome structure analysis using Massively Parallel Signal Sequencing (MPSS; see www.lynxgen.com). MPSS is a high volume, low cost, method that simultaneously generates 15-30 nucleotide sequence reads from two million DNA molecules from a single sample. This technology can be used for comprehensive genome-wide analyses of any target genome. Approximately 70% of short sequence reads from mouse or human genomic DNA map to unique loci, and approximately 96% of sequence reads from cDNA map to unique transcripts.

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 below the level of detection for microarray technology. Based on an MPSS analysis of the transcriptome of human monocytes and immature dendritic cells, Vasicek concluded 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 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 sequencing methodology is well-established, accurate and reliable; however, for many specific applications of MPSS, it is the method of sample preparation that requires development and improvement.

For mapping large scale polymorphisms, MPSS was adapted to carry out BAC end sequencing. This 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 sequence reads can be used to identify novel SNPs. The estimated false positive rate for SNPs identified twice in these sequence reads is 6 per million bp.

Bioinformatics tools in germ cell analyses
   Michael Primig, Biozentrum & Swiss Institute of Bioinformatics
Michael Primig described GermOnline, a bioinformatic knowledgebase focused on genes and annotation relevant to mitosis, meiosis, germline development, gametogenesis and fertility in yeast and higher eukaryotes (see 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 Basle, Switzerland and the Swiss Institute of Bioinformatics. It has mirror sites in Europe, Asia and the USA to ensure worldwide continuous access.

GermOnline adopted the mechanism of community-based curation in order 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; www.geneontology.org). Thus, in most areas of GermOnline, data entry is restricted to GO keywords and terms.

GermOnline is a cross-species integrated knowledgebase that provides access to data curated in other molecule and species-specific databases. Other examples of integrated databases are Uniprot and Swissprot. GermOnline is unique 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 knowledgebase.

Future goals for GermOnline include increasing funding and adding capacity to curate data on protein-DNA interactions and SNPs as well as video and other types of image data.

**Session 7: Lingering Puzzles in Mutagenesis and Inheritance**

**Session Chair:** Heinrich Malling (National Institute of Environmental Health Sciences)

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

*James Crow, University of Wisconsin—Madison*

James Crow presented an overview of past and current studies on the effect of parental 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 the number of affected offspring increases more quickly than expected when the age of the male parent, but not the female parent increases. One could infer from this result that the germ line mutation rate increases with age in parental males but not in parental females 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 the 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 and achondroplasia, hotspot mutations tend to occur only in males and in some cases, they increase dramatically with paternal age. However, hotspot mutations are gene and sequence-specific and are therefore only relevant to paternal age effect 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. Indels show no increase with parental age. Crow argued that base substitutions show a slight paternal bias and a smaller but significant paternal age effect than hotspot mutations. Thus, the actual paternal age effect in a specific gene reflects the relative
contribution of base substitution, indel and hotspot mutations to the disease phenotype. Paternal age effect is thus predicted to vary significantly from one gene to another.

**Radiation and germ line mutation at repeat sequences: Are we in the middle of a paradigm shift?**

Bryn Bridges, University of Sussex, UK

Bryn Bridges described several hypotheses that have been proposed to explain the estimated 10-fold excess of childhood cancer among inhabitants of Seascale between 1950 and 1990. Seascale is in the vicinity of the Sellafield nuclear power plant, and inhabitants of Seascale are known to have been exposed to relatively high doses of radiation, in some cases exceeding 100 mSv. Although initial studies correlated paternal preconception dose to exposed Seascale fathers with the number of affected children (the PPI hypothesis), a similar effect has not be reproduced in studies of other exposed fathers. 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 trans-acting and/or 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 a viable explanation for the excess cancer in Seascale has not yet been discovered. 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 may have been insufficiently comprehensive, because they did not evaluate subtle post-birth effects that manifest later in development.

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

Yuri Dubrova, University of Leicester, UK

Yuri 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 repeat (ESTRs). The spontaneous mutation rate in microsatellite and ESTR loci is several orders of magnitude higher than in the rest of the human genome, affecting up to 15% of all gamete genomes. ESTRs appear to have a high mutation rate in human somatic cells and germ line cells, so they may be ideal for studying induced mutations in the human germ line. Most mutations in ESTRs are repeat gain or loss events, suggesting that they arise via replication slippage. Importantly, because of the high mutation rate in ESTRs and microsatellite sequences, fewer samples are needed to detect exposure-induced mutations in these sequences than in single copy genes. Thus, Dubrova estimated a minimum sample size of 240, 2,400 or 240,000 is sufficient to measure induced mutations in human ESTR, microsatellite or single copy genes, respectively.

Initial studies compared the dose response in ESTRs or the 7-locus test in mice exposed to acute X-rays. 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 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 4 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 correlation between paternal but not maternal exposure and mutation rate. Dubrova explained the negative result for atomic bomb survivors on three accounts: first, this population received smaller radiation doses than the other exposed populations; second, the number of individuals studied may have been too small; 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 germ line. 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 germ line. 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.

Heritable Mitochondrial Mutagenesis
Eric Shoubridge, McGill University, Montreal

Eric Shoubridge discussed mouse and human mitochondrial genetics and the impact of mutations in the mitochondrial genome on fertility and disease. The mitochondrial genome is a small dsDNA genome that has 13 protein coding genes, most of which play essential roles in oxidative phosphorylation. The replication of mtDNA is not under cell cycle control, but the number of copies of mtDNA per organelle is regulated and maintained at 2-10 copies. mtDNA is transmitted to embryos exclusively from the cytoplasm of the maternal germ line. Thus, its inheritance is non-Mendelian and strictly maternal. Mitochondria have the capacity to repair DNA lesions, but the mutation rate in mtDNA is ≈10-fold higher than in genomic DNA. In most cases, mtDNA is homoplasmic, but in some cases, more than one mtDNA variant co-exist in a single organelle.

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

Shoubridge used a mouse model to analyze the segregation of mtDNA variants during oocyte development and early embryogenesis. Most mice 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 founder heteroplasmic female in which ≈4% of the total mtDNA was the NZB variant. 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 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 very low, and proposed that this in effect 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 Sommer, City of Hope, Molecular Genetics

Germ Cell Methylation in Mutagenesis
Jaquetta Trasler, Montreal Children’s Hospital Research Institute

Jaquetta 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 is 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 germ cells begin to be methylated before birth and continue to be methylated at some sites after birth. In contrast, female germ cells are primarily reprogrammed 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 DNA methylase transferases (DNMT) that play roles in de novo 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 de novo methylation during DNA reprogramming. Knockout mice lacking DNMT1 show 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 de novo methylation in the mouse germ line. DNMT3B knockouts have no defect in gametogenesis, 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 line 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 Transgenerational Actions of Endocrine Disruptors on the male germ line and fertility
Michael K. Skinner, Washington State University
Michael Skinner described environmentally-induced transgenerational changes in methylation in male germ cells in the mouse. Skinner discovered this phenomenon while testing whether the endocrine disruptor, vinclozolin, has adverse effects on testis development and germ cell differentiation in male rat embryos. When rats were exposed to vinclozolin in utero from E7 to E15, 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. These effects were only observed when the embryos were exposed during stages E13-E15, which is the most critical period for testes development in the mouse embryo. Approximately 10-15% of the exposed male pups were completely infertile. Somewhat unexpectedly, Skinner discovered that these exposure-induced symptoms were transmitted to the offspring of the affected males. Thus, the exposed animals acquired a heritable phenotype, which can be transmitted through at least four generations.

Skinner proposed and tested the idea that vinclozolin exposure had disrupted methylation patterns in the male germ line of exposed rats. Skinner characterized methylation patterns in the genomic DNA of affected males, identifying 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 known genes included some STAT-like transcription factors. These results led Skinner to propose that transient exposure to vinclozolin during stages E13 to E15 caused a permanent epigenetic reprogramming of specific genes in male germ cells. This led to an exposure-induced epigenetic transgenerational phenotype including adverse effects on male fertility. One possible mechanism for the genetic reprogramming might be vinclozolin-induced changes in transcription during stages E13-E15.

Subsequent characterization of affected males revealed significant adverse effects in older animals. The late stage 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 demonstrates that environmental agents can cause transgenerational effects by an epigenetic mechanism. It also confirms the existence of gender-specific windows of susceptibility in which germ cells and/or germ line progenitor cells are susceptible to environmentally-induced adverse effects.

**Transgenerational Effects of Low Dose Mutagenesis**

Diana Anderson, University of Bradford, UK

Diana Anderson discussed evidence that low dose environmental exposures, including radiation and chemicals, can induce heritable adverse effects in humans and animals. The evidence for such effects in humans is very 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 also failed to provide evidence of adverse effects, including a study of the rate of birth defects among the offspring of ≈18,000 cancer survivors.

More data is available to support low dose exposure effects in rodents. Nevertheless, the outcome is more ambiguous than for high dose exposures, which cause abundant birth defects in rodents. However, Anderson emphasized the value of rodent models for detecting the effects of low or high dose exposure by monitoring adverse birth outcome or chromosomal aberrations.

Anderson reviewed studies of adverse effects in animals and humans exposed to environmental chemicals including cyclophosphamide, 1,3 butadiene and urethane. The offspring of male rodents
Acute exposure to cyclophosphamide had numerous birth defects as well as chromosome abnormalities and increased tumor incidence. Anderson characterized this system as a useful animal model demonstrating male-mediated teratogenicity, and went on to examine several low dose exposures. For example, chronic low dose 1,3-butadiene, increased the frequency of adverse pregnancy outcomes. Some recent human epidemiological data supports the possibility that occupational exposure to 1,3 butadiene has adverse impacts in humans. In contrast, low dose urethane increased the rate of liver tumors in exposed males, but did not cause birth defects.

In closing, Anderson emphasized that animal model systems can be used to differentiate the outcome of exposure to different environmental chemicals at different doses. She recommended additional use of these models to evaluate whether environmental exposures have potential adverse impacts on 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 Hanawalt, Stanford University

DNA is continually damaged by many exogenous and endogenous agents, some of which induce lethal or mutagenic DNA lesions. Cells express DNA repair systems to prevent the deleterious effects of this DNA damage. There are four major DNA repair pathways: nucleotide excision repair (NER), base excision repair, mismatch repair and recombination repair, each of which is targeted to different types of DNA damage. 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 including yeast. Base excision repair may be essential in mammalian species, since it has not been possible to isolate viable mutants with defects in this pathway in mice.

The NER pathway is targeted primarily to large bulky helix-distorting lesions, but also to several non-bulky lesions. NER is a complex pathway involving the concerted action of ~25 proteins and subpathways that carry out either global genome repair (GG-NER) or repair targeted to the transcribed strand of active genes (TC-NER or TCR). Human hereditary defects in nucleotide excision repair (NER) cause at least three distinct disease syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). These syndromes are characterized by marked sun sensitivity, a striking clinical and genetic heterogeneity and pleiotropic features including developmental and neurological abnormalities and variable signs of premature aging. Human XP patients but not other NER-deficient patients have a 1000-fold increase in skin cancer predisposition. The effect of XP mutations on susceptibility to skin cancer is much less pronounced in mice.

Complex mechanisms have evolved to coordinate the cell's response to DNA damage. These include cell cycle checkpoints and intricate signaling pathways. One element of the DNA damage response is linked to the process of RNA transcription, which can bypass some, but not all, types of DNA damage. When RNA polymerase stalls at an unrepaired DNA lesion, a cellular stress signal is induced. Through intricate signaling networks, cell cycle arrest is evoked, and TC-NER DNA repair proteins are recruited to the transcribed DNA strand. If the lesion goes unrepaired and RNA polymerase remains stalled, additional signaling pathways are activated and the cell becomes irreversibly committed to programmed cell death. TC-NER is thought to be less efficient in mice than in humans, because
mouse cells express a low level of p48/DDB, a protein specifically required for TC-NER. This may explain the fact that mice are a poor model for XP-associated human cancer.

Other 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. Defective genes linked to Bloom syndrome and Werner syndrome express multifunctional proteins with DNA helicase activity. Defects in human mismatch repair proteins are linked to a hereditary form of colon cancer and to sporadic colon and endometrial cancer.

Clinical and Economic Aspects of Mutation Detection
Robert Erickson, University of Arizona Health Science Center

Robert Erickson reviewed current issues in implementing molecular genetic assays in clinical medicine. High throughput molecular genetics has changed the face of research medicine 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 chip methodology to screen for any of 1000 possible mutations that cause cystic fibrosis. However, the logistics of 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 chip technology will eventually emerge and be widely used to rapidly screen for genetic defects, but this will only occur after many existing DNA patents have expired and chip costs have dropped 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 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 Boice, Vanderbilt University Medical Center

John Boice reviewed epidemiological studies of heritable disease phenotypes in exposed human populations. The exposed populations include atomic bomb survivors, radiation workers, individuals exposed to diagnostic X-rays, individuals exposed to high environmental radiation, and cancer survivors. Many indicators were scored in these studies (i.e., adverse pregnancy outcome, sex ratio, childhood cancer, death of offspring, cytogenetic abnormalities and minisatellite mutation rate) and ≈80,000 individual offspring were included in the study of atomic bomb survivors alone. On the whole, the results were clearly negative. In their 2001 report, the 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 also 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.
In contrast, there is evidence of radiation-induced chromosome aberrations in exposed individuals, and although offspring of cancer survivor cohorts do not show increased cancer rates, cancer survivors themselves show strong evidence of exposure-induced cancer. One explanation for these results is that epidemiological studies to date of exposed human populations may have lacked power, had insufficient dose range, or failed to measure appropriate outcomes. In addition, affected offspring may elude detection because they may be eliminated by natural biological processes such as early miscarriage.

Epidemiologists are continuing to look for evidence of effects on the rate of heritable genetic disease in offspring of human cancer survivors. A very large scale international collaboration has been formed and is currently conducting a demonstration project focused primarily on adverse pregnancy outcomes. Molecular methods including minisatellite characterization and analysis of chromosome or chromatid aberrations will also be carried out in offspring and in selected trios. Efforts have been made to precisely calculate the gonadal dose that was given to each exposed individual. When this study nears completion and data is thoroughly analyzed, we may have a more definitive answer to lingering questions about the integrity of the germline in exposed human populations.

Risk Assessment

K. Sankaranarayanan, Leiden University Medical Centre

K. Sankaranarayanan presented several novel concepts that could be used to develop a new or modified paradigm for assessing human genetic risk associated from exposure to ionizing radiation. Sankaranarayanan pointed out that the existing paradigm is based on the assumption that adverse effects of radiation will manifest in progeny of exposed individuals as genetic diseases similar to those that occur naturally in the population. Because human data is extremely limited, risk is generally estimated using three factors: the doubling dose for radiation-induced germ cell mutations in mice, the background rate of “spontaneous” genetic disease in humans and population genetic theory. Recent estimates suggest that the genetic risk associated with chronic/low dose irradiation is \( \approx 4,000 \) 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).

Recent studies in experimental systems demonstrate that most radiation-induced mutations are DNA deletions encompassing multiple genes. Because of this, 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 multi-system developmental abnormalities.

In the second part of his presentation, 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 (NHAJ), homologous recombination (HR) and non-homologous end-joining (NHEJ). Sankaranarayanan proposed that germ cells favor NHAJ-dependent DSB repair and that low copy repeats 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 aborted human fetuses or perinatal deaths. 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**

Joe Gray (Lawrence Berkeley National Laboratory), Sally Perrault (EPA) and Colin Collins (University of California, San Francisco) led an open discussion session on *Integrating New Technologies in Germ Cell Mutagenesis*. 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.

To begin the session, Gray presented a spreadsheet/matrix of nucleic acid technologies and biological parameters that could potentially be measured or analyzed with those technologies. (The technologies included PCR based sequencing, PCR-based conformation analysis, sequencing by hybridization, end-sequence profiling (ESP), primer extension, FISH, CGH, high throughput LOH, optical mapping, genome subtraction, expression arrays, SAGE (RNA & DNA), ChIP, protein lysate arrays, 1- & 2D gel electrophoresis, mass spectrometry and computational biology. The biological parameters that might be analyzed included genome-wide mutation rate, chromosome aberrations, multigenic disorders, locus-specific mutation rate, DNA methylation, genetic mapping, gene function and gene expression profiling.) Gray asked meeting participants to consider how best to use these technologies to move the field forward.

The ensuing discussion, although useful, did not identify a well-defined project or specific technologies that are particularly relevant to current issues in germ cell mutagenesis. However, several important points were raised during the discussion which may prove useful in future discussions and planning sessions within the germ cell mutagenesis community. The main discussion points were:

- There was fairly strong consensus that the scientific community should design and execute a collaborative project that would attempt to measure induced mutation rate in human germ cells. It was emphasized that the project should be carried out in parallel in human subjects (or samples) and in a mouse model system. Such a "matched mouse/human study" was considered essential, because of the relative ease of manipulating experimental conditions in mice, the relative difficulty of determining exposure and controlling variables in human subjects, the relative difficult of obtaining human samples, and the difficult of extrapolating animal data to human populations. Although no specific technology was identified for use in such an experiment, it was suggested that different technologies could be implemented by different researchers, all using a single set of samples, if those samples were chosen *a priori* and a central source of samples was established (*i.e.*, a biobank; see below). It was also recommended that a single mutagenic agent be selected as a focus of this project. Because of the extent of the knowledgebase on radiation exposure in animals and humans, it was suggested, but not firmly agreed, that the study should focus on the mutagenic effects of radiation exposure.

- The background spontaneous mutation rate in the human genome (and other genomes) is approximately $1 \times 10^{-7}$ per cell per generation on average. To detect induced mutations, a technology must be sensitive to at least $1 \times 10^{-7}$ and must have an error rate lower than $1 \times 10^{-7}$ per bp. Unfortunately, most available technologies including high fidelity DNA sequencing are not sufficiently accurate for this purpose. Although a relatively high induced mutation rate in an exposed animal population can be readily detected by DNA sequencing-based methods, small
changes in mutation rate are difficult to detect with confidence using existing molecular methods.

- Potential germ cell mutagens should be assessed for their ability to cause hereditary phenotypes by both genetic and epigenetic mechanisms. Molecular studies should identify various types of mutations including single base substitutions, chromosome aberrations and aneuploidy.

- It will be difficult to assess or measure the significance and/or impact of genetic alterations in non-coding regions of the genome.

- Subfertility and infertility should be considered possible phenotypic consequences of mutagen exposure.

- Offspring of childhood or adolescent cancer survivors should be exploited as a human population with known mutagen exposure. Many of these individuals are in the medical system and many are also likely to be willing to participate as subject in studies of germ cell mutagenesis in humans.

- There are advantages to measuring mutation rate directly in sperm DNA. However, such experiments should be coupled with functional analysis to determine the phenotypic implications of germ line mutations identified in sperm DNA. In addition, it should be recognized that results deduced from experiments with sperm may not extrapolate to oocytes.

- Many members of the clinical community are reluctant to consider data from animal model systems.

- Meeting participants agreed that a biobank should be established as a research resource for the germ cell mutagenesis community. Additional and/or improved database resources were also recommended. Some participants anticipated difficulty in funding such efforts.

- Meeting participants disagreed over whether a transgene in a genetically-engineered mouse model is a representative mutagenic target and on whether data from transgenic mouse models should be considered valid measures of the mutagenicity of the entire mouse germ cell genome.

- End Sequence Profiling (ESP) is a relatively new technology that can clone and map many kinds of chromosomal defects (i.e., 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 very useful to the germ cell mutagenesis community. The two main disadvantages of ESP are its high cost and that it requires expertise in BAC library construction.

**Open Discussion Session 2**
Research Recommendations for Assessing Mutations in the Post-Genome Era

John Mulvihill (University of Oklahoma) led a two-hour open discussion on *Research Recommendations for Assessing Mutations in the Post-Genome Era*. This session was devoted to developing recommendations and proposals for future activities in germ cell mutagenesis research. In his opening comments, Victor McKusick (Johns Hopkins) 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.
One set of recommendations, a funding initiative and a white paper were proposed during this discussion session. Resources and technologies of value to future research efforts were discussed, experimental designs for studying the human germ cell mutagenesis were proposed and the merits of all these ideas were debated extensively. The main points of the discussion are summarized in the following paragraphs.

**Proposed Recommendations**

John Mulvihill presented the following recommendations for meeting participants to consider:

- Assure continued funding for human mutation research and related studies.
- A lead federal agency should be made responsible for tracking progress, facilitating interchange of information, and encouraging coordinated interdisciplinary studies.
- A repository of biologic materials from high risk individuals, their spouses, and children for studies of somatic and heritable mutations.
- Improved technologies for specimen storage.
- Specific funding to develop promising original methods.
- Proposed studies of human germ cell mutagenesis or proposals for developing novel technologies should be reviewed by an independent expert panel to determine project feasibility.
- Animal studies should be carried out to define mechanisms of mutagenesis and elucidate the relationship between mutagenic potency in animals and humans.

After presenting these recommendations, Mulvihill disclosed that they were produced at an earlier meeting on the topic of Germ Cell Mutagenesis and published in a 1987 report from the Office of Technology Assessment. Nevertheless, many of the same concerns and ideas surfaced during this 2004 meeting, and this list of recommendations appear still to be largely relevant today. This suggests that the field may not have been ready or able to implement these goals and recommendations when they were formulated in 1987. Mulvihill asked meeting participants to assess if that conclusion is correct, or if the current state of the field points to alternate ways or approaches to advance knowledge and understanding of human germ cell mutagenesis.

**Proposed Funding Initiative**

David DeMarini (EPA) read part of a draft letter, excerpted below, proposing a large scale Human Germ Cell Mutagenesis Program (HGCMP).

"An international program should be instituted before the end of CY 2005 to determine those systems that can be used to detect induced germ cell mutations in humans. The process of beginning such a program should use the recently completed Human Genome Program (HGP) as a model....to speed implementation. The purpose of the proposed program will not be of the magnitude of the HGP, but its work and purpose will be as important if not more important for human health purposes."
DeMarini indicated his intention to circulate and/or publish the letter, seek input and feedback, and organize a committee to evaluate and/or implement future actions based on this proposal. This committee would also explore potential sources of funding for the proposed Program. There was considerable discussion about all aspects of this proposed initiative, including attempts to define its goal, its endpoint and its name. General reaction to the proposal was mixed, including strongly positive support as well as strongly cautionary responses. McKusick expressed significant skepticism and cautioned that the proposal lacked definition. He also wondered how its funding could be justified, if one considered the relative merits of a program on the human germ cell vs a program on the human brain or other tissue/organ of the human body.

Richard Woychik (Jackson Laboratory) made a strong case for modulating the scope and concept of the proposal. Woychik first emphasized the complexity of germ cell biology and the need to understand cell-type-, species- and exposure-dependent differences in mutagenic outcome. Woychik argued that this complexity dictates an integrated approach incorporating different approaches and strategies to understanding and analyzing human germ cell mutagenesis. Woychik also emphasized the importance of distinguishing the proposed program from other genomics-based programs with implications for human mutagenesis (i.e., why this program and why now?). Lastly, Woychik suggested that the goals of the Germ Cell Mutagenesis community may not be "endpoint-driven." Based on that observation, Woychik proposed generating a white paper on the mutability of the human germ line, whose raison d'etre would be to increase awareness of issues and concerns in this field.

Several alternative titles were suggested for the proposed "Human Germ Cell Mutagenesis Program," including the Human Germ Cell "Variation" Program, the Human Germ Cell "Risk" Program and the Human Genome "Integrity" Program. The suggested alternate titles reflect the fact that meeting participants wanted to minimize emphasis on mutagenesis per se. The putative goal/endpoint of the proposed program was discussed extensively, but the precise goal remained ill-defined. In general, it was agreed that the program goal would be to demonstrate a link between mutagen exposure, genetic alteration in human germ cells and human hereditary genetic disease. A secondary goal expressed during the discussion was to quantify and/or reduce the magnitude of genetic risk associated with exposure to specific mutagens. Additional discussion failed to establish a clear endpoint or deliverable that could be produced by the proposed program within a fixed time frame.

Several specific experimental approaches were proposed that could increase our understanding of human germ mutagenesis. Jack Taylor (NIEHS) re-iterated an idea presented earlier in the conference, proposing that 100 offspring of childhood cancer survivors and their parents (i.e., 100 trios) should be used as a target population for SNP analysis. In this experiment, ≈100 genes would be resequenced in 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 parent DNA, or if some of them could be attributed to a mutation in a parental germ cell.

Harvey Mohrenheiser (University of California, Irvine) 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 reiterated and emphasized that one of the benefits of studying childhood cancer survivors and their offspring is that the amount and conditions of exposure are precisely known from patient medical records; this information greatly increases the potential value of analyzing this exposed population over most other exposed human populations.
Bryn Bridges (University of Sussex, U.K.) 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 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.

General discussion points

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

• A repository of biological samples from exposed populations and unexposed controls would be a valuable resource to the scientific community studying human germ cell mutagenesis.

• The Complex Trait Consortium (www.complextrait.org) is generating 1000 recombinant inbred mice 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 mechanistic understanding of many aspects 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 to understanding the mutability of human germ cells. This should include understanding of germ cell stage-dependent or sex-specific variation in the genetic risk associated with environmental exposure.
A rapid method to screen for mutations in restriction enzyme cleavage sites was used to estimate the rate of radiation-induced mutation in the mouse.

Single molecule PCR revealed increased mutation rates in non-exposed offspring of irradiated male mice.

Transgenerational effects were observed in offspring from irradiated mouse sperm. An induced, heritable genomic instability was postulated.

Array CGH was used to identify 12 rare inherited variants in 40 children of A-bomb survivors.

SNP analysis was used to identify cross-over hotspots and analyze linkage disequilibrium in regions of the human genome. This technique facilitates mapping of disease markers and haplotype analysis.

Batch sperm typing strategies are more efficient and provide better resolution than family pedigree analysis or single sperm typing to study the distribution of recombination events in the human genome.

Sensitivity to restriction digestion by MboI was used to enrich for mutant alleles of FGFR2, the gene that causes Apert Syndrome. Data generated with enriched DNA samples suggest that mutation in FGFR2 confers a selective advantage to sperm. Clonal expansion of germ cells carrying the FGFR2 mutation could explain the apparent "paternal age effect."

The sperm chromatin structure assay (SCSA) and improved methods for collecting semen samples could facilitate field studies of exposed human populations.

Automated methods for analyzing sperm FISH will increase throughput, reduce cost and improve assay reliability.

Immunological methods can be used to detect aberrant DNA methylation in 1- and 2-cell rat embryos.

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