Germ Cell Exposures and Heritable Effects: Is Our Regulatory Paradigm Failing to Protect Future Generations?

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Outline

- Discovery of germ cell mutagens starting in 1927
- EMS/EMGS founded in 1969 to eliminate germ cell mutagens
- 85 rodent germ cell mutagens in 2020, none in humans
- No absolute requirement for germ-cell effects by EPA or FDA
- Should this paradigm change?

Germ Cells are NOT Somatic Cells

- Completely different genetic processes (meiosis, crossing over, spermiogenesis, etc.)
- Completely different epigenetic processes (including reprogramming, imprinting, chromatin remodeling; windows of vulnerability)
- Significant differences in cellular and molecular content in M v F germ cells
- Dependent on the health and signals of adjacent support cells (e.g., Sertoli cells in the males, granulosa cells in the females)
- Minor defects in germ cells could blueprint major defects in offspring development

Brief History of Germ Cell Mutagenicity

1927-1936: Radiation (X-rays & UV light) fruit flies, corn, barley. Muller, Stadler, Altenburg

1937: X-rays in mice (chromosomal effects). Snell, Hertwig, Brenneke

1942-1946: Chemicals (Mustard gas & mustard oil) fruit flies. Auerbach

1951: X-rays, first germ cell gene mutagen in mice: Bill Russell, Oak Ridge Natl Lab

1950s: *Many untested chemicals introduced after World War II*

1955: **Joshua Lederberg** raised concern of germ cell mutagens to NAS

1968: **James Crow** published report of NIH germ cell mutation conference

1968: Sam Epstein showed environmental chemicals were germ cell mutagens in mice

1969: Epstein & Legator proposed in U.S. govt report to test pesticides for mutagenicity

1969: **EMS/EMGS founded** to eliminate germ-cell mutagens; deep concern about intellectual and physical health of future generations

[DeMarini, Environ Mol Mutagen 61:8-24, 2020]

Today: What Are the Rodent Germ-Cell Mutagens?

- There are 85 proven germ-cell mutagens in rodents
- Most are cancer chemotherapeutic agents
- Environmental germ-cell mutagens are side-stream and main-stream cigarette smoke, the particulate fraction of outdoor air pollution, and diesel exhaust

[Marchetti et al., Environ Mol Mutagen 61:42-54, 2020]

What Are the Declared Human Germ-Cell Mutagens?

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No absolute requirement for testing for germ-cell effects by EPA or FDA

How Did Germs Cells Fall Off the Radar?

- Focus was on ionizing radiation after World War II
- Focus of EMS/EMGS shifted in 1970s away from germ cell to cancer due to Ames assay & “War on Cancer”
- Research & funding for research reflected this shift
- Regulatory requirements for testing reflected this shift
- Failure to appreciate epigenetic germline effects
- Assumption of randomness in *de novo* mutation

Why No Human Germ Cell Mutagens?

- No agency to declare a human germ-cell mutagen other than the Atomic Bomb Survivors' Commission
- Wrong population exposure (atomic bomb survivors; acute vs chronic)
- Wrong genes (coding versus non-coding, and single-gene versus complex multi-genic traits)
- Wrong tools (next-gen DNA sequencing and gene expression analyses needed)
- Difficult to study human female germ cells and early-stage male germ cells
- Regulatory agencies do not regulate based on mutagenicity: air pollution and cigarette smoke are mutagenic but are not regulated on that: air on mortality and smoking on cancer

Are There Really No Human Germ-Cell Mutagens?

Agent	Rodent			Human		
		Mutagenic			Mutagenic	
	Carcino	Somatic	Germ	Carcino	Somatic	Germ
Ionizing Radiation	+	+	+	+	+	+?
Chlorambucil	+	+	+	+		?
Chlornaphazine	+	+	+	+		?
Cyclophosphamide	+	+	+	+	+	?
Melphalan	+	+	+	+	+	?
Myleran	+	+	+	+	+	?
Air Pollution	+	+	+	+	+	+?
Tobacco Smoke	+	+	+	+	+	+?

[Shelby, Environ Mol Mutagen 23 (Suppl 24):30-34, 1994; DeMarini, Environ Mol Mutagen 53:166-172, 2012]

Evaluation of Agents as Human Germ-Cell Mutagens As IARC Evaluates Human Carcinogens

	Animal				Human	
Agent	Mouse	Rat	Bird	Evidence	Mechanism	Evaluation
Ionizing Rad	+,+	+		Sufficient	Strong	+
Chemotherapy	+,+	+		Sufficient	Strong	+
Smoking	+,+			Sufficient	Strong	+
Air pollution	+,+		+	Sufficient	Strong	+

[DeMarini, Environ Mol Mutagen 53:166-172, 2012]

Strong Evidence That Paternal Smoking Is a Germ Cell Mutagen

- Impaired male fertility
- Increased DNA damage in sperm
- Increased mutation in sperm
- 1.3 million extra cases of aneuploid pregnancies per generation globally
- Increase in specific cancers in children of smoking fathers

[Beal et al., Mutation Res 773:26-50, 2017]

Mutagenicity Testing History

- Mutagenicity testing of pesticides by EPA began in 1976 under TSCA.
- EPA published its first Guidelines for Mutagenicity Risk Assessment in 1986, focusing on transmissible germ cell genetic risk (Dearfield et al., Mutation Res 521:121-135, 2002)
- Efforts in 1990s to classify agents as human mutagens, but no criteria set
- A classification scheme & criteria proposed for human mutagens based on IARC's carcinogen classification scheme (DeMarini, Environ Mol Mutagen 53:166-172, 2012)

Mutagenicity Testing Requirements of Regulatory Agencies Worldwide

- First test is for gene mutation, typically the Ames assay in bacteria.
- Second test is for chromosomal mutation, typically the micronucleus assay either in cells in culture or in the mouse (bone marrow).
- If chemical analysis shows that the chemical is present in the germ cells of the animal, then any of the following assays could be done in the mouse: dominant lethal assay or gene/chromosome mutation in sperm
- Thus, germ cell assays are rarely done and if so, only in males for mutation, not gene expression, and not for phenotypic effects

[Cimino, Environ Mol Mutagen 47:362-390, 2006; Dearfield et al., Mutation Res 521:121-135, 2002; Dearfield et al., Environ Mol Mutagen 52:177-204, 2011; Heflich et al., Environ Mol Mutagen 61:34-41, 2020]

International Agreements on Germ Cell Testing

- Most members of the United Nations ascribe to the Global Harmonization System (GHS) to classify hazardous chemicals
- GHS considers human germ cell mutation to be equal to cancer and reproductive effects but does not provide a method for determining if an agent is a human germ cell mutagen
- REACH in Europe & European Chemicals Agency do not require germ cell testing but imply that somatic cell mutagens in vivo are germ cell mutagens

Guidance for Industry S2(R1): Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use [U.S. FDA, page 6, 2012]

"Results of comparative studies have shown that, in a qualitative sense, most germ cell mutagens are likely to be detected as genotoxic in somatic cell tests so that negative results of in vivo somatic cell genotoxicity tests generally indicate the absence of germ cell effects."

Not All Germ Cell Mutagens Are Detected in Mouse Somatic Cell Mutation Assays

- N-Hydroxyacrylamide (Witt et al., Environ Mol Mutagen 41:111-120, 2003)
- Cigarette smoke (Marchetti et al., PNAS 108:12811, 2011)
- Various Chemotherapy Drugs (Waters et al., Mutation Research 341:109-131, 1994; Yauk et al., Mutation Res 783:36-54, 2015; Client et al., Mutation Res 292:105-111, 1993)

The Future of Mutagenicity Testing

- Somatic and germ cell mutation should be assessed in a single animal and integrated into standard repeat-dose toxicology studies
- Mutation as an endpoint should eventually replace carcinogenicity assays
- In utero exposures should be assessed for genetic mosaicism
- Next-gen DNA sequencing & changes in gene expression should be done

[Godschalk et al., Environ Mol Mutagen 61:55-65, 2020;
Heflich et al., Environ Mol Mutagen 61:34-41, 2020;
Marchetti et al., Environ Mol Mutagen 61:42-54, 2020]

Epigenetic Testing Not Done at All

- Epigenetic modifications, which alter gene expression, can be heritable and stable and produce long-term changes to genomic architecture and function, including changes in heritable DNA methylation and histone modifications (Singer & Yauk, Environ Mol Mutagen 51:919-928, 2010)
- Mice exposed to particulate air pollution have hypermethylation of sperm DNA that persists through sperm maturation (Yauk et al., PNAS 105:605-610, 2008)
- Paternal irradiation results in hypermethylation of thymus of children (Filkowski et al., Carcinogenesis 31:1110-1115, 2010)
- Research on this is reported at this Beyond Genes conference

Conclusions & Recommendations

- Current testing regulations are insufficient for germ cell effects.
- Mutagenicity testing should include the Ames assay, mouse LIVER micronucleus assay, and sperm mutation assay, which should be integrated into standard repeat-dose tox studies.
- A standard gene expression assay in somatic and germ cells should be developed and included with mutagenicity testing.
- Mosaicism evaluated in reproductive toxicity assays.
- Molecular epi studies should be done to assess mutations and gene expression changes in children and sperm of men exposed to smoking, drugs, and other environmental agents.

Conclusions & Recommendations (Cont.)

- An international agency should evaluate agents for germ cell effects in humans.
- Funding agencies should support research on germ cell effects in exposed male and female rodents and in humans.
- EPA should reconsider its planned reduction & possible elimination of animal tox testing, which is needed to assess germ cell effects.
- CompTox (EPA) and Tox21 (NIEHS) high throughput testing does not include mutagenicity, carcinogenicity, or germ cell endpoints and are insufficient for the safety evaluation of chemicals for human health effects, especially of future generations.