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Commentary

In utero Exposure to Genotoxicants Leading to Genetic Mosaicism: An Overlooked Window of Susceptibility in Genetic Toxicology Testing?

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In utero development represents a sensitive window for the induction of mutations. These mutations may subsequently expand clonally to populate entire organs or anatomical structures. Although not all adverse mutations will affect tissue structure or function, there is growing evidence that clonally expanded genetic mosaics contribute to various monogenic and complex diseases, including cancer. We posit that genetic mosaicism is an underestimated potential health problem that is not fully addressed in the current regulatory genotoxicity testing paradigm. Genotoxicity testing focuses exclusively on adult exposures and thus may not capture the complexity of genetic mosaicisms that contribute to human disease. Numerous studies have shown that conversion of genetic damage into mutations during early developmental exposures can result in much higher mutation burdens than equivalent exposures in adults in certain tissues. Therefore, we assert that analysis of genetic effects caused by *in utero* exposures should be considered in the current regulatory testing paradigm, which is possible by harmonization with current reproductive/developmental toxicology testing strategies. This is particularly important given the recent proposed paradigm change from simple hazard identification to quantitative mutagenicity assessment. Recent developments in sequencing technologies offer practical tools to detect mutations in any tissue or species. In addition to mutation frequency and spectrum, these technologies offer the opportunity to characterize the extent of genetic mosaicism following exposure to mutagens. Such integration of new methods with existing toxicology guideline studies offers the genetic toxicology community a way to modernize their testing paradigm and to improve risk assessment for vulnerable populations. Environ. Mol. Mutagen. 61:55–65, 2020.

Key words: genetic mosaicism; *in utero* exposure; regulatory toxicology

INTRODUCTION

The concept that all cells in an individual are genetically identical, because they originate from a single fertilized egg, is incorrect. Although DNA replication during cell division occurs with high fidelity, mistakes that escape proofreading or DNA repair occur regularly. As each replicating cell accumulates new mutations, most cells contain their own unique genotype. Cells with divergent genomes can subsequently clonally expand into distinct subpopulations carrying different mutational loads. The result is a genetically mosaic individual (De 2011; Campbell et al. 2015).

Genetic mosaicism is defined as the presence of two or more populations of cells with different genotypes in one individual (De 2011; Forsberg et al. 2017). In humans, genetic mosaicism is found in up to 70% of cleavage-stage embryos and 90% of blastocyst-stage embryos derived from *in vitro* fertilization (Taylor et al. 2014); therefore, mosaicism is the rule rather than the exception. These

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mutations can vary from single nucleotide polymorphisms, to large structural variants, and aneuploidies (Youssoufian and Pyeritz 2002). Genetic mosaicism is increasingly recognized as being involved in many diseases depending on the type of mutation, the location, and the number of affected cells in an organ (Youssoufian and Pyeritz 2002). Clearly, the causes and consequences of genetic mosaicism deserve further attention.

All life stages may be susceptible to genetic mosaicism; for example, early windows of development are vulnerable because of high rates of cellular proliferation; whereas, much later life stages experience declining DNA repair capabilities (Machiela and Chanock 2013). Deleterious mutations that occur very early in development may be embryonically or prenatally lethal. However, mutations occurring during periods of organogenesis may have an increased probability of clonal expansion and adverse phenotypic impacts (Machiela and Chanock 2013). Somatic mutations that occur early in development can expand to populate entire organs or other anatomical structures (Fig. 1). Although not all adverse mutations will impact tissue structure and function, there is growing evidence that clonally expanded mosaics can contribute to adverse human health effects. It is also important to note that genes may be switched on or off when cells start to differentiate, and the impact of a mutation may only become apparent if it is present in a gene that is switched on and relevant for that particular tissue and life stage. Overall, our understanding of the role of somatic mosaicism in human health is growing at a rapid pace, and mosaicism arising early in development has now been implicated in many diseases from cancer to developmental and neurological disorders (Youssoufian and Pyeritz 2002).

There is a general consensus that critical windows of susceptibility to genotoxicants exist and that genotoxicity *in utero* may lead to cancer later in life (Anderson et al. 2000; Godschalk and Kleinjans 2008; Hleyhel et al. 2019; Troisi et al. 2019). However, there is very little understanding of the role that exposure to genotoxic agents during development plays in the induction of genetic mosaicism. *In utero* development represents a potentially sensitive window for the induction of mutations by environmental mutagens that can lead to genetic mosaicism in the growing fetus. Although biologically plausible, very little research has been conducted to explicitly explore this hypothesis. We contend that this area of genetic toxicology warrants significant attention given the increased awareness of the prevalence of mosaicism in the population, its established impact on disease, and our ethical obligations to address such vulnerable populations during risk assessment.

To this end, a subgroup of the Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute summarized and analyzed the available evidence for environmentally induced genetic mosaicism during *in utero* development. Herein we: highlight potential health effects (beyond cancer) associated with genetic mosaicism;

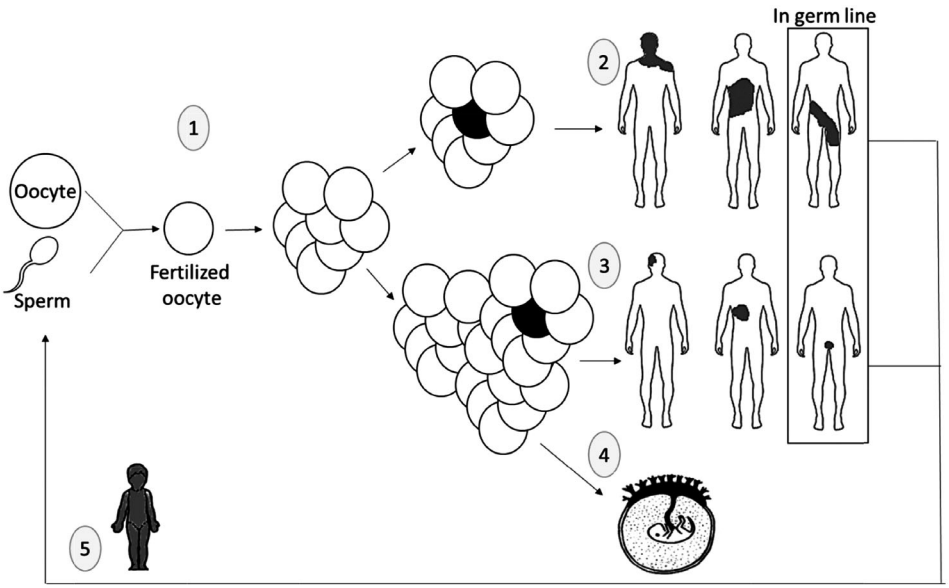

Fig. 1. In genetic mosaicism, the mutation does not occur before conception (1), but at later stages in development. Depending on the timing of the somatic mutation during embryogenesis, the area of tissues/organs or the number of affected cells may vary (2, 3). A mutation may actually be confined to one single organ, including the placenta (4), with the latter mutations not occuring within the offspring itself. Mosaicism may also occur in germ cells, in which case children of the mosaic individual may inherit the mutation (5).

discuss the types of mutational endpoints that should be studied; summarize the evidence supporting the role for environmental mutagenesis in genetic mosaicism; and address the potential implications to the current genetic toxicology testing paradigm. Until now, regulatory application of genetic toxicology data has been mostly limited to hazard identification. Genetic toxicology is presently moving away from a simple yes/no answer regarding the mutagenicity of a compound. Instead, major emphasis is now being placed on quantitative responses, including potency comparisons and deriving margins of exposure. Thus, under this emerging paradigm, it is important to study mutagenic potency under conditions where the cells/tissues are most susceptible to mutation-induction. Since mutagenicity highly associates with cell turn-over, the *in utero* phases serve as potential highly vulnerable periods for mutation induction and may become more relevant in future testing strategies. However, consideration of susceptible windows for mutation induction would only become important if the paradigm actually changes to an approach in which mutations are considered quantitatively as a risk, rather than just identifying mutagens.

TABLE I. Examples to Illustrate Widespread Presence of Genetic Mosaicism in Humans

Type of disease	Manifestation of disease	Examples in which genetic mosaicism is detected
Monogenic diseases	Metabolic derangement	Lesch–Nyhan disease
	Immune dysfunction	Adenosine deaminase deficiency
	Clotting disorder	Hemophilia A and B
	Skeletal disorder	Marfan syndrome
	Muscular disorder	Duchenne muscular dystrophy
	Skin disorder	Incontinentia pigmenti
	Endocrine disorder	Androgen insensitivity
	Chromosomal instability	Bloom syndrome
	Aneuploidy	Klinefelter syndrome (47, XXY)
Complex diseases	Tumor suppressor mutations	Cancer
	Chromosomal instability	Cancer susceptibility
	Brain development	Schizophrenia and bipolar disorders
Others	Aging	Accumulation of mutations with age
	Immune response variability	V(D)J recombination in lymphocytes
	Mitochondria	Heteroplasmy

Note: The diseases/conditions in this table are a selection to illustrate the involvement of genetic mosaicism in many diseases/conditions. Examples were derived from references: (Youssoufian and Pyeritz 2002; Dadi et al. 2009; Sakai et al. 2015; Aryaman et al. 2018; Lichtenstein 2018; Risques and Kennedy 2018; Verheijen et al. 2018; Keefe 2019; Nicolas and Veltman 2019). More examples can be found in the scientific literature.

HEALTH EFFECTS OF GENETIC MOSAICISM

Monogenic Diseases

The literature describing the presence of genetic mosaicism in human diseases is extensive (Youssoufian and Pyeritz 2002; De 2011; Freed et al. 2014; Campbell et al. 2015; Forsberg et al. 2017). Clinical recognition of genetic mosaicism in human disease originally involved easily observable traits, which included pigment variations and other abnormalities of the skin (Campbell et al. 2015; Kromann et al. 2018). With the development of more sophisticated molecular diagnostic techniques, it was recognized that genetic mosaicism is involved in many monogenic diseases (Youssoufian and Pyeritz 2002; Forsberg et al. 2017) and that the effect of mosaic mutations is dependent on the pattern of the distribution of mutations in the organism. The involvement of genetic mosaicism in many monogenic disorders is now generally accepted, and includes disorders with various clinical manifestations, including metabolic imbalances, immune dysfunction, musculoskeletal disorders, and endocrine disorders (Table I).

Mosaicism in Complex Diseases

Mosaic mutations have been associated with complex diseases, such as predisposition to cancer and the development of neurological disorders (Lichtenstein 2018; Nicolas and Veltman 2019), that are influenced by a combination of mutations in multiple genes and environmental factors. Genomic instability is a hallmark of cancer: tumors are genetically heterogeneous because cells accumulate mutations while the tumor grows. Therefore, it seems plausible that cells that already carry a specific set of somatic mutations will influence the age of onset of cancer. However, it is unknown how important the contribution of early developmental mutations is to the total cancer burden. The strength of the association with cancer susceptibility will likely vary among tissues and tumor types (Helleday et al. 2014; Lichtenstein 2018).

Although neurological/psychiatric disorders such as schizophrenia and bipolar disorder are generally considered to be caused by complex gene–environment interactions, recent advances in genomic analyses demonstrate that genetic mosaicism may explain how developmental events in combination with environmental insults alter the risk for the manifestation of a psychiatric disorder (Bushman and Chun 2013; Sakai et al. 2015; Leija-Salazar et al. 2018; Rohrback et al. 2018; Nicolas and Veltman 2019). For neurodegenerative diseases, the association between the frequency of somatically mutated cells and disease remains unknown. Neurodegenerative diseases are heterogeneous and, therefore, one must be careful about generalizing from one example to the broader problem.

Mosaicism in Other Processes

Genetic mosaicism has been detected in "normal" processes, such as cellular senescence and aging (Risques and

Kennedy 2018), the generation of immune cell diversity (Dadi et al. 2009), and mitochondrial heteroplasmy (Aryaman et al. 2018) (See Table I). Heteroplasmy is the presence of more than one type of mitochondrial DNA (mtDNA) within a cell, tissue, or individual. Individual cells contain thousands of mitochondria and each mitochondrium contains multiple copies of mtDNA. Sequential mitotic events can lead to the clonal expansion of mitochondrial mutations, even within a single cell. Mutated mtDNA may eventually dominate the mtDNA pool, resulting in physiological consequences. Recent progress in mitochondrial genetics has demonstrated a relationship between mutations in mtDNA and disease phenotypes (Aryaman et al. 2018). However, to date, there is no evidence of chemically induced mutations in mtDNA (Valente et al. 2016). Thus, it will not be further discussed in this article.

Special Forms of Mosaicism

Genetic mosaicism can affect the soma, the germline, or the placenta (Fig. 1). Germline or gonadal mosaicism is a special form of mosaicism wherein some gametes carry a mutation, but the rest of the germ cells are normal (Acuna-Hidalgo et al. 2015). These mutations can subsequently be transmitted to offspring as seemingly *de novo* germline mutations, but in fact, the mutation did not occur during spermatogenesis/oogenesis, but much earlier in the development of the primordial germ cells of the parents. Mutations may be present in only some of the stem cells that give rise to gametes and, therefore, only some offspring may be affected. For example, application of next-generation sequencing (NGS) in several human families showed that 3.8% of mutations were mosaic in the parental germline, leading to 1.3% of mutations shared by siblings (Rahbari et al. 2016). A separate study found that nearly 10% of *de novo* mutations are post-zygotic and present in both somatic and germ cells (Sasani et al. 2019). These data suggest that the mutation rate per cell division is highest during early embryogenesis and differentiation of primordial germ cells, and is reduced in postpubertal spermatogenesis, indicating that these early developmental stages are critical to potential induction of mutations in germ cells.

Another special type of mosaicism is confined placental mosaicism, first described by Kalousek and Dill (1983). In this type of mosaicism, mutations can be detected in chorionic villi; whereas, only normal cells can be found in the child by a subsequent prenatal or postnatal test. Most pregnancies with confined placental mosaicism experience no complications and the child develops normally. However, in some cases prenatal or perinatal complications may occur, because the type of mutation interferes with normal development of the placenta or the number of affected cells interferes with placental function (Kalousek and Vekemans 1996) (see Fig. 1).

CAN EXPOSURE TO CHEMICALS DURING DEVELOPMENT INDUCE GENETIC MOSAICISM?

Spectrum of Mutations Involved in Genetic Mosaicism

A variety of genetic changes are associated with human diseases. These include mutations affecting single bases, up to DNA rearrangements involving a few to thousands of bases or entire chromosomes (Stenson et al. 2017). All of these types of mutations are also observed in genetic mosaicism (Youssoufian and Pyeritz 2002; Forsberg et al. 2017). The most common form of mosaicism involves aneuploidy. For example, a large proportion of patients with Turner syndrome (45, X) are actually mosaics having both cells that are normal (46, XX) and aneuploid (45, X) (Rasouli et al. 2019). Aneuploid mosaicism may be the result of a nondisjunction event during early embryonic divisions and generally leads to a milder phenotype, because the mutation is not present in all cells. Another frequent type of genetic mosaicism is copy number variation (CNV), which may be induced by incorrect repair of DNA damage and somatic recombination. CNVs are increasingly recognized as causative factors in human diseases (Zhang et al. 2009) and evidence is accumulating that chemical exposures can induce these types of events. Thus, the ability of environmental exposures to cause genetic mosaicism during *in utero* development requires the analysis of a broad spectrum of genetic changes, some of which are not currently detected by tests that are routinely used in genetic toxicology.

Maternal Exposure and Mosaicism

Since the thalidomide disaster in the 1960s, it has been apparent that environmental chemicals and pharmaceuticals can cross the placenta to cause adverse effects in the fetus. In many cases the fetus is more sensitive to the toxicant exposure than the mother (Gupta and Gupta 2017). The dose eventually reaching the fetus after maternal exposure depends on many factors, including passive and active transfer through the placenta and physicochemical properties of the chemical such as molecular weight, lipophilicity, polarity, and ionization. It should also be noted that the mother and the placenta itself are able to biotransform chemicals, thus allowing them to cross the placental barrier based on the properties of the metabolites (Myllynen et al. 2007). For more information about transplacental xenobiotic transfer, we direct the reader to reviews on this topic (Myllynen et al. 2007; Giaginis et al. 2009; Gupta and Gupta 2017; Koren and Ornoy 2018).

Consistent with the above, there is clear evidence to support that the fetus can be exposed to DNA-damaging xenobiotics present in the peripheral circulation of the mother following environmental exposures. For example, tobacco smoke-related compounds have been detected in the umbilical cord blood, placenta, and urine of neonates of smoking

mothers (Mercelina-Roumans et al. 1996; Godschalk et al. 2005). Tobacco smoke contains many genotoxic agents that pass the placenta and may induce DNA damage and subsequent mutations in the developing child. Indeed, increased levels of hypoxanthine-guanine phosphoribosyltransferase (*hprt*) mutations have been detected in cord blood of neonates of smoking mothers, compared to neonates of nonsmokers (Godschalk et al. 2005). Similarly, chromosomal aberrations (Bocskay et al. 2005), DNA adducts, and *hprt* mutations (Perera et al. 2002) have also been observed in cord blood samples from newborns of mothers prenatally exposed to carcinogenic polycyclic aromatic hydrocarbons in urban air pollution. Overall, the results suggest an association between somatic mutations in the newborn and transplacental exposure to air pollutants, providing mechanistic support to link early life exposures to airborne genotoxicants and increased cancer risk. Although these examples of transplacental mutagenesis do not necessarily reflect the induction of genetic mosaicism, they do indicate that the *in utero* phase of development is relevant for mutagenesis, and it is likely that mutations in early life clonally expand to result in a larger proportion of cells carrying the same mutation.

It has been hypothesized that early-life exposure to chemicals may contribute to pediatric leukemias (Lau et al. 2009). An adverse outcome pathway has been developed that provides empirical evidence supporting that DNA damage incurred during *in utero* toxicant exposure (in this case *via* chemicals that bind to topoisomerase II enzymes) can result in DNA double-strand breaks and subsequent oncogenic protein fusions that initiate the pathway to leukemia (Barjesteh van Waalwijk van Doorn-Khosrovani et al. 2007; Vanhees et al. 2011; Hernández and Menéndez 2016). Consistent with a role for exposure to clastogenic compounds in this pathway, Lau et al. (2009) demonstrated that *in utero* exposure to the carcinogen benzene led to increased micronucleus and DNA recombination events in fetal and postnatal hematopoietic tissues in mice. The findings led these authors to hypothesize that these may be molecular initiating events in the etiology of childhood leukemias caused by benzene. Overall, the role of potential clonal expansion of mutation-bearing cells following mutagen exposure and its involvement in childhood cancers is unclear and warrants further research.

Numerous other studies in experimental animals suggest that differentiating tissues are susceptible to mutation induction. For example, Mei et al. (2005) showed that early postnatal exposures to N-ethyl-N-nitrosourea (ENU) were more mutagenic in livers of BigBlue transgenic mice than exposures during adulthood. In contrast, Dobrovolsky et al. (2012) found that acute *in utero* exposure to ENU resulted in higher red blood cell (RBC) *Pig-a* mutant frequencies in exposed BigBlue dams than their offspring. Extremely variable responses in RBC *Pig-a* mutant frequencies were observed in the offspring, even among littermates. The authors suggested that this variability results from a switch from hepatic to predominantly bone marrow erythropoiesis that occurs during early development. Although there is no doubt that ENU passes the placenta to induce transplacental carcinogenesis (Rice 1973), ENU is a direct-acting genotoxic agent and, therefore, it is possible that the dose that reached the fetus is lower than the exposure the dams experienced. These data emphasize the critical influence of metabolism of carcinogens in maternal, placental, and fetal tissues, and the transplacental passage of carcinogens and their localization within the fetus on the changing susceptibilities of different tissues during embryonic and fetal development for mutation induction.

An elegant example of critical windows of susceptibility for mutagenic effects was provided by Chawanthayatham et al. (2015). In their study, pregnant *gpt* delta C57BL/6J mice were given aflatoxin B1 (AFB1) *via* intraperitoneal injection or oral gavage on gestational day (GD) 14. DNA adducts were measured 6-h postexposure in the liver DNA of mothers and embryos. A parallel cohort gave birth and the livers of the offspring were analyzed for *gpt* mutations at 3 and 10 weeks of age. The data revealed that AFB1-derived DNA adducts in GD14 embryos were 20-fold more potent inducers of mutagenesis than DNA adducts in the adults. The increased sensitivity to mutagenesis was correlated with the proliferative potential in the developmentally exposed rodents. The authors concluded that "early life exposure, especially during the embryonic period, is strikingly more mutagenic than treatment later in life." Unfortunately, DNA sequencing data were insufficient to draw conclusions as to the extent of somatic mosaicism in each animal, although the authors noted there was no evidence of increased jackpot mutations.

Despite these compelling examples that mutagenicity arises following *in utero* exposure, and that fetal and newborn tissues can exhibit increased sensitivities to mutagenicity, only a handful of studies have specifically explored the association between developmental exposures and genetic mosaicism. Russell et al. (1988) found that exposure of mouse zygotes to ENU caused an increase in mutation rate that was an order of magnitude greater than that induced by the same ENU exposure (50 mg/kg) to spermatogonial stem cells. Remarkably, of the 11 mutants recovered from the exposed zygotes, eight were mosaics. Meier et al. (2017) applied a novel NGS strategy to sequence thousands of mutant *lacZ* genes collected from male transgenic mice exposed *in utero* to increasing doses of benzo[*a*]pyrene (BaP) from GDs 7–16. They found an increased burden of both somatic and germ cell mutations in the adult offspring, especially in organs that in adult life no longer proliferate, particularly the brain. Indeed, *in utero* exposures led to a far higher burden of mutations per mg/kg BaP exposure in proliferating tissues than was observed in similarly exposed adult males. Sequencing demonstrated that a large proportion of the increased

mutant load could be attributed to mutations of clonal origin and that the embryos of exposed female mice exhibited higher proportions of genetic mosaicism than exposed adults. Furthermore, the induced mutations had the hallmark DNA sequence changes of BaP exposure, demonstrating that the effects were the direct consequence of transplacental chemical exposure and not due to indirect mechanisms (Meier et al. 2017). Mutation analysis in the female offspring of these dams in a separate study showed highly similar results (Luderer et al. 2019). These mutations may not only lead to transplacental carcinogenesis but may also increase cancer susceptibility of offspring, because expanding the population of cells that already carry mutations may provide a "jump start" to carcinogenicity. Other health-related effects from these mutations would be associated with mutation localization and the extent to which the mutations populate the tissue. Overall, the two studies on BaP provide compelling evidence to demonstrate the extent of clonal expansion of mutations that can occur following developmental exposures and the downstream impact on total mutation burden.

A variety of other examples of increased mutagenicity in animals exposed *in utero* exist, too numerous to describe herein, but include exposures to dioxin-like compounds (Pedersen et al. 2010), diesel exhaust particle (Ritz et al. 2011), and radiation (Barber et al. 2009). Thus, transplacental induction of genetic mosaicism has been documented; additional research in this area to strengthen the link between genetic mosaicism and adult diseases is of great importance for the protection of human health.

Paternal Exposure and Mosaicism

As discussed above, exposure during pregnancy can induce genetic mosaicism in offspring. Less intuitive is that paternal exposure to genotoxic agents may also lead to genetic mosaicism. Mutations present in sperm will of course not result in genetic mosaicism, because all cells of the offspring will carry these mutations. However, preconceptional exposure of males induces DNA damage in sperm that may not necessarily compromise conception (Verhofstad et al. 2010). This DNA damage is subsequently transmitted to the oocyte during fertilization, and if not repaired in time before the onset of DNA replication, may induce mutations expressed in later developmental stages. For example, BaP-related DNA damage was detectable by immunohistochemistry in sperm and up to the 8-cell stage of the blastocyst in human embryos derived from *in vitro* fertilization (Zenzes et al. 1999). There is little evidence that paternal exposure induces somatic mosaicism. However, DNA damage in sperm of exposed fathers can be converted into mutations after fertilization, which may result in an individual with mutations that are not present in all cells, and therefore, can be defined as genetic mosaicism.

Paternal exposure to BaP has been studied to detect the induction of germline mutations. In the course of these experiments, genetic mosaicism was detected, but was not taken into account, and often not reported. For example, Godschalk et al. (2015) investigated the ability of BaP to cause mutations in endogenous expanded simple tandem repeat (ESTR) sequences (*Ms6-hm* and *Hm-2*) in the male mouse germline and whether these were transmitted to offspring. In some instances, the offspring appeared to have two different paternal alleles (the original paternal allele and another mutated paternal allele), which could indicate the presence of mosaicism. In fact, the frequency of paternally derived genetic mosaicisms was 4.7-fold higher in the offspring of exposed fathers compared to controls ($P < 0.05$, unpublished data). Another recent study using BaP exposed MutaMouse males confirmed the ability of paternal exposures to cause mutations in the early embryo. Beal et al. (2019) applied whole-genome NGS to pedigrees derived from males exposed for 28 days to 100 mg/kg body weight/day BaP. They found that BaP exposure during postmitotic periods of spermatogenesis led to a statistically significant increase in embryonic mutations with respect to nonexposed controls. These mutations were present at a variant allele fraction of ~0.25, which is indicative of the induction of genetic mosaicism because mutations were present in only a portion of the cells in the organism (Beal et al. 2019).

These studies also point to the importance of the oocyte's active DNA repair machinery that is tasked with repairing paternally derived DNA damage before the onset of DNA replication during the first embryonic cell divisions (Marchetti et al. 2007). Therefore, it would be interesting to study in more detail how DNA lesions in sperm can affect genetic instability (Laubenthal et al. 2012) and mutagenesis in offspring, including the induction of somatic and germline mosaicisms.

HOW TO DETECT GENETIC MOSAICISM?

The induction of spontaneous as well as chemical- or radiation-induced mosaicism has been largely neglected in the scientific literature, probably because most of the available assays were unable to distinguish between unique and clonally expanded mutations and most research efforts focused on mutagenesis in adults. Thus, the relative contribution of mosaicism to the overall mutation burden is largely unknown.

Russell et al. (1988) and Russell and Russell (1992, 1996) used phenotypic markers in the specific-locus test to investigate the prevalence of germline mosaicism and they showed that the frequency of mosaics is probably higher than whole body mutants. Later studies using cytogenetics, specific point mutations (e.g., *hprt* mutations) in humans, or ESTR and transgene mutations in rodents, did detect

potential genetic mosaics, but these were not taken into account or reported, because they did not contribute to the original research question. More recently, advances in genomic technologies enable the analysis of genetic information at a genome-wide level, which has revived interest in genetic mosaicism. Genomic technologies, including NGS, RNA sequencing, single-nucleotide polymorphism microarrays, and comparative genomic hybridization microarray analysis have provided unprecedented opportunities to assess various types of genomic variation between, and increasingly within, individuals (Biesecker and Spinner 2013; Dou et al. 2018). The use of these techniques will inevitably increase because the costs of DNA sequencing are falling quickly.

A caveat of deep sequencing bulk cellular DNA is the limitation of NGS technologies to accurately detect very low-frequency genetic changes. The ability to detect rare variants is dependent on the number of DNA reads obtained during sequencing, the rate of technical sequencing errors, and the amount of starting genetic material. Conventional bulk deep sequencing is, thus, generally limited to the detection of mutations with variant allele frequencies greater than ~1% (Salk et al. 2018; Salk and Kennedy 2020). As such, it is not feasible, or cost-effective, to ultra-deep sequence genomes using the canonical NGS methods to explore low levels of mosaicism following chemical exposures. However, it should be noted that a mosaic that affects a relatively large proportion of cells is not exclusively the result of the mutations occurring at an early developmental stage (thus having the opportunity to clonally expand to relatively high frequencies), but could also originate from a cell clone with a fitness advantage. This advantage in a proliferating cell population would drive all mutations that were originally present in that clone to higher allele frequencies. These mutations would be detectable by bulk sequencing.

The degree and type of genetic mosaicism may differ between organs within the same individual/animal. Therefore, an analysis of multiple organs is needed to detect mosaicism effectively, which of course will increase analytical costs. A recent study investigated the frequency of post-zygotic mosaicism in 27 organs obtained from five healthy donors (Huang et al. 2018). It was found that multiple organs shared high-frequency post-zygotic mosaics, which probably arose during early embryogenesis. Germline mosaicism can also be studied effectively by sequencing technologies in both laboratory rodents as well as humans. In humans, sperm samples can be obtained noninvasively and germline mutations can already be detected in single sperm cells (Hinch et al. 2019; Tran et al. 2019), illustrating its potential use for studies in which *in utero* exposures to genotoxic agents are involved.

Recently, error-corrected sequencing technologies, such as duplex sequencing, have been developed that may allow the reliable detection of variant frequencies that are ~100-fold lower than what can be detected with standard NGS (Salk et al. 2018). These technologies promise to revolutionize how mutagenicity testing is conducted and to allow the investigation of genetic mosaicism following either adult or *in utero* exposure. Overall, more work is needed to fully understand the origin and spatial distribution of post-zygotic mosaicism during normal human development and after exposure to genotoxic agents. This knowledge would help in selecting relevant tissues and sequencing technologies that will facilitate this analysis.

IMPACT OF GENETIC MOSAICISM FOR THE CURRENT REGULATORY PARADIGM

In the previous sections, we summarized the evidence showing that *in utero* development is a sensitive stage for the induction of genetic mosaicism with broad implications for human health. Thus, we encourage the research community in general, and EMGS members in particular, to contribute to improve our understanding of the impact of environmental exposures on the genesis of genetic mosaicism.

Another important aspect to consider is whether a change is necessary to incorporate the routine analysis of *in utero* genetic mosaicism into the current regulatory testing paradigm. *In vivo* genotoxicity and mutagenicity analyses form a critical component of regulatory hazard identification of therapeutic drugs, agrochemicals, industrial compounds, food additives, natural toxins, and nanomaterials. At this moment, these tests are performed in adult animals only. Is it time to revisit this paradigm? Are we overlooking a critical period of development in regulatory testing for genotoxicity effects? A straightforward response to these questions is not currently available.

It could be said that there is no reason to modify the current testing paradigm, because there is no available evidence that the normal battery of tests conducted in adult animals would miss chemicals that would induce genetic mosaicism when the exposure happens *in utero*. Moreover, uncertainty factors are included during risk assessment to account for the higher sensitivity of certain life stages to assure the acceptable exposure levels are also protective of the most sensitive individuals. On the other hand, the present focus of genotoxicity testing on predicting carcinogenesis is narrowly focused, as it is widely acknowledged that mutations (and genetic mosaicism) play a role in many diseases other than cancer (Youssoufian and Pyeritz 2002; Nicolas and Veltman 2019). Furthermore, the present push for considering mutation in and of itself as an adverse outcome, and not just as a proxy for cancer (Heflich et al. 2019) makes a compelling case for improved experimental designs, where mutations are more likely to: (1) be detected; (2) have a variety of health effects; and (3) occur in both male and female germ cells, and all somatic tissues. Measuring the induction of mutations after *in utero* exposures

would also provide a more tangible measure of the differential sensitivity of developing embryos versus adults and contribute to reduce the uncertainties around the estimate of an acceptable exposure level.

A design in which the maternal animal is exposed, rather than an adult, has been proposed previously in a report from the International Workshops on Genotoxicity Testing (Yauk et al. 2015). This design was developed to address the potential to integrate germ cell testing within the current International Conference on Harmonization reproductive toxicology testing strategies. We note that such a design also provides a reasonable solution to the issue of differences in mitotic indices in adult tissues, potentially enabling more meaningful comparisons of mutagenic effects across tissues. An example of such an experimental model might involve the exposure of dams over the course of gestation to birth. Tissues can be collected at any time following birth, as mutations will clonally expand over the course of development and will manifest as genetic mosaicism. This approach enables the measurement of both mutation frequency and mosaicism. Moreover, mutation induction could be measured in both adult (dam) and developing rodents. We note that *in utero* exposure is also directly relevant to female germ cell mutagenesis, encompassing both mitotic and meiotic periods of oogenesis. This is another research and testing gap that is not addressed in the current testing paradigm; there are testing methods available for the male germline, but mutagenicity in the maternal germline is not studied at all, because the proliferative stage of female germ cells occurs *in utero*. Although the reliable study of oocyte mutagenesis may not be feasible with present technologies, because of the inability to retrieve sufficiently large numbers of eggs, highly accurate single-cell whole-genome sequencing technologies are expected to be developed in the future and could be applied for this purpose (Blanshard et al. 2018; Qian et al. 2019).

As described in the Introduction, it is critical to note that genotoxicity testing is currently transitioning from a qualitative hazard identification approach (is a compound mutagenic? yes or no) toward quantitative evaluations of hazard using dose–response data. Dose–response data are used for the determination of a genetic toxicity point-of-departure, which can subsequently be used to derive reference doses and margin of exposure values that are more useful for evaluating human risk and regulatory decision-making. It is unclear at this moment whether BMD modeling of genotoxic responses using assays in adults is protective enough for the developing embryo. Dose-setting could be challenging and the dose reaching the fetus is unclear. Top doses should be determined based on maternal and fetal survival to birth, and given that fetal germ cells may be exquisitely sensitive to genotoxicity (Meier et al. 2017; Luderer et al. 2019), will also need to consider sperm and oocyte counts from animals exposed *in utero* to ensure sufficient retrieval of cells for mutation analysis.

Finally, the current *in vivo* mutagenicity tests focus only on a small part of the genome (Thybaud et al. 2017), whereas sequencing technologies have the potential to screen the whole genome. Currently, mutation frequencies are too low to be detected by standard NGS assays. If, however, mutations have the time/chance to clonally expand *in vivo* in the developing fetus, these technologies may open new doors for genotoxicity testing.

CONCLUSIONS AND FUTURE DIRECTIONS

There is an increased understanding of the critical role that genetic mosaicism plays in human disease. There is also a renewed and growing recognition that mutations themselves are adverse and, thus, could be used as a regulatory endpoint in human health risk assessment (White and Johnson 2016; Heflich et al. 2019). This is certainly true for germ cell mutations that are transmitted to offspring and cause genetic disease (Marchetti et al. 2020). This is also arguably true for somatic genetic mosaicism occurring in early development that can cause various diseases (see Table I). However, existing experimental designs for regulatory genetic toxicology focus on evaluating the carcinogenic potential of a chemical through a mutagenic mode of action. Moreover, the design of current regulatory experiments for mutagenicity assessment *in vivo* focuses exclusively on adult exposures and does not capture the complexity of some of the genomic changes that are known to contribute to human disease.

Herein, we argue that developmental windows are an overlooked life stage that should be considered as new genotoxicity testing paradigms evolve to address mutation as a regulatory endpoint in human health risk assessment. The timing is right, as recent developments in sequencing technologies offer practical tools and analytical pipelines to detect and study all types of genetic mosaicism. These tools are already applied in the clinic at unprecedented scales to diagnose genetic disorders associated with mosaicism, and it is time for the genetic toxicology community to modernize our testing paradigms to align with the medical community.

The major obstacle to such a paradigm change is the limited amount of data currently available on the ability of various classes of chemicals that induce mutagenicity in rodents exposed *in utero*. Therefore, prior to implementing any such changes, further experiments would be required assessing chemicals spanning a range of mutagenic modes of action and potencies to establish the sensitivity and specificity of such an approach. Given that the placenta, maternal metabolism, and maternal toxicokinetics may limit exposure for some chemicals, both maternal adult and fetal tissues could be evaluated for mutagenicity. Overall, it is clear that a significant amount of research, with associated costs, would be required to support such a paradigm

change. The protocol may also, in and of itself, be somewhat more costly than the approaches currently recommended, since the design would require mating and conception prior to exposure. However, this cost may be offset by an improved strategy to identify chemically induced mutagenicity in somatic tissues in parallel with both male and, in the future, female germ cells (including progenitors and stem cells), reducing the need to have an additional set of animals for the sole purpose of male spermatogonial stem cell assessment. Furthermore, an integrated approach with other types of toxicity testing (e.g., reproductive and developmental toxicology) could be considered that would enable the simultaneous assessment of other endpoints and concomitant reduction of animals in regulatory testing. This is particularly true now that new error-corrected sequencing technologies are available that readily enable the measurement of mutation in any tissue/species without the need for transgenic rodents [e.g., Duplex sequencing (Salk et al. 2018; Salk and Kennedy 2020)]. This provides new opportunities to integrate genotoxicity testing with two-generation developmental toxicity studies, an approach that would not add additional animal tests. Therefore, this approach is worth serious consideration.

Conflict of Interest

The authors have no conflict of interest.

Author's Contributions

All authors (R.W.L.G., C.L.Y., G.R.D., J.v.B., and F.M.) contributed to the ideas presented in this commentary. R.W.L.G drafted the manuscript and all other authors (C.L.Y., G.R.D., J.v.B., and F.M.) critically revised the manuscript. All authors approved the final version of the manuscript.

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