WOMEN IN REPRODUCTIVE SCIENCE

Errors and insight: intentional and accidental studies of human chromosome abnormalities

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Abstract

Perhaps every career makes sense in retrospect. I have spent mine facing a series of accidental environmental exposures that derailed our studies but provided new insight. Although at times I have felt more catalyst than scientist, the journey has been extraordinary, and the problem I have spent my career studying – human aneuploidy – has taken on new significance with growing evidence of the sensitivity of the germline to the environment.

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A question big enough for two

Genetics and chromosomes – it was love at first sight – both figuratively and literally. I was hooked on genetics after an undergraduate class at Michigan State University. I'd like to say I pursued my graduate studies at the University of Hawaii because it afforded an opportunity to work with renowned human cytogeneticist, Patricia Jacobs. It's true I wanted to work with her, but Terry Hassold – the man I met while working part-time in a human genetics clinic laboratory (and would eventually marry) – was the compelling factor. Terry was embarking on a postdoc in Patricia Jacobs' Laboratory at the University of Hawaii and I followed. Dr Jacobs rightfully thought romance had no place in the laboratory, so I attempted to pursue my degree in a different department. But my graduate studies began in earnest when she finally took me into her laboratory a year later and subjected me to the rigorous boot camp she thought appropriate for graduate education. It was grueling, but I emerged a fledgling scientist with a question that has driven my entire career.

When I joined the lab in the 1970s, Pat Jacobs was engaged in a population study of human miscarriage. Human pregnancy loss is exceedingly common – on the order of one in five recognized pregnancies ends in first or early second trimester miscarriage. Pat was interested in the role of chromosome abnormalities in this loss and the genesis of the errors. Our study in Honolulu was complemented by similar surveys by Dorothy Warburton

in New York City, Margaretta Mickelson in Denmark and Joelle and Andre Boué in Paris. Data from these studies of human miscarriage provided an understanding of the incidence and etiology of human aneuploidy and led Terry Hassold to generate the now famous plot of the incidence of trisomic conceptions by maternal age – or, as my female colleagues call it, 'the dreaded age curve' (Fig. 1). This J-shaped curve and the data behind it captivated me: The incidence of errors is high (50% of miscarriages) and, although not affected by ethnicity, is strongly influenced by age (Hassold *et al.* 1978). The meiotic error rate in humans is at least an order of magnitude greater than in other, well-studied organisms such as mice, flies or yeast (Fraser & Maudlin 1979, Koehler *et al.* 1996, Chu & Burgess 2016). Intriguingly, errors are increased at both extremes of our reproductive life, with a precipitous rise from the mid-thirties on. This intriguingly complex problem has fed both Terry Hassold's and my research careers for over 40 years. A brief personal profile along with some important papers published is presented in Box 1.

The mouse: terrible model but powerful tool

The aneuploidy problem was captivating, but I found humans a limiting model organism. My desire for an experimental approach led me to Anne McLaren's MRC Mammalian Development Unit in London. Armed with an NIH postdoctoral fellowship, I worked with Paul Burgoyne and learned to make chromosome

Box 1: Patricia Ann Hunt, Ph.D.

I have had many wonderful colleagues throughout my career, but the lessons of two, my graduate mentor, Patricia Jacobs and postdoctoral advisor, Eva Eicher allowed me to succeed in the face of adversity. Pat taught me to think about the data I was seeing, not the data I expected. Eva taught me that the pure pleasure of using your brain and following your heart should – and must – outweigh the slings and arrows thrown at a good woman in the course of duty. Both impressed upon me the importance of not simply writing up my findings but placing them in context, speculating and outlining data needs. By agreeing and disagreeing with my speculations and picking up the gauntlet to address our knowledge gaps through their own studies, my colleagues have driven the field of aneuploidy forward. Meanwhile, I have been privileged to meet and count among my colleagues a wonderful group of scientists from different fields who also became focused on the effects of BPA and other endocrine-disrupting chemicals. My work with these remarkable, socially responsible scientists has convinced me that the next generation of scientists must be better advocates for science and view sharing their findings and concerns with the general public an essential part of their job. The playing field has changed, and the battle has taken on new urgency: The development of the new field of developmental origins of adult health and disease (DOHAD) and growing evidence of transgenerational effects gives new importance to the field of reproductive biology.

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preparations of single cleavage-stage embryos and devise complex breeding schemes to obtain sex chromosome abnormalities that boggled the minds of others. During his career, Paul made many important contributions to this field, and his student, James Turner and others – most notably Monika Ward – have picked up where he left off and continue to provide insight to the unique features of sex chromosomes.

Paul's interest in sex chromosomes expanded the foundation laid by Pat Jacobs, and I found myself comparing and contrasting human and mouse data. I returned to the United States after 2 years armed with

a repertoire of mouse developmental and reproductive techniques, convinced of the power of mouse models, but lacking an understanding of mouse genetics. To address this, I went straight to the source – The Jackson Laboratory (TJL) – for a year of postdoctoral training with one of the all-time best mouse geneticists and a great scientific mentor, Eva Eicher. My training with Eva and my continued connection with Dorcas Corrow, a manager in TJL production, not only armed me with the mouse knowledge I needed but proved instrumental in solving the problems I encountered when I established my own laboratory.

Figure 1 The dreaded age curve. The frequency of trisomic conceptions among spontaneous abortions plotted by maternal age shows the rapid increase in errors in the decade preceding menopause as well as an increase in pregnancies occurring in young women. Adapted from (Hunt 2017).

When I joined the Department of Genetics at Case Western Reserve University as a young assistant professor, I remained focused on human age-related aneuploidy. I was convinced the mouse was a useful asset despite its significant shortcomings; the incidence of meiotic errors (1–2% of eggs) and the effect of maternal age (a doubling in older females) are extremely modest by comparison with the human. But I also recognized the importance of human studies and worked to establish clinical connections that would provide access to human eggs.

To facilitate studies of meiotic chromosome behavior, we developed new techniques for visualizing meiotic chromosomes. By comparison with today's live cell imaging technology, our method for fixing and probing intact oocytes seems crude but was instrumental in studies that defined my career. We pioneered the technology using XO female mice and tracked the meiotic segregation of the single X (Hunt *et al.* 1995). These studies led to an important realization: The chromosome behaviors we were seeing should trigger cell arrest and death but did not. This led us to conclude cell cycle control in the oocyte must differ in a fundamental respect from control mechanisms in spermatocytes and somatic cells (LeMaire-Adkins *et al.* 1997). This reframed my view of aneuploidy. It suggested

the high error rate in oocytes was not simply a matter of making more mistakes but of allowing cells to survive and become viable eggs – albeit ones that would give rise to aneuploid embryos. I assumed this cell cycle control difference would become the focus of my research, but serendipitous events turned our efforts in another direction, while others unraveled the unique features of the mammalian oocyte that allow cells with errors in chromosome attachment and alignment to become viable gametes (Homer *et al.* 2005, McGuinness *et al.* 2009, Kolano *et al.* 2012, Mihajlovic & FitzHarris 2018).

The early twentieth century geneticist, William Bateson, understood the power of mutations, and I took his advice to ‘*treasure your exceptions*’ to heart. Despite its low meiotic error rate and weak maternal age effect, I was convinced that the available mutants and ease of gene targeting made mice a tractable model for meiotic studies. In the course of our studies, Terry Hassold and I have compared and contrasted results of meiotic studies in males and females and in mice and humans. In addition to differences in the tolerance of aberrant chromosome behavior and error propensity, we have found important differences in the earliest meiotic events, including the response to errors (Hunt & Hassold 2002), onset of synapsis (Gruhn *et al.* 2016), placement of sites of recombination (Lynn *et al.* 2005, Gruhn *et al.* 2013) and chromatin conformation (Lynn *et al.* 2002, Baier *et al.* 2014). Sex-specific differences in meiotic chromosome cohesion uncovered in the course of our studies (Hodges *et al.* 2001) led to collaborative studies with Rolf Jessberger and Ekaterina Revenkova of the SMC1 β -knockout mouse and provided evidence that loss of cohesion is an important mechanism of error (Revenkova *et al.* 2004, Hodges *et al.* 2005). Several laboratories subsequently provided compelling evidence of the role of age-related loss of chromosome-associated cohesins in human and mouse eggs (Liu & Keefe 2008, Chiang *et al.* 2010, Lister *et al.* 2010). Although several features of human aneuploidy argue against cohesin loss as ‘the’ underlying cause of the striking age-related increase in meiotic errors (Nagaoka *et al.* 2012), it is clear that, in addition to altered cell cycle control mechanisms in the oocyte, loss of cohesion is an important factor in meiotic aneuploidy.

Our studies in mice also provided evidence that the propensity for errors in chromosome segregation extends beyond the meiotic divisions to the embryonic cleavage divisions. By the late 1980s, it was clear certain human chromosomes (e.g. chromosome 16) are more prone to error than others (Hassold *et al.* 1991). To try to understand the features of a chromosome that make it nondisjunction-prone we turned to the BALB/cWt mouse. Eva Eicher's laboratory demonstrated that the incidence of hermaphroditism on this inbred strain was due to loss of the Y chromosome that created sex chromosome mosaics (Eicher *et al.* 1980). We assumed the Y chromosome (WtY) had a centromere defect that

made it nondisjunction prone but, when Terry Hassold's graduate student, Chris Bean, conducted meiotic studies, he found no evidence of increased Y nondisjunction. When he analyzed embryos from BALB/cWt females in my lab, however, he found nearly all errors made by the WtY chromosome occurred during the first three cleavage divisions (Bean *et al.* 2001). The idea that the early cleavage divisions, like female meiotic divisions were prone to error, was both fascinating and clinically relevant. Fluorescence *in situ* hybridization (FISH) studies of human embryos derived through assisted reproductive technologies (ARTs) were suggesting an astonishingly high frequency of mosaicism. Since mosaics were rare in spontaneous abortion studies (Hassold 1982), this raised concern that the high incidence in ART might be, at least in part, attributable to the methodology. When we evaluated the effect of *in vitro* fertilization on WtY segregation, we found error frequency was increased and we could modulate it by changing culture conditions (Bean *et al.* 2002). Although the error-prone nature of the early cleavage divisions also appears to be a feature of the human embryo (Vanneste *et al.* 2009, McCoy 2017), mosaicism remains a contentious issue in the ART world (e.g. Capalbo *et al.* 2017). Evidence of variation among centers (Munne *et al.* 2017) suggests minor protocol differences (e.g. ovarian stimulation and/or culture protocols) may play a significant role. Improvements in analytical techniques (e.g. array comparative genomic hybridization, SNP arrays, quantitative PCR and next-generation sequencing) make it possible to obtain detailed information on human embryos. Although discussion of the merits of these different approaches has dominated in the field for several years, the use of any of them to compare the incidence of cleavage errors under different stimulation and culture protocols could prove a powerful tool in refining and improving ART technology.

It's all about us: the value and travail of human studies

My graduate training with Pat Jacobs proved a great boon when I approached clinicians at Case Western Reserve University's affiliated hospital, University Hospitals of Cleveland, in the hope of using our enhanced technology to understand the effect of age on the human oocyte. I knew I could only obtain the precious human oocytes needed by personally handling as much of the legwork as possible and minimizing effort required on the part of my clinical colleagues. Before I obtained funding to hire a very talented nurse coordinator, I consented patients myself, donned a surgical gown, gloves and mask, and hovered in the operating room trying to be inconspicuous while waiting for the surgeon or fellow to aspirate follicles visible on the surface of the patient's ovary and deliver the fluid into the tube incubating in my hand. I also relentlessly badgered the pathologists for surgical specimens of ovarian tissue removed during

surgery *before* the tissue was fixed in formalin and tried never to lose hope when specimens were fixed before I could intervene. Over the years, research colleagues have bemoaned the failure of their attempts at clinical studies and blamed clinicians. Most assumed clinicians should and would go out of their way to assist research. Thanks to Pat, I knew it was entirely up to me to make it happen: I understood the necessity of persuading my clinical colleagues of the importance of the proposed studies, keeping them apprised and acknowledging their crucial contributions.

The human oocytes we obtained were aspirated from large antral follicles, but we had no control over their maturation or health status. We cultured them for 24–48 h and analyzed those with a visible first polar body indicating that they resumed and completed the first meiotic division. Because studies of trisomies suggested most errors had their genesis during this division (Hassold *et al.* 2007), my goal was to analyze eggs from women of different ages to determine how age affected the types of errors that occurred. Our analysis, however, provided unanticipated insight: We saw a strong age-associated effect on the ability to build a second meiotic spindle and organize the chromosomes on it. Although captivated by the effect of age in our data, I feared the defects might simply reflect oocyte immaturity. As I fulminated over our growing data, a strange thing happened. A former graduate student colleague, David Battaglia, now an ART clinic laboratory director, reported remarkably similar findings. David was studying 'spare' (unfertilized) oocytes from women subjected to ovarian stimulation during the course of infertility treatment. Despite the differences between our studies, our data were remarkably similar, showing an age-related increase in the number of eggs with spindle aberrations and misaligned chromosomes (Battaglia *et al.* 1996, Volarcik *et al.* 1998). Recent data from studies in mice in the FitzHarris Laboratory have provided new insight to these old data, suggesting that age-related changes in spindle dynamics predispose to defects in kinetochore attachment that lead to segregation errors (Nakagawa & FitzHarris 2017).

A career-altering mistake

Finding gross disturbances in meiotic chromosome behavior in eggs from older donors made me view the age problem differently. Could subtle changes in the hormonal signals that orchestrate oocyte growth and maturation be responsible for the increase in aneuploid eggs at both ends of the maternal age spectrum? I could think of several mouse models to test this. Using them, we found defects remarkably similar to those in human eggs from older donors and were able to determine that failure to form a normal first meiotic spindle and orchestrate chromosome alignment was not simply a symptom of oocyte immaturity. Importantly, phenotypically identical

defects were evident in the eggs from mice in which either communication between the oocyte and soma of the follicle (XY females) or the endocrine control of oocyte growth (LH hypersecretion) were defective (Hodges *et al.* 2002). Aberrant chromosome behavior did not delay the onset of anaphase (providing further support for oocyte-specific differences in cell cycle control), but was correlated with an increase in chromosome segregation errors, especially the premature separation of sister chromatids (Hodges *et al.* 2002). Although these data suggested subtle changes in the ovarian environment might be responsible for the increase in meiotic errors at both ends of the human maternal age spectrum, publication of our findings was delayed by a serendipitous event that provided compelling support for our hypothesis.

The story of this part of my career has been told many times. In essence, while we were testing the hypothesis that hormonal fluctuations during the late stages of oocyte growth cause aneuploidy, mice in our colony were inadvertently exposed to the endocrine-disrupting chemical bisphenol A (BPA) via damaged caging materials. The sudden onset of this accidental exposure was immediately evident as a significant increase in defects in oocytes from control females (Hunt *et al.* 2003, Koehler *et al.* 2003). Although it is easy to recount the experience, it is difficult to convey its career-altering impact. Just as pieces of the aneuploidy puzzle seemed to be coming together, we suddenly had compelling evidence of the vulnerability of the mammalian oocyte to environmental exposure. But, further studies in mice were impossible until we could eliminate the exposure, obtain normal baseline control data, and experimentally test the effects of BPA exposure.

Several years later when we finally published data from the inadvertent exposure and subsequent controlled experiments establishing BPA as the culprit, it caused a media firestorm: The link between BPA exposure and aneuploid eggs suggested that a chemical used in a wide range of consumer products had the potential to induce pregnancy loss and birth defects. As I struggled to coherently explain our findings to nonscientists, each interview became a learning experience. The biggest lesson I learned is that the greatest impact does not come from the data but from our ability to clearly and simply articulate the findings and their significance. Almost all baby bottles on the market at the time were polycarbonate, which I found unsettling. Thus, at the end of most interviews, when asked if there was anything I wanted to add, I invariably voiced my concern that – via baby bottles and sippy cups – we were placing this chemical into the hands of the most vulnerable segment of our population. Imagine my surprise when I received a phone call from a baby bottle manufacturer several weeks later and my astonishment as I watched baby bottles made of polymers other than polycarbonate come onto the market within months.

My interest in BPA was intensified by data from our next experiments. Studies in our laboratory and by others had established a link between the placement of sites of meiotic crossing over along the length of human chromosome and segregation errors leading to trisomic conceptions (Hassold & Hunt 2001). This led Terry Hassold to postulate the ‘two hit model of aneuploidy’ in which events occurring at the onset of meiosis in the fetal ovary set the stage for mistakes in chromosome segregation decades later when the meiotic divisions were completed (Hassold & Sherman 2000).

Oocyte growth and maturation in the adult ovary is orchestrated by complex endocrine signals, so it was easy to understand how spurious signals from endocrine-disrupting chemicals (EDCs) might have an effect, but there was no *a priori* reason to suspect that the complex events of meiotic prophase in the fetal ovary could be similarly influenced. However, when Martha Susiarjo (then a student in my lab, now an assistant professor at the University of Rochester studying the epigenetic mechanisms underlying environmental effects) assessed the effect of maternal BPA exposure coinciding with the onset of meiosis in the fetal mouse ovary, she found a stunning increase in levels of meiotic recombination. More importantly, when she examined eggs and embryos from adult females exposed *in utero*, Martha found that the subtle changes induced by exposure at the onset of meiosis dramatically increased the incidence of aneuploidy (Susiarjo *et al.* 2007).

By comparison with our first BPA exposure effect, the implications were far more serious: Brief BPA exposure during fetal development could affect three generations simultaneously – the mother, her unborn female fetus and the oocytes that would give rise to her grandchildren. Importantly, because all oocytes initiate development prenatally, maternal exposure could affect the entire cohort of eggs ovulated by an exposed female fetus. This type of grandmaternal effect would be nearly impossible to document in humans. Thus, when Cathi VandeVoort suggested studies in the rhesus monkey, the power of this model was immediately obvious. By sharing tissues with investigators interested in BPA effects on other organ systems, this small set of monkeys provided a wealth of data. Our studies of the fetal ovary not only recapitulated the prophase effect we had described in mice but also provided compelling evidence that maternal BPA exposure in late gestation could disrupt follicle formation (Hunt *et al.* 2012). This was not unexpected since the disruptive effect of perinatal estrogenic exposure on follicle formation in mice was well documented but, combined with the effect on the periovulatory oocyte we had observed, it provided evidence that at least three distinct stages of oocyte development were vulnerable to the effects of BPA.

The wild world of EDCs

Entering the world of EDCs was like stepping into a fun house; our view of the world became distorted and environmental effects suddenly were everywhere. After publishing our studies of prenatal BPA exposure two things happened in close succession: First, we had trouble generating timed matings for follow-up studies and began to see an increase in birth defects and late-stage fetal deaths. Eventually, we traced these effects to a disinfectant used in the facility that contained two quaternary ammonium compounds (Quats or QACs). Although the effects on reproduction and fetal development were significant, the results of our meiotic studies were unaffected, so I merely reported our findings at a meeting but did not publish them. Brendan Mahr happened to hear the talk and covered the story in *Nature* (Hunt 2008), leading Terry Hrubec to contact me when her studies of neural tube defects were complicated by a sudden increase in defects in control animals. She wondered if the increased incidence was a QAC effect. I shared our data with her, and she translated our initial collaboration into a series of studies demonstrating the reproductive and developmental effects of this family of chemicals that, like BPA, has crept into our daily lives seemingly without our knowledge (Melin *et al.* 2014, 2016, Hrubec *et al.* 2017).

After QAC exposure was eliminated and we resumed BPA studies, we began to notice variability in our results, with expected exposure effects in some groups of females but not others. The data changes were temporal and, our heightened awareness of variables in the mouse room allowed us to rapidly link the data shifts to different lots of animal feed. This led to studies unraveling the meiotic effects of yet another environmental variable, dietary phytoestrogens (Muhlhauser *et al.* 2009).

As reports of adverse effects induced by BPA increased and industry fought to discredit new findings and minimize concern, I found myself collaborating with researchers from different disciplines to rebut industry talking points, explain the strange nonmonotonic effects being reported and provide cogent arguments about why traditional toxicology testing methods were insufficient for EDCs (vom Saal *et al.* 2007, 2010, Myers *et al.* 2009). In response to one industry argument – that the risk posed by BPA was minimal because it was rapidly metabolized – Fred vom Saal, Cathi VandeVoort and I designed a pharmacokinetic study in the rhesus monkey (Taylor *et al.* 2011). Our subsequent studies of pregnant females demonstrated that pregnancy altered BPA metabolism and that rapid maternal metabolism did not preclude passage of BPA to the fetal compartment (vom Saal *et al.* 2014, VandeVoort *et al.* 2016). These data clearly increased the importance of our human studies, but our attempts to link maternal BPA levels to

meiotic effects in oocytes in the developing fetal ovary were stymied for two reasons: First, in response to criticism about the reliability of data from biomonitoring studies, a Round Robin study was designed by NIEHS to assess the consistency of measurements made in different laboratories (Vandenberg *et al.* 2014). Until measurement issues were resolved there was no point in subjecting precious human samples to BPA analysis. At the same time, increased public awareness led to the enactment of BPA bans in several states, and ‘BPA free’ products were being rapidly introduced. Because this was accomplished through the substitution of replacement bisphenols, BPA was no longer the only culprit.

Effects in males too and, oh my goodness, déjà vu

Although our human BPA studies were thwarted, data from continuing studies in mice provided compelling evidence that the germline in both sexes is sensitive to environmental effects. In asking whether BPA induced similar effects on meiotic recombination in the male, data from a student in the lab, Lisa Vrooman, provided evidence that brief perinatal exposure to BPA or other exogenous estrogens altered the developing spermatogonial stem cell (SSC) lineage, permanently altering meiotic recombination levels in the adult male (Vrooman *et al.* 2015). Additionally, Lisa identified an effect that was intrinsic to the testicular environment, as she found that the age of the male affected recombination – with the lowest levels in the first wave of cell that initiate spermatogenesis, a rapid increase to adult levels, and an additional increase with advancing paternal age (Vrooman *et al.* 2014). Another student, Tegan Horan, built upon Lisa’s studies of estrogenic exposures, demonstrating that effects on the testis induced by perinatal exposure are transgenerational and increase in severity with successive generations of exposure (Horan *et al.* 2017).

The mechanisms underlying the effects of BPA on meiotic recombination are clearly sex specific. In females we have been able to pinpoint the window of sensitivity to the onset of meiosis and are currently working to understand how BPA induces changes in chromatin compaction. In contrast, in males, recombination effects are a downstream consequence of changes induced in the developing SSC population. Understanding the epigenetic changes responsible in both sexes will provide important insight to how meiotic recombination levels are set in mammals and influenced by the environment. However, mechanistic studies in our lab have been delayed by the most recent in the series of unfortunate events that have dogged my research. In a bizarre twist, nearly exactly 20 years after the inadvertent BPA exposure of our mice launched us into the world of EDCs, we again fell victim to an environmental

exposure – this time to replacement bisphenols. Details of this latest misadventure – exposure to replacement bisphenols – and the insight provided by our studies to identify and eliminate the contaminants recently have been published (Horan *et al.* 2018, Gorence *et al.* 2019). These data demonstrate that, like BPA, several common replacement bisphenols induce meiotic effects in both males and females. These findings confirm the need to obtain information not only on BPA but all bisphenols to which humans are now being exposed. They also provide a ray of hope in the dismal world of BPA and replacement bisphenols, suggesting that if we could eliminate these pervasive environmental contaminants, meiotic effects resulting from exposure would persist for several generations, but ultimately return to baseline levels (Horan *et al.* 2018).

Epilogue

Some scientists are visionaries whose work challenges our perceptions and forever changes how we think. Others of us serendipitously provide important insight. I've tried to detail the path that has led me to conclude that the germline in both sexes is strongly influenced by the environment. Since it represents the future of a species, the germline might be expected to occupy a protected niche. However, because meiotic recombination is highly responsive to a range of environmental effects, and it is a process that increases genetic diversity, it could be argued that the subtle germline changes induced by the environment act as an evolutionary driver to facilitate species adaptation. This is fascinating basic biology but, given the introduction of a wide and ever-increasing variety of manmade EDCs, it is also a cause for alarm. My studies of EDCs have convinced me the major impact a scientist can have is not through her publications but by sharing her findings and concerns with other scientists, clinicians, legislators and the general public.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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