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UCRL-JRNL-207365

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October 20, 2004

Cytogenetic and Genome Research

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Mechanisms and Chemical Induction of Aneuploidy in Rodent Germ Cells

Cytogenetics and Genome Research

July, 2004

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Keywords: aneuploidy, germ cells, mechanisms.

Abstract

The objective of this review is to suggest that the advances being made in our understanding of the molecular events surrounding chromosome segregation in non-mammalian and somatic cell models be considered when designing experiments for studying aneuploidy in mammalian germ cells. Accurate chromosome segregation requires the temporal control and unique interactions among a vast array of proteins and cellular organelles. Abnormal function and temporal disarray among these, and others to be **inidentified,** biochemical reactions and cellular organelles have the potential for predisposing cells to aneuploidy. Although numerous studies have demonstrated that certain chemicals (mainly those that alter microtubule function) can induce aneuploidy in mammalian germ cells, it seems relevant to point out that such data can be influenced by gender, meiotic stage, and time of cell-fixation post-treatment. Additionally, a consensus has not been reached regarding which of several germ cell aneuploidy assays most accurately reflects the human condition. More recent studies have shown that certain kinase, phosphatase, proteasome, and topoisomerase inhibitors can also induce aneuploidy in rodent germ cells. We suggest that molecular approaches be prudently incorporated into mammalian germ cell aneuploidy research in order to eventually understand the causes and mechanisms of human aneuploidy. Such an enormous undertaking would benefit from collaboration among scientists representing several disciplines.

Significance of aneuploidy

Aneuploidy, a condition in which the chromosome number is not an exact multiple of the haploid number, is the greatest genetic affliction of man. Approximately 10% of human embryos, 40-60% of early spontaneous abortuses, and 0.31% of human newborns have an abnormal number of chromosomes (Bond and Chandley, 1983; Hook, 1985; Hecht and Hecht, 1987). Although numerous hypotheses and etiologies have been proposed for human aneuploidy, the only consistent findings remain its positive correlation with maternal age (Bond and Chandley, 1983; Hook, 1985; Chandley, 1987) and its more frequent occurrence during female meiosis I (Hassold, 1985; Hassold and Sherman, 1993).

Accurate chromosome segregation requires the coordinated interaction among protein kinases and phosphatases, microtubules, motor proteins, centrosomes, kinetochores and their associated proteins, spindle checkpoint proteins, anaphase promoting complex, proteasomes, and the securin-cohesion complex. Abnormal function and temporal disarray among these biochemical reactions and cellular organelles have the potential for predisposing cells to aneuploidy. Presently, little is known about the numerous potential molecular mechanisms of aneuploidy in any cell type.

The intent of this limited review is twofold: (1) to present information about the current state of rodent germ cell aneuploidy research, and (2) to suggest that the advances being made in understanding the molecular mechanisms of chromosome segregation be incorporated into mammalian germ cell aneuploidy research.

Oogenesis & Spermatogenesis

Oogenesis

Oogonia enter meiotic prophase and progress to dictyate (diplotene) during fetal development. These primary oocytes remain in dictyate until meiosis resumes in response to gonadotrophins or release from follicles and in vitro culture. Upon re-initiation of meiosis, the intraoocyte titer of cyclic adenosine monophosphate (cAMP) decreases. This results in reduced cAMP-dependent protein kinase activity and a shift in the enzyme equilibrium toward cAMP-dependent phosphatase and maturation promoting factor (MPF) activities (Bornslaeger et al., 1986; Schultz, 1988; Downs et al., 1989). MPF is composed of a 34 kDa serine-threonine kinase subunit ($p34^{cdc2}$) and a 45 kDa cyclin B regulatory subunit (Gautier et al., 1991; Strausfeld et al., 1991). In addition to MPF, mitogen-activated protein kinases (MAPKs) and the product of the c-mos protooncogene Mos kinase also have pivotal roles during oocyte maturation (Paules et al., 1989; Hashimoto, 1996; Murray, 1998).

Oocyte maturation (OM) encompasses the nuclear and cytoplasmic modifications that occur during the transition from the dictyate stage of meiosis I to metaphase II (MII) (Schultz, 1986; Racowsky, 1993). OM is regulated by specific post-translational phosphorylation and dephosphorylation reactions coupled with proteasome-mediated proteolytic events that regulate signal transduction pathways. During anaphase I, homologous chromosomes randomly undergo bipolar segregation resulting in a haploid oocyte and a first polar body. Following a brief prophase without DNA synthesis, the secondary oocyte remains in MII for a limited time period until fertilization, spontaneous activation, or atresia. If fertilization occurs, anaphase II ensues and sister chromatids segregate to form an oocyte pronucleus and a second polar body. Subsequently, the haploid male and female pronuclei fuse to form a diploid one-cell zygote.

Spermatogenesis

Spermatogenesis is a complex differentiating system that is regulated by strict controls of the expression of genes encoding proteins that play essential roles during specific periods of germ cell development (Hecht, 1998; Grootegoed et al., 2000; Inselman et al., 2003). Spermatogenesis is initiated from stem cells through division of spermatogonia to form spermatocytes (meiotic cells). Spermatocytes undergo two meiotic divisions and give rise to haploid round spermatids (postmeiotic cells) that undergo major morphological changes that culminate in the formation of mature spermatozoa and their release into the lumen of seminiferous tubules (spermiogenesis) **(Kleen** 2001). Released sperm undergo a final process of maturation within the epididymis where they acquire motility and the ability to fertilize the egg.

The kinetics of spermatogenesis are well established for men and several mammalian species and are remarkably constant within species (Adler 1996). It takes approximately 35 days in mice and 64 days in humans for germ cells to develop from spermatogonia to spermatozoa. Meiotic prophase lasts about two weeks and is followed by the first and second meiotic divisions, which occur within 24 hr of each other, while spermiogenesis takes about three weeks. This means that if one is to assess the aneugenic effects of a chemical on male germ cells by analyzing sperm, at least three weeks have to pass from exposure to collection of sperm to give time to the germ cells that were undergoing meiotic divisions at the time of treatment to mature and reach the ejaculate. The longer the time interval between treatment and collection, the earlier in spermatogenesis the effects are being sampled.

<u>Molecular mechanisms of chromosome segregation: an overview</u>

Several reviews provide insight about the roles of unique proteins and control mechanisms associated with chromosome separation and segregation (Nasmyth, 2001; Lee and Orr-Weaver, 2001; Uhlmann, 2001; 2003a). Although the bulk of such information has been derived from non-mammalian somatic cells, it is clear that chromosome segregation during meiosis largely depends on the same machinery used during mitosis (Nasmyth, 2001).

The metaphase-anaphase transition (MAT) represents a highly **intergraded** process culminating in a point of no return. Chromosome segregation in eukaryotes utilizes a bipolar spindle formed by microtubule arrays that integrate kinetochores. Microtubule-based motors energized by ATP hydrolysis and microtubule depolymerization are needed for chromosome movement to the spindle poles (Inoue and Salmon, 1995; Biggins and Walczak, 2003). These motors consist of kinesin, kinesin-related proteins (Wordeman and Mitchison, 1995; Walczak et al., 1996), dynein (Pfarr et al., 1990; Steuer et al., 1990) and the kinetochore protein CenP-E (Yen et al., 1992). Even though the MAT appears straightforward from a cytogenetic viewpoint, it is actually a complex series of events involving the coordination of independent processes that depend on prior checkpoint release and anaphase promoting complex (APC) activation. Chromatid arm separation and centromere separation (anaphase A) are independent events with different mechanisms (Sluder and Rieder, 1993; Rieder and Salmon, 1998), and separation does not initiate poleward movement of chromatids (anaphase B) (Zhang and Nicklas, 1996). Also, sister chromatids can separate in the absence of spindle attachment (Rieder and Palazzo, 1992).

During prometaphase, a spindle-assembly checkpoint utilizes unique proteins to delay anaphase if proper kinetochore-microtubule attachment and tension are lacking (Li and Benezra, 1996; Taylor et al., 1998; Zhou et al., 2002; Musacchio and Hardwick, 2002; Bharadwaj and Yu, 2004). Although this checkpoint acts as a safeguard for the timely initiation of anaphase and the orderly segregation of chromosomes, it can be overridden. Anaphase can still occur following exposure of cells to microtubule disrupting drugs, in the absence of normal spindle bipolarity, and in the presence of unattached kinetochores and abnormal chromosome orientation (Rieder and Palazzo, 1992; Rieder et al., 1994).

In mouse oocytes, phosphorylated Bub1 checkpoint protein was found at kinetochores during metaphase I and II (Brunet et al., 2003) and a functional Mad2-dependent spindle checkpoint was identified during meiosis I (Wassmann et al., 2003). Besides spindle checkpoint proteins, Mps 1 kinase (Abrieu et al., 2001), CENP-E motor protein (Chan et al., 1998; Abrieu et al, 2000), the dynein interacting proteins Zw10 and Rod (Basto et al., 2000; Chan et al., 2000), and the chromosome passenger proteins Aurora B and survivin also have important roles during

spindle checkpoint signaling and in correcting abnormal kinetochore-spindle fiber attachments (Carvalho et al., 2003; Lens and Medema, 2003).

When proper kinetochore-microtubule attachment and tension have been attained, the spindle checkpoint is released and the APC can be activated. The APC represents a large protein complex that ubiquinates mitotic cyclins and other regulatory proteins that are destined for timely proteolysis by proteasomes (Kotani et al., 1999; Glickman and Ciechanover, 2002). Proteasomes consist of multicatalytic 26S proteases and a 20S central core catalytic subunit bordered by two 19S components that hydrolyze C-terminal peptide bonds to acidic, basic, and hydrophobic amino-acid residues (Goldberg, 1995; Coux et al., 1996; Glickman and Ciechanover, 2002). The proteolysis of securins by proteasomes enables the protease separase to inactivate cohesin proteins (Jallepalli et al., 2001; Agarwal and Cohen-Fix, 2002; Uhlmann, 2003b).

In eukaryotes, chromosome cohesion is mediated by the multisubunit protein complex cohesin whose subunit composition differs among species, mitosis, and meiosis. The mitotic Scc1 cohesion subunit is replaced by the meiosis-specific Rec8 cohesion protein (Parisi et al., 1999; Watanabe and Nurse, 1999) which is needed for maintaining sister centromere cohesion during meiosis I (Molnar et al., 1995; Klein et al., 1999). Rec8 can be cleaved by separase and Rec8 mRNA has been found in mouse oocytes (Buonomo et al., 2000; Lee et al., 2002). In addition to Rec8, Spo13, Slk19p, Sgo1,and Sgo2 proteins also appear essential for preventing sister chromatid separation during meiosis I in yeast (Kamieniecki et al., 2000; Shonn et al., 2002; Kitajima et al., 2004).

Most cohesins are removed from mammalian chromosome arms during prophase and prometaphase, while a lesser amount is removed from kinetochores during anaphase (Lee and Orr-Weaver, 2001; Nasmyth, 2001; 2002). During meiosis I, centromeric cohesion between sister chromatids must be preserved to insure that they segregate to the same pole and later removed during meiosis II. Although separase may not be needed for sister chromatid arm separation, it is needed for removing centromeric cohesin during anaphase onset in mouse oocytes (Waizenegger et al., 2002; Terret et al., 2003; Herbert et al., 2003).

Precocious loss of cohesins can lead to premature centromere separation (PCS) of sister chromatids and homologues during mitotic and meiotic divisions (Sonada et al., 2001; Hoque and Ishikawa, 2002; Uhlmann, 2003a). Several groups have proposed that PCS predisposes cells to aneuploidy (Angell, 1991; Mailhes et al., 1997; Yin et al., 1998a; Soewarto et al., 1995; Pellestor et al., 2002; Cupisti et al., 2003). Also, preovulatory (Mikamo and Hamaguchi, 1975; Kamiguchi et al., 1979) and postovulatory oocyte aging (Rodman, 1971; Webb et al., 1986;

Mailhes et al., 1998, Fissore et al., 2002) have been associated with reduced kinase activity, spindle abnormalities, PCS, and aneuploidy.

Protein kinases & phosphatases

Abnormalities in the function of protein kinases, phosphatases, microtubules, motor proteins, centrosomes, kinetochores and their associated proteins, spindle checkpoint proteins, anaphase promoting complex, proteasomes, and the securin-cohesin complex each have the potential for predisposing cells to aneuploidy.

Protein **kinses** and phosphatases exert pivotal roles during mitosis and meiosis. Defects in these enzyme activities can lead to temporal perturbations during OM and abnormal chromosome segregation (Kotani et al.,1999). 2-aminopurine and 6-dimethylaminopurine represent kinase inhibitors that can disrupt $p34^{cdc2}$ kinase and MAPK activities in mouse oocytes (Rime et al., 1989; Szollosi et al., 1993) and induce spindle checkpoint override in mammalian cells (Schlegel et al., 1990; Andreassen et al., 1996). The tyrosine phosphatase inhibitor vanadate has been shown to induce aneuploidy in mouse oocytes and polyploidy in bone marrow cells (Mailhes et al., 2003a). Okadaic acid is a phosphatase 1 and 2A inhibitor that can inactivate MPF, result in spindle checkpoint override, and induce spindle and kinetochore abnormalities and aneuploidy in mammalian oocytes (Schwartz and Schultz, 1991; De Pennart et al., 1993; Mailhes et al., 2003b).

Aurora B kinase performs numerous tasks during cell division. Its activity is needed for: (1) chiasmata resolution and chromosome condensation (Shannon and Salmon, 2002); (2) kinetochore assembly (Tanaka et al., 2002; Adams et al., 2001a); (3) kinetochore-microtubule interaction and spindle checkpoint signaling (Kallio et al., 2002; Hauf et al., 2003); (4) microtubule dynamics (Murata-Hori and Wang, 2002; Kallio et al., 2002); (5) chromosome congression, alignment, and bipolar chromosome orientation (Kallio et al., 2002; Lens and Medema, 2003); (6) chromosome cohesion (Rogers et al., 2002; Sumara et al., 2002); (7) chromosome segregation (Kallio et al., 2002; Katina et al., 2002;); and (8) cytokinesis (Adams et al., 2001a; Hauf et al., 2003). Aurora B forms a cell-cycle regulated protein complex with the passenger proteins survivin and inner centromere protein (InCenP) (Adams et al. 2001b; Bolton et al., 2002). Survivin appears needed for both proper chromosome segregation and cytokinesis (Uren et al., 2000) and stable association of the checkpoint protein BubR1 to kinetochores in response to lack of kinetochore-spindle fiber tension (Lens and Medema, 2003). Abnormalities involving spindle geometry, spindle checkpoint targeting and function, chromosome alignment

and segregation, and cytokinesis were noted when mammalian somatic cells were exposed to the aurora B kinase inhibitors hesperadin (Hauf et al., 2003) and ZM447439 (Ditchfield et al., 2003).

Polo-like kinases (Plks) represent a family of serine/threonine protein kinases that also are active during mitosis, meiosis, fertilization, and cytokinesis (Golsteyn et al., 1994; Glover et al., 1998; Tong et al., 2002, Dai et al., 2003). They enhance dissociation of cohesins from chromosome arms during prophase and prometaphase (Losada et al., 2002; Sumara et al., 2002). The mammalian homolog Plk1 is expressed during rodent OM (Matsubara et al., 1995) and participates in microtubule organization and separase-independent removal of cohesins from chromosome arms during prophase (Tong et al., 2002; Sumara et al., 2002; Fan et al., 2003). Plk1 was detected on spindle poles during metaphase and was later translocated to the spindle midzone during anaphase of mouse oocytes (Wianny et al., 1998). Plk mutants in budding yeast (Sharon and Simchen, 1990) and Drosophila (Sunkel and Glover, 1988) exhibited meiotic arrest, spindle abnormalities, and chromosome missegregation.

Although a myriad of kinase and phosphatase reactions occur during the orderly progression of the metaphase-anaphase transition, very few of the enzyme chemical inhibitors mentioned above are specific for a unique kinase or phosphatase. Instead of focusing on a particular biochemical pathway, the majority of chemically-induced germ cell aneuploidy data has been generated from compounds that alter microtubule kinetics.

Chemically-induced germ cell aneuploidy

Traditional cytogenetic analysis involves analyzing chromosomes for both numerical and structural aberrations; whereas, most contemporary analyses involve counting the number of fluorescent spots in a cell, which are interpreted to represent a specific chromosome(s). Thus, these newer assays detect aneuploidy for a specific chromosome(s) and not the entire genome. False positive estimates of hypoploidy can result from the overlap or fusion of DNA hybridization regions and inefficient probe penetration (Eastmond and Pinkel, 1990). Micronuclei assays detect chromosome loss events; however, the number of kinetochore-labeled micronuclei can be reduced by chemical treatment, chromosome bridges, apoptosis, and phagocytosis (Heddle et al., 1991; Miller et al.,1991)

Most mammalian germ cell aneuploidy studies have concentrated on descriptive aspects primarily involving chemicals that damage microtubules (Adler, 1990; Miller and Adler, 1992; Mailhes, 1995; Eichenlaub-Ritter et al.,1996), assay development (Mailhes and Marchetti, 1994;

Eastmond et al., 1995; Parry et al., 1995), and gender differences (Eichenlaub-Ritter, et al., 1996; Wyrobek et al., 1996). Variation in the degree of chemically-induced germ cell aneuploidy exists between cell types and the end-points measured (Aardema et al., 1998). Differences in follicular and seminiferous tubule anatomy, chronology and biochemistry of germ cell maturation, and the cellular barriers through which a compound must traverse to reach the target cell are thought to influence differences between oocytes and spermatocytes. Although more chemicals have been studied in spermatocytes than oocytes, female germ cells appear more sensitive when treated with similar doses of the same chemical (Adler, 1993; Mailhes, 1995). The literature is practically devoid of information about the molecular mechanisms of mammalian germ cell aneuploidy.

female germ cells

Reviews for chemically-induced aneuploidy for female germ cells can be found in Mailhes et al., 1986; Pacchierotti, 1988; and Mailhes and Marchetti, 1994. Most chemically-induced aneuploid germ cell studies have utilized spindle inhibitors such as colchicine (Mailhes and Yuan, 1987), vinblastine sulfate (Russo and Pacchierotti, 1988; Mailhes et al., 1993b), nocodazole (Generoso et al., 1989; Eichenlaub-Ritter and Boll, 1989), griseofulvin (Marchetti and Mailhes, 1994; Tiveron et al., 1992; Mailhes et al., 1993a), and benomyl (Mailhes and Aardema, 1992). In addition to inducing aneuploidy, these compounds also resulted in transient cell cycle arrest.

Since 1994, the following spindle disrupting compounds have been reported to induce aneuploidy in mammalian oocytes: podophyllotoxin (Tateno et al.,1995), nocodazole (Everett and Searle, 1995), carbendazim (MBC) (Zuelke and Perreault, 1995; Jeffay et al., 1996), chloral hydrate (Eichenlaub-Ritter and Betzendahl, 1995), griseofulvin (Marchetti et al., 1996), thiabendazole (Mailhes et al., 1997), diazepam (Yin et al., 1998a), trichlorfon (Yin et al., 1998b), and taxol (Mailhes et al., 1999). Additionally, topoisomerase inhibitors have been shown to increase the frequencies of aneuploidy and structural aberrations in rodent oocytes (Mailhes et al., 1994; Tateno and Kamiguchi, 2001; 2002) and aneuploidy in mouse spermatocytes (Attia et al., 2002). The oxidizing agent tertiary hydroperoxide (Tarin et al.,1996) and the proteasome inhibitor MG-132 induced temporal perturbations during mouse oocyte maturation in vitro and predisposed oocytes to chromosome missegregation (Mailhes et al., 2002).

male germ cells

During the last few years the investigation of chemically induced aneuploidy in male germ cells has moved from using the classic cytogenetic chromosome count in metaphase II spermatocytes or zygotes (Allen et al., 1986; Pacchierotti, 1988; Miller and Adler, 1992; Adler, 1993; Kallio and Lahdetie, 1998; Marchetti et al 2001) to using FISH in sperm (Schmid et al., 1999, Sun et al., 2000, and Adler et al., 1997; 2002). Although sperm FISH allows the relatively fast analysis of thousands of cells thereby increasing the statistical power of detecting small effects, several technical factors are critical to the reliability of the assay (Schmid et al 2001) and can greatly **infuence** the outcome. However, the use of strict scoring criteria, rigorous blinding of scores using procedures that normalize cell numbers on slides from treated and control animals and replicating findings, especially when the effects are small, can improve the effectiveness of the assay and assure valid measurements of sperm aneuploidy.

Using sperm FISH, two classes of agents have been shown to induce aneuploidy in male germ cells: the spindle disrupting compounds colchicine (Schmid et al 1999), diazepam (Schmid et al 1999), and griseofulvin (Shi et al 1999); and the topoisomerase inhibitors etoposide and merbarone (Attia et al 2002).

Conclusion

Decades of aneuploidy research have been devoted to proposing etiologies, identifying some of the causative agents, and expounding the virtues of different assays. Although considerable progress has been made in our understanding of mammalian germ cell biology and chromosome segregation in non-mammalian species, we are still faced with the eventual need to comprehend the molecular mechanisms and potential differences of chromosome segregation in male and female germ cells. We suggest that information about the temporal order of biochemical pathways and their interactions with organelles responsible for chromosome congression and segregation during meiosis be acquired. Based upon such fundamental knowledge, the **intracies** of aneuploidy can then be further investigated. One broad approach for deciphering some of the many potential molecular mechanisms of aneuploidy is to transiently expose cells in vitro to cell-permeable, reversible compounds that have been reported to impede the function of specific organelles or biochemical pathways required for chromosome congression and segregation. Examples for oocytes include the proteasome inhibitor MG-132 (Mailhes et al., 2002), the protein phosphatase 1 and 2A inhibitor okadaic acid (Mailhes et al., 2003), and the Eg5 kinesin inhibitor

monastrol (Mailhes et al., 2004). Although gene knockout, siRNA, and microinjection technologies appear applicable for disrupting specific events during oocyte maturation, their present use seems limited due to the unavailability of specific knockouts and siRNAs plus the need for analyzing hundreds of germ cells from each treatment group while minimizing random error. Certainly, the need for collaborative research designed to understand the various mechanisms responsible for aneuploidy is a prerequisite for any endeavor attempting to reduce the incidence of human aneuploidy.

Acknowledgements

Work performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48 with funding support from NIEHS IAG Y1 ES 8016-5 (FM).

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