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Chromosomal instability in mammalian pre-implantation embryos: potential causes, detection methods, and clinical consequences

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Abstract

Formation of a totipotent blastocyst capable of implantation is one of the first major milestones in early mammalian embryogenesis, but less than half of in vitro fertilized embryos from most mammals will progress to this stage of development. Whole chromosomal abnormalities, or aneuploidy, are key determinants of whether human embryos will arrest or reach the blastocyst stage. Depending on the type of chromosomal abnormality, however, certain embryos still form blastocysts and may be morphologically indistinguishable from chromosomally normal embryos. Despite the implementation of pre-implantation genetic screening and other advanced in vitro fertilization (IVF) techniques, the identification of aneuploid embryos remains complicated by high rates of mosaicism, atypical cell division, cellular fragmentation, sub-chromosomal instability, and micro-/multi-nucleation. Moreover, several of these processes occur in vivo following natural human conception, suggesting that they are not simply a consequence of culture conditions. Recent technological achievements in genetic, epigenetic, chromosomal, and non-invasive imaging have provided additional embryo assessment approaches, particularly at the single-cell level, and clinical trials investigating their efficacy are continuing to emerge. In this review, we summarize the potential mechanisms by which aneuploidy may arise, the various detection methods, and the technical advances (such as time-lapse imaging, “-omic” profiling, and next-generation sequencing) that have assisted in obtaining this data. We also discuss the possibility of aneuploidy resolution in embryos via various corrective mechanisms, including multi-polar divisions, fragment resorption, endoreduplication, and blastomere exclusion, and conclude by examining the potential implications of these findings for IVF success and human fecundity.

Keywords

Aneuploidy; Chromothripsis; Fragmentation; Micronuclei; Pre-implantation embryo

Introduction

The formation of a blastocyst is one of the first major landmarks in early mammalian development and yet, only 30–50 % of pre-implantation embryos from the majority of mammals will typically reach this stage following in vitro fertilization (IVF; Fig. 1; Alper et al. 2001). In contrast, approximately 80 % of mouse embryos will form blastocysts when cultured in vitro since developmental arrest prior to this stage is far less frequent in this species. Although the cause(s) of embryo arrest may differ between species, an abnormal number of whole chromosomes, or aneuploidy, is thought to be a primary determinant of whether a human embryo will progress in development (Munne et al. 1994). Additional evidence from other mammals more closely related to humans suggests that the incidence of aneuploidy is not unique to human pre-implantation development (Dupont et al. 2010), although it may occur at a higher frequency in human embryos (Vanneste et al. 2009a, 2009b; Johnson et al. 2010b; Chavez et al. 2012; Chow et al. 2014). With the exception of trisomy 13, 18, and 21 and abnormalities in the X- or Y-chromosome, the vast majority of embryonic aneuploidies involving whole chromosomes are incompatible with live birth. Notably, high infant mortality rates are also associated with trisomy 13 (Patau syndrome) and trisomy 18 (Edwards syndrome), with only a small number of cases of survival past 1 year. For IVF-derived embryos, those with aneuploidy will probably arrest prior to transfer to the mother, fail to implant if transferred, or spontaneously abort early in gestation (for a review, see Delhanty 2005). In the case of in vivo human conceptions, an aneuploid embryo might undergo arrest even before a woman knows that she is pregnant. The introduction of pre-implantation genetic screening (PGS), first by DNA fluorescent in situ hybridization (DNA-FISH; Munne and Weier 1996), then via microarray-based approaches, and more recently using next-generation sequencing (NGS; Hou et al. 2013; Fiorentino et al. 2014; Wang et al. 2014), have provided a sufficient means for aneuploidy detection in IVF embryos. However, several challenges remain, including the potential detriment of the embryo following biopsy for PGS (Scott et al. 2013a, 2013b), chromosomal mosaicism between various cells or cell populations (Kuo et al. 1998; Daphnis et al. 2005; Baart et al. 2006; Mastenbroek et al. 2007), and the effects of sub-chromosomal aberrations (Vanneste et al. 2009a, 2009b; Johnson et al. 2010b; Chavez et al. 2012; Chow et al. 2014) on subsequent development. Recent identification of common genetic variants, or DNA sequence variation, near genes in patients that are at high-risk for producing aneuploid embryos further complicates the already complex process of pre-implantation chromosomal instability (McCoy et al. 2015). Moreover, despite the higher probability that aneuploid embryos will arrest at the cleavage stage, embryos with chromosomal abnormalities can still form blastocysts and are often morphologically indistinguishable from chromosomally normal (euploid) embryos, especially when evaluated by static observations (Baltaci et al. 2006). Thus, alternative approaches are needed for embryo selection, such as time-lapse imaging and the genetic, proteomic, or metabolomic analysis of culture media or other non-invasive methods following embryo culture for the potential detection of biomarkers that are

associated with aneuploidy (Chavez et al. 2012; Seli et al. 2007; Wong et al. 2010). In this review, we summarize the methods used to distinguish euploid from aneuploid embryos, the complex mechanisms underlying chromosomal instability, and how aneuploidy is dealt with once it arises across diverse mammalian species. We also discuss the clinical consequences of aneuploidy generation, particularly in the context of IVF and other assisted reproduction technologies (ARTs), and the potential implications for early embryogenesis in natural human conceptions.

Methods for the detection of aneuploidy

DNA florescent in situ hybridization

In the past, the most frequently used method for diagnosing aneuploidy was PGS of day-3 biopsied blastomeres via DNA-FISH (Munne and Weier 1996). However, DNA-FISH can result in false positive or negative results due to procedural artifacts and is limited to the simultaneous analysis of only a select number of chromosomes, typically between five and twelve of the most frequently affected (Kuo et al. 1998; Daphnis et al. 2005; Baart et al. 2006; Mastenbroek et al. 2007). Thus, several studies initially underreported the incidence of human embryonic aneuploidy since all 24 chromosomes can potentially be affected, particularly in cases of mitotic errors (Chavez et al. 2012; Chow et al. 2014). In addition, cleavage-stage human embryos exhibit high rates of mosaicism between blastomeres (Kuo et al. 1998; Baart et al. 2006), which can complicate the interpretation of ploidy results if embryos contain a mixture of aneuploid and diploid cells. As the number of cells that can be biopsied from patient embryos is limited, supernumerary cleavage-stage embryos donated with informed consent for research have provided an alternative source for aneuploidy assessment. However, fresh embryos donated to research at the cleavage stage are usually of poorer quality than embryos that were selected for transfer and thus, not representative of the embryo population as a whole.

Microarray-based technologies

More recent studies with array comparative genomic hybridization (aCGH) or single nucleotide polymorphism (SNP) arrays to evaluate all 24 chromosomes in high quality, whole, human embryos have demonstrated that 50–80 % of human embryos at the cleavage stage have one or more blastomeres that are indeed aneuploid. In addition, the incidence of aneuploidy in cleaving human embryos appears to be irrespective of fertility status, maternal age, or whether a fresh versus frozen/thawed IVF cycle, since a similar frequency is observed in embryos from fertile couples, young women, and following cryopreservation (Vanneste et al. 2009a, 2009b; Johnson et al. 2010b; Chavez et al. 2012; Chow et al. 2014). Moreover, the incidence of aneuploidy in vitro is thought to reflect the situation in vivo considering that 20–30 % of natural human conceptions are estimated to result in a live birth (Zinaman et al. 1996; Macklon et al. 2002; Slama et al. 2002) and chromosomal abnormalities have been reported in the majority of spontaneous miscarriage cases (Fritz et al. 2001; Benkhalifa et al. 2005). This is in contrast to lower mammalian species, particularly the mouse, which is estimated to exhibit approximately 1 % embryonic aneuploidy rates, depending on the strain (Lightfoot et al. 2006). However, it should be noted that microarrays for the detection of chromosomal copy number are far more utilized

for humans than for other mammalian species due, in large part, to PGS of human embryos in IVF clinics and genetic testing centers. Thus, the overall aneuploidy rates via “whole-genome” cytogenetic methods in pre-implantation embryos from non-human species, especially those more closely related to humans, remains to be determined. Aneuploidy analysis via DNA-FISH of rhesus macaque embryos produced in vitro (Dupont et al. 2010) and the frequent observations of abnormal nuclear structure in blastomeres from rhesus embryos as compared with other mammalian species (Chavez et al. 2012) does suggest that the incidence of chromosomal abnormalities in non-human primates is similar to that in humans.

Given the high incidence of mosaicism in cleavage-stage human embryos shown by aCGH (Vanneste et al. 2009a, 2009b; Chavez et al. 2012; Chow et al. 2014) and the potential detriment of polar body and blastomere biopsies on embryonic development, alternative approaches have recently been implemented to evaluate aneuploidy. These include extended culture of embryos to the blastocyst stage and analysis of chromosomal status via trophectoderm biopsy, wherein multiple extra-embryonic cells are removed from the outer layer of the embryo. Whereas human blastocysts are thought to exhibit less aneuploidy than cleavage-stage embryos (Magli et al. 2000), the presence of two or more cell populations with a different chromosomal make-up has been reported at the blastocyst stage and may be more prevalent in embryos from women of advanced maternal age (Fragouli et al. 2011; J. Liu et al. 2012). In addition, it is important to differentiate between the proportion of aneuploid cells in an embryo versus the percentage of embryos with mosaicism, as the latter increases at the blastocyst stage (van Echten-Arends et al. 2011). Besides mosaicism percentage, there are additional potential risks associated with prolonged embryo culture such as the introduction of epigenetic changes, monozygotic twinning, and other factors that may disrupt embryo integrity in humans and other mammalian species (Peramo et al. 1999; Khosla et al. 2001; Fernandez-Gonzalez et al. 2009; Katari et al. 2009; Kallen et al. 2010). Nevertheless, extended culture may prevent the selection of cleavage-stage embryos that are destined to arrest, which is why blastocyst transfer with or without the use of PGS has increased (Munne et al. 1994; Alper et al. 2001; Schoolcraft et al. 2010).

Quantitative polymerase chain reaction and DNA sequencing

In response to claims that DNA-FISH or whole-genome amplification in conjunction with SNP arrays (Northrop et al. 2010; Treff et al. 2011; Macaulay and Voet 2014) is inaccurate, time consuming, and not conducive to fresh embryo transfers, particularly at the blastocyst stage, fluorescent real-time quantitative polymerase chain reaction (qPCR) has been developed as an alternative to microarray-based approaches for PGS (Treff and Scott 2013). Whereas qPCR provides a more rapid method for ploidy assessment, the multiplex amplification and relative quantitation of only 96 presumably single copy loci (four on each chromosome) does not allow for the identification of copy number variants in specific chromosomal segments, termed segmental aneuploidy, in embryos. Given that certain sub-chromosomal aberrations, such as homozygous or hemizygous deletions, large duplications or amplifications, and regions of de novo uniparental isodisomy may induce embryonic/fetal lethality and are one of the major causes of developmental disorders in offspring that survive to birth (Hochstenbach et al. 2011; Vanneste et al. 2012), it is important to evaluate both

whole and sub-chromosomal instability in embryos. Moreover, as this technology is still impacted by mosaicism and has only been applied to trophectoderm biopsies (Scott et al. 2013a, 2013b; Werner et al. 2014; Rienzi et al. 2015), which generally consist of between 5 and 10 cells, we do not yet know whether qPCR can reliably detect aneuploidy in single cells.

Next-generation whole-genome DNA sequencing (NGS or DNA-Seq) has also provided an additional platform besides qPCR and array-based methods to assess aneuploidy comprehensively in human embryos, including at the single-cell level (Hou et al. 2013; Fiorentino et al. 2014; Wang et al. 2014). Since aCGH covers approximately 30 % of the genome and can only detect gross chromosomal abnormalities at a lower threshold of approximately 1 MB (Bi et al. 2012), sub-chromosomal aberrations that are smaller than this resolution and missed by aCGH would be detectable by NGS in theory. In addition to examining copy number variation at a single nucleotide level, DNA-seq is also more cost-effective than aCGH since 48–96 samples or more can be simultaneously sequenced with high coverage by multiplexing with barcoded adapters. Furthermore, DNA-Seq does not require *a priori* knowledge of the genome being sequenced and thus, can be used for de novo genome assembly of multiple species (Hurd and Nelson 2009). Lastly, NGS also allows for the analysis of repetitive DNA sequences (Gisselsson et al. 2001), balanced chromosomal rearrangements, and an initial map of insertion and deletion (INDEL) variation in the genome (Talkowski et al. 2011). Until bioinformatics pipelines and NGS analysis software are developed and simplified for use in IVF clinics, however, microarray-based technologies are likely to continue to dominate the PGS field (Van der Aa et al. 2013).

Non-invasive methods for aneuploidy detection

Time-lapse imaging

Despite increased accessibility of PGS for IVF patients, several randomized control trials (RCTs) using DNA-FISH to evaluate the ploidy status of a select number of chromosomes have demonstrated no benefit or a significant reduction in live birth rates from IVF cycles with PGS versus those without PGS (Mastenbroek et al. 2007; Hardarson et al. 2008; Staessen et al. 2008). More recent studies suggest that PGS via qPCR, microarrays, or NGS may improve IVF outcomes for certain patients, but this has not been evaluated by an RCT (Harper and Sengupta 2012). Nonetheless, embryo biopsy for PGS is invasive and potentially detrimental to subsequent development; this has led to the exploration of alternative non-invasive means of accessing embryo quality and ploidy. One such approach has been the implementation of time-lapse imaging to monitor embryos throughout development rather than at specific time points that may not reveal subtle differences in embryo behavior (Fig. 1). Whereas initial studies focused on the utility and safety of time-lapse monitoring (TLM; Payne et al. 1997; Hardarson et al. 2002; Lemmen et al. 2008; Mio and Maeda 2008; Nakahara et al. 2010), once TLM was determined not to be detrimental to embryo development, several groups began to investigate whether there were morphological, spatial, and/or temporal correlates between imaging behavior and embryo quality (Wong et al. 2010; Cruz et al. 2011; Meseguer et al. 2011; Fig. 2). In 2010, Wong and colleagues demonstrated that TLM could be used to predict blastocyst fate prior to embryonic genome

activation (EGA) by measuring the duration of the first cytokinesis and the time between the second and third mitotic divisions (Wong et al. 2010). Since this initial report, other studies have confirmed the importance of early cleavage divisions and identified additional imaging parameters predictive of developmental success (Cruz et al. 2011; Meseguer et al. 2011; Azzarello et al. 2012; Cruz et al. 2012; Dal Canto et al. 2012; Hashimoto et al. 2012; Hlinka et al. 2012; Meseguer et al. 2012; Rubio et al. 2012; Desai et al. 2014; Y. Liu et al. 2014a, 2014b, 2015; Stensen et al. 2015; Fig. 2). Whether the first three mitotic divisions are similarly prognostic for mammalian species other than the human remains to be determined, but an examination of early mitotic timing in murine, bovine, and rhesus monkeys has suggested that this may be the case (Pribenszky et al. 2010; Sugimura et al. 2012; Burrue et al. 2014).

In a follow-up study, Chavez et al. (2012) determined that the timing of the first three mitotic divisions, in conjunction with an assessment of a dynamic process called cellular fragmentation, might also be used largely to distinguish euploid from aneuploid human embryos at the cleavage stage. It is important to note, however, that approximately 12 % of aneuploid embryos from this study exhibited normal parameter timing and no cellular fragmentation, indicating that certain embryos can appear euploid at least up to the 4-cell stage. Upon further examination, these embryos all had incurred mitotic errors with 4 chromosomes or fewer affected, suggesting that if they were allowed to progress in development, perhaps additional later imaging parameters might be used to distinguish chromosomally normal and abnormal embryos. Indeed, other imaging parameters such as the time to 5th cell, initiation of cavitation, and the completion of blastulation may also differentiate between euploid and aneuploid cleavage-stage human embryos and blastocysts (Campbell et al. 2013a, 2013b; Basile et al. 2014; Yang et al. 2014; Fig. 2). Although it is known that aneuploidy often results in the absence of or delayed blastulation (Campbell et al. 2013a, 2013b; Kroener et al. 2012), no study has yet observed a direct correlation between the first cell division in aneuploid embryos and blastocyst formation, because this parameter was either not evaluated or not measured from a shared start point such as the time from insemination (Basile et al. 2014; Yang et al. 2014; Rienzi et al. 2015). This is imperative since meiotic errors are likely to cause a delay in the first mitotic division. In addition, cellular fragmentation, which frequently occurs at the 1- to 2-cell stage, appears to be associated with spindle formation and progression through meiosis and mitosis (Chavez et al. 2012; Stensen et al. 2015). Recently, Vera-Rodriguez and colleagues (2015) demonstrated that the duration of the first mitotic division is predictive of embryo chromosomal status up to approximately the 8-cell stage, but whether this imaging parameter is similarly predictive beyond 8-cells is unknown. Additional studies have also reported a significant difference in the duration of, or synchrony between, the second and subsequent mitotic divisions in euploid versus aneuploid embryos that reach the blastocyst stage, although these findings are not entirely clear given that others have observed no such correlation (Campbell et al. 2013a, 2013b; Basile et al. 2014; Yang et al. 2014; Rienzi et al. 2015). Taken together, these data suggest that aneuploidy can have differential effects on pre-implantation development and probably depends on the number of abnormal chromosomes and at which stage the error(s) occurred. Perhaps, future studies with high-resolution live-cell imaging of chromosome

dynamics will more precisely determine how chromosomal mis-segregation impacts cell division and embryo behavior.

Of course, clinical validation of these findings is required, and recent retrospective or prospective registered clinical trials of patient embryos have confirmed the importance of certain cell cycle parameters in predicting blastocyst formation and/or aneuploidy risk (Conaghan et al. 2013; Kirkegaard et al. 2013; Athayde Wirka et al. 2014; Fig. 2). While there is some debate as to whether TLM is actually beneficial for embryo selection (Kaser and Racowsky 2014) and the assessment of ploidy status (Rienzi et al. 2015), the first RCT evaluating implantation rates, ongoing pregnancy, and early pregnancy loss suggests that TLM is more effective than conventional IVF techniques (Rubio et al. 2014). However, whether dynamic imaging analysis can also positively impact live birth rates, particularly in cases of single embryo transfers (SETs), remains to be determined. Furthermore, it is important to note that when comparing these time-lapse studies, any discrepancy in findings may be due to differences in the stage of pre-implantation development evaluated, whether only a portion of or whole embryos were analyzed, and how the imaging parameters were measured. Indeed, the use of a common procedural start point such as the time of intra-cytoplasmic sperm injection can have confounding effects on the measurement of other overlapping parameters, as recently shown by a study that implemented a biological start point instead (Y. Liu et al. 2014a). Lastly, which method(s) of ploidy assessment used is also a consideration, since array-based and sequencing approaches are far more comprehensive than qPCR and DNA-FISH of limited targets which might influence the interpretation of results. Nevertheless, despite evidence that the measurement of developmental kinetics can differentiate a large proportion of aneuploid embryos from those that are euploid, chromosomally normal and abnormal embryos that appear and behave similarly may be indistinguishable and still require PGS and/or additional screening (Chavez et al. 2012; Yang et al. 2014).

Based on observations that the measurement of imaging parameters can be diverse, subjective, and rather time-consuming, especially in cases of abnormal cell divisions, several studies have investigated the use of computer-assisted technologies for embryo selection (Campbell et al. 2013a, 2013b; Conaghan et al. 2013; Basile et al. 2014; VerMilyea et al. 2014; Molder et al. 2015; Fig. 2). The initial focus of these reports was on the implementation of algorithms for automated cell tracking and/or aneuploidy risk models for embryo classification. In addition, Conaghan and colleagues (2013; indicated by asterisk in registered clinical trials) also demonstrated that morphology plus automated cell tracking improved embryo selection particularly among high quality embryos and reduced inter-individual variability when used adjunctively. Additional studies have concentrated on the correlation between computer-derived embryo scoring and clinical pregnancy rate with promising results as well as on other algorithms for the classification of embryo potential or ploidy status (Campbell et al. 2013a, 2013b; Basile et al. 2014; VerMilyea et al. 2014; Molder et al. 2015). Given the inherent diversity in embryo kinetics and the vast differences in IVF routine between clinics, it will be important to determine whether clinic-specific or even patient-specific algorithms are necessary for embryo classification.

Embryo spent media

Alternatively, other non-invasive approaches, including the analysis of the media that was used for embryo culture, known as “spent media”, have also been explored in order to potentially diagnose the molecular health of developing embryos. The goal of these studies has been to identify biomarkers in spent media using advanced genetic, proteomic, or metabolomic technologies and to improve the assessment of embryo viability and/or ploidy status. If spent media analysis can support or replace embryo biopsies, which are technically challenging, invasive, and pose a risk for misdiagnosis because of chromosomal mosaicism, then perhaps the non-invasive screening of spent media could reduce implantation failures and increase IVF success. Whereas initial embryo metabolism studies evaluated specific molecules such as glucose, lactate, and pyruvate (Hardy et al. 1989; Gott et al. 1990; Conaghan et al. 1993; Turner et al. 1994; Gardner et al. 2001), more recent studies have performed metabolomics profiling of culture media (Seli et al. 2007, 2008, 2010b; Picton et al. 2010). However, since all of these studies used methods capable of identifying a limited number of metabolites involved in known cellular processes, the current focus has been on the discovery of important and yet to be characterized metabolomics pathways that allow for the simultaneous analysis of several hundred metabolites (Nadal-Desbarats et al. 2013). In a similar targeted-based approach, protein assessment of embryo spent media first concentrated on specific molecules such as HOXA10 (Sakkas et al. 2003) and leptin (Cervero et al. 2005), but more recent studies have been extended to the analysis of multiple known and unknown molecules by protein microarrays and mass spectrometry, respectively (Katz-Jaffe et al. 2006; Dominguez et al. 2010). By combining mass spectrometry with liquid chromatography for greater sensitivity in high-throughput samples, an additional study identified nine proteins (including lipocalin-1) that were differentially expressed between euploid and aneuploid blastocysts, but this finding has not yet been clinically validated (McReynolds et al. 2011). Thus, whether there is a clear association between a specific metabolomic or proteomic signature in spent media and ploidy status in embryos remains to be determined.

In addition to proteomic and metabolomic profiling, genetic analysis of spent media following embryo culture has also been evaluated as a non-invasive means for aneuploidy and mutation assessment. Notably, both nuclear and mitochondrial DNA have been detected in media from embryos that undergo blastomere or cellular fragmentation breakdown (Stigliani et al. 2013; Assou et al. 2014). In another study by Wu et al. (2015), the quantification of cell-free DNA (cfDNA) was measured in the spent media of embryos from couples at risk of the inherited disorder, α -thalassemia-SEA and shown to have a high efficiency for the detection of α -thalassemia-SEA in cfDNA at the blastocyst stage. Stigliani and colleagues (2013) also found that mitochondrial DNA quantity in culture media is significantly correlated with maternal age and the degree of cellular fragmentation and thus, may be an indication of poor embryo quality and aneuploidy. Lastly, “-omics” profiling of follicular fluid and genetic analysis of the oocyte-surrounding cumulus cells has been investigated for the purpose of pre-implantation screening and is reviewed extensively elsewhere (Assou et al. 2010; Seli et al. 2010a; Krisher et al. 2015). Taken together, we suggest that although these non-invasive aneuploidy detection methods may not serve as a substitute for PGS, especially when used alone, the analysis of a panel of biomarkers in

conjunction with other approaches such as cfDNA or TLM analysis may assist in embryo diagnostics.

Potential mechanisms of aneuploidy generation

Introduction to meiosis and mitosis

To determine the possible causes of aneuploidy in pre-implantation embryos, research has primarily focused on the cellular and molecular players involved in the fidelity of correct chromosome segregation during meiosis and/or mitosis. As a brief summary, meiosis begins in spermatocytes and primary oocytes, wherein the DNA replicates to form pairs of homologous chromosomes (one paternal, one maternal), each containing sister chromatids. During the synapsis stage of prophase I, homologous chromosome pairs coalesce tightly through a zipper-like structure called the synaptonemal complex (Yuan et al. 2002; Page and Hawley 2004), and at the pachytene stage, non-sister homologous chromatids crossover, recombine, and exchange maternal and paternal chromosome regions at chiasmata loci. When all homologs have completed their crossover events, and once the meiotic recombination cell cycle checkpoints are fulfilled (Hochwagen and Amon 2006), the cell cycle progresses to the diplotene stage, during which the synaptonemal complex disassembles to leave bivalents tethered at chiasmata loci (Kleckner 2006). At this stage of meiosis, primary oocytes in neonatal ovaries undergo dictyate arrest and will not resume meiosis until just prior to ovulation after they are recruited during a menstrual cycle (Jones 2008). Upon exiting prophase I and progressing into metaphase I, homologous chromosome pairs still connected at chiasmata loci align on the metaphase plate. The protein structures called kinetochores at the centromeres of homologous pairs form amphitelic microtubule attachments, whereby microtubules emanating from centrioles on opposite spindle poles attach at sister kinetochores and create an evenly spaced bi-polar “tug” across the centromere of the chromosomes. Once tension is detected on both homologous kinetochores of all chromosome pairs, the microtubules shorten, the chiasmata are eliminated as the crossovers resolve, and the two homologs separate to opposite poles (Petronczki et al. 2003). For the oocyte, the first cytokinesis is highly asymmetrical, resulting in the extrusion of a small diploid polar body that is not able to participate in fertilization, whereas meiosis I spermatocytes divide symmetrically and have the potential to form functional gametes. The second meiotic division (MII) is similar to mitosis in that the now genetically unique sister chromatids become separated upon degradation of securin by the anaphase-promoting complex (APC) and dissolution of the glue-like cohesion protein complexes by the separase protease (Petronczki et al. 2003; Peters 2006). After clearing the spindle assembly checkpoint, the sister chromatids are pulled apart at the kinetochore by opposing amphitellically attached microtubules, and since the DNA was not replicated in MII, the resulting daughter cells are haploid (Petronczki et al. 2003). Whereas four identical round haploid spermatids will go on to differentiate into highly specialized spermatozoa, the extrusion of the haploid second polar body in MII oocytes does not occur until fertilization by a mature sperm (Saunders et al. 2002; Fan et al. 2003).

Maternal origins of aneuploidy

Given the complexity of meiosis during oogenesis as compared with spermatogenesis, it is not surprising that the majority of meiotic errors can be traced back to the maternal chromosomes (Hassold and Hunt 2001). Indeed, cytogenetic analysis of spontaneous miscarriages, stillbirths, and new-borns determined that up to 70 % of meiotic aneuploidies originated from the oocyte, depending on which chromosome(s) was affected (Lamb et al. 2005; Sherman et al. 2006; Handyside 2012). This percentage, of course, does not take into account pregnancies that spontaneously aborted during the first few weeks of gestation and therefore, were not detected. Nonetheless, IVF and other ART procedures have suggested that meiotic error rates in women are relatively high in comparison to other mammalian species, with approximately 20–25 % of human oocytes identified as aneuploid (Hassold and Hunt 2001). Estimates of aneuploidy in mouse oocytes, on the other hand, are typically between only 0.05 and 1 %. The incidence of human aneuploidy also increases with maternal age, since as many as 50 % of pregnancies are diagnosed as aneuploid in women over the age of 40 (Munne et al. 1995; Nagaoka et al. 2012). However, mosaic aneuploidies, which are mitotically derived, are generally not correlated with advanced maternal age (Silber et al. 2003; Munne 2006; Munne et al. 2007), except in cases of mitotic non-disjunction (Munne et al. 2002).

Although meiotic errors can occur during either fetal oogenesis or the resumption of meiosis several decades later, the considerable time between the initiation and the completion of meiosis is likely to contribute to the alteration or degradation of factors important for chromosome fidelity. Since the major wave of EGA does not occur until approximately the 4- to 8-cell stage in human embryos, the first cell divisions are highly dependent on the large pool of maternal mRNAs and proteins provided by the oocyte (Braude et al. 1988). This is supported by findings that the oocyte cytoplasm alone, which is devoid of nuclear content, is sufficient to reprogram a somatic cell capable of development to the blastocyst stage (Tachibana et al. 2013). Thus, defective maternal resources, together with the relaxation or absence of cell cycle checkpoints discussed below, may contribute to aneuploidy and allow embryos to proceed in mitosis (Harrison et al. 2000; Los et al. 2004; Mantikou et al. 2012). By definition, these factors are termed maternal-effect genes given that they were initially transcribed during oogenesis. In the mouse, several maternal-effect genes have been identified (for a review, see Li et al. 2010) and include factors associated with remodeling sperm chromatin, EGA, and de novo DNA methylation (Li et al. 2010; Fig. 1). As remodeling of the male genome is a complicated process that can lead to DNA breaks (Derijck et al. 2006), the maternal-derived ubiquitin-conjugating enzyme, UBE2A (Roest et al. 2004), repairs sperm chromatin in preparation for EGA. Other maternal-effect genes such as ZFP36L2 ring finger protein-like 2 (Ramos et al. 2004) and autophagy protein ATG5 (Tsukamoto et al. 2008) are involved in maternal mRNA and protein degradation, respectively.

Notably, the expression of certain maternal RNAs and proteins can persist beyond the oocyte-to-embryo transition. To complicate matters, particular maternal-effect genes may not be expressed until after EGA, making it difficult to differentiate between maternal and embryonic effects (Li et al. 2010). Multi-protein complexes of maternal origin, such as the

sub-cortical maternal complex (SCMC), have also been shown to be important for embryonic progression beyond the 2-cell stage, at least in mouse embryos (Li et al. 2008; Fig. 1). The SCMC is a group of at least five proteins, including FLOPED/Ooep, MATER/Nlrp5, TLE6, Padi6, and FILIA/Khdc3, which localize to cytoplasmic lattices in mouse oocytes (Yurttas et al. 2008). Of these, Mater/Nlrp5 was one of the first proteins to be identified (Tong et al. 2000) and its depletion prevents SCMC formation, resulting in pre-implantation embryonic lethality in both mice (Li et al. 2008) and rhesus monkeys (Wu 2009). Similarly, embryonic lethality is also observed in embryos from mice lacking Floped/Ooep (Tashiro et al. 2010). Depletion of FILIA, on the other hand, in mouse oocytes does not lead to embryonic lethality, but rather to mitotic aneuploidies. This is most likely due to the role of FILIA in assembling key spindle components such as AURKA, PLK1, and γ -tubulin to the microtubule-organizing center as well as MAD2 to the kinetochores (Zheng and Dean 2009). In a study by Yu et al. (2014), the SCMC was also shown to control symmetrical divisions in mouse zygotes by influencing the central position of the mitotic spindle via Cofilin, a key regulator of F-actin assembly. These observations may be explained by evidence that *Tle6*-null mice exhibit unequal-sized blastomeres and cellular fragmentation, a phenotype more often observed in higher mammals such as cattle, rhesus monkeys, and human. To our knowledge, however, only a few maternal-effect genes have been studied in relation to mitotic aneuploidy in humans, even though SCMC members appear to be conserved across mammalian species (Li et al. 2010; Ohsugi et al. 2008; Akoury et al. 2015; Pierre et al. 2007). Since SCMC proteins play a role in maintaining both euploidy (Zheng and Dean 2009) and symmetrical divisions (Yu et al. 2014) in mouse pre-implantation embryos, it will be important to determine whether they have analogous functions in other mammalian species (Mantikou et al. 2012).

In addition to the potential inheritance of aberrant maternal RNA and proteins, there is also evidence that suboptimal maternal genetic variants or deleterious mutations within meiosis- or mitosis-associated genes and/or their regulators may also lead to abnormal chromosome segregation and cell division. Recently, McCoy and colleagues (2015) identified a genetic variant of *polo-like kinase 4* (*PLK4*), a gene known to cause aneuploidy upon disruption of the centrosome cycle (Ko et al. 2005); this variant occurred at higher frequency in women who produced aneuploid embryos solely due to mitotic errors. Whether there are other genetic variants associated with mitotic errors remains to be determined, but this haplotype resides in a region of low recombination on chromosome 4 and includes additional genes such as *INTU*, *SLC25A31*, *HSPA4L*, *MFSB8*, *LARP1B*, and *PGRMC2* (McCoy et al. 2015). Although these genes do not have known functions in maintaining proper chromosome segregation, it will be important to determine how their established roles in cell polarity, heat shock, and ATP translocation might relate to chromosomal instability in embryos (Chen et al. 2012). Perhaps, extensive research in the cancer field will lend insight into the key signaling pathways that are dysregulated to induce embryonic chromosomal instability and aneuploidy (Orr and Compton 2013).

Lastly, potential accumulation of maternal mitochondrial DNA (mtDNA) mutations over the several years between fetal oogenesis and meiotic resumption may also lead to defective mitochondria and possible aneuploidy generation. Whereas an association between maternal mtDNA and chromosomal instability is still speculative at the moment, oocytes from women

of advanced maternal age have been shown to exhibit increased rates of mtDNA mutations (Keefe et al. 1995) and lower ATP production (Van Blerkom et al. 1995). Moreover, a significant decrease in mitochondrial activity has been documented in mosaic human embryos with chaotic profiles in comparison with euploid or mosaic embryos with non-chaotic profiles (Wilding et al. 2001) and in individual aneuploid versus euploid blastomeres from the same embryo (Picton et al. 2010). Since ATP production is required for the movement of microtubules during anaphase and cytoskeletal actin filament contraction during cytokinesis (Wilding et al. 2001; Eichenlaub-Ritter et al. 2004), maternal mtDNA mutations might indirectly impact chromosome segregation and cell division. Thus, the replacement of dysfunctional mitochondria with healthy mitochondria by nuclear transfer into an enucleated oocyte may provide an alternative means, especially for women of advanced maternal age, to successfully conceive as previously shown in both mice and cattle (Takeuchi et al. 2005; Chiaratti et al. 2011).

Paternal contribution to aneuploidy

Although maternal meiotic errors are far more frequent than those of paternal origin and increase with age, mitotically derived mosaic aneuploidies in embryos are generally not associated with advanced maternal age. This is supported by studies that determined that mitotic errors are just as frequent, if not more frequent, than meiotic errors in embryos from women of average maternal age (Vanneste et al. 2009a, 2009b; Chavez et al. 2012; Chow et al. 2014). Thus, when mitotic mosaicism is detected in embryos, it may be due to defects in the sperm used for fertilization (Silber et al. 2003; Munne 2006; Munne et al. 2007). Aside from providing the paternal genome and phospholipase C-zeta, which releases the oocyte from its MII arrest by activating calcium oscillations (Whitaker 2006; Yoon et al. 2008), the sperm also contributes its centrosome in the majority of mammalian species (Fig. 1). In contrast to all mammalian species studied to date, the centrosome is maternally inherited in the mouse embryo (Schatten et al. 1991), a finding that may help explain the relatively low aneuploidy rates observed in mouse embryos (Lightfoot et al. 2006). The sperm centrosome, consisting of two centrioles, duplicates and separates around the time of pronuclear syngamy in zygotes to generate spindle microtubules that pull the chromosomes apart during the first embryonic divisions (Sathananthan et al. 1991; Palermo et al. 1994). Palermo et al. (1994) demonstrated that the presence of two sperm in human zygotes led to mosaicism in embryos, whereas monospermic embryos and those that inherited an extra haploid set of chromosomes from the mother were euploid. Moreover, dispermic embryos also exhibit multi-polar mitotic spindles, and disruption of the sperm centrosome induces mosaicism in embryos (for a review, see Palermo et al. 1997). As further proof that the centrosome is paternally derived, the spindle complex in mature human oocytes has been shown to lack both centrioles and asters at the poles (Sathananthan et al. 2006). Studies have also found that certain forms of male infertility are due to centriole abnormalities (Rawe et al. 2002) and may contribute to mosaicism in embryos (Obasaju et al. 1999). Additional evidence is provided by observations of increased mitotic mosaicism in embryos fertilized by testicular sperm extraction from men with non-obstructive azoospermia and suggests that non-ejaculated spermatozoa may be immature and less effective in organizing the first mitotic spindle (Silber et al. 2003). Taken together, these studies suggest that further consideration should be given to paternal age and the potential inheritance of an age-related dysfunctional

centrosome, similar to how an association between advanced maternal age and meiotic errors is regarded (Nagaoka et al. 2012).

Despite substantial evidence that the centrosome is paternally contributed in the majority of mammalian species, an apparent contradiction to this notion are findings of mitotic divisions following parthenogenetic activation. Indeed, parthenotes can develop to the blastocyst stage and are a source of highly proliferative parthenogenetic embryonic stem cells (pESCs; for a review, see Daughtry and Mitalipov 2014). Data from Brevini et al. (2009, 2012) have demonstrated that porcine parthenotes do indeed contain centrioles, but in an excessive number, suggesting that de novo centriole generation is dysregulated. Similar findings were observed in both porcine and human pESCs as well as observations of multipolar spindles and high rates of aneuploidy when evaluated by DNA-FISH. Furthermore, Q. Liu et al. (2014) showed that blastomeres from porcine parthenogenetically activated embryos were predominately aneuploid or haploid when tested by karyotyping. As further support for this, all biopsied blastomeres in spontaneous parthenogenetically activated embryos from an infertile patient were identified as aneuploid by DNA-FISH and SNP-microarray (Combelles et al. 2011). This suggests that, although the source of centrioles in parthenotes appears to be maternal, paternally inherited centrosomes are more proficient in early mitotic divisions.

In addition to the transfer of defective centrosomal components, the lack of and/or inheritance of aberrant paternal mRNAs, proteins, or other factors may also influence the chromosomal integrity of embryos (Xanthopoulou et al. 2012; Burrue et al. 2014; Vera-Rodriguez et al. 2015) (Fig. 1). Since sperm are generally considered transcriptionally silent, RNAs detected in the paternal gametes were initially assumed to be either products of transcript degradation or simply contaminants from surrounding cells in the testes or epididymis (Pessot et al. 1989). However, more recent studies have shown that sperm may actually contain up to thousands of different RNAs that retain both coding and specific non-coding RNAs, some of which have known roles in mitotic progression and the prevention of aneuploidy (Taylor et al. 1997; Dawlaty et al. 2008; Sandler et al. 2013). One of these microRNAs, miRNA-34c, is detected in zygotes, but not parthenotes, indicating it is inherited from the sperm. By blocking paternal miR-34c in the zygote, W.M. Liu and colleagues (2012) showed that the first cleavage division was significantly suppressed, at least in mouse embryos. Male mice deficient in all three members of the miR-34c family, however, are reported to be both viable and fertile, although the latter data was not shown (Concepcion et al. 2012). Nevertheless, additional studies also suggest that a small population of paternal transcripts inherited from human sperm is important for sustaining early embryogenesis beginning at the 3- to 4-cell stage in human pre-implantation development (Taylor et al. 1997; Sandler et al. 2013). The presence of functional sperm RNA indicates that, analogous to maternal-effect genes, males also assist in the oocyte-to-embryo transition. Determination as to which sperm RNAs persist and how their integrity might be influenced by ageing or environmental factors will be important.

Anaphase lagging and non-disjunction of chromosomes

Even with the presence of parental factors for the maintenance of euploidy, meiosis and mitosis are obviously complex cellular processes with several points during which errors

could arise and impact subsequent development. As discussed above, if chromosomal mis-segregation occurs during meiosis, zygotes will inherit meiotic-based aneuploidies from the parental gamete(s) (Yuan et al. 2002), whereas chromosomal errors that take place in the first cell divisions lead to the propagation of mitotic-induced aneuploidies in the cleaving embryo. Regardless of whether meiotic or mitotic in origin, the majority of mis-segregation errors are thought to be the result of two different mechanisms: either lagging chromosomes during anaphase or non-disjunction (Fig. 1). Anaphase lagging occurs when a chromatid fails to connect or is dissociated from the spindle apparatus and is lost upon cytokinesis, resulting in a monosomy in one of the two daughter cells during meiosis or mitosis. For meiosis, this might occur as early as the MI stage if the spindle assembly checkpoint is bypassed, leading to precocious centromere division and abnormal chromatid allocation to the first polar body and MII oocyte (Angell 1997). Lagging chromosomes in anaphase may be due to the depletion and/or reduced stringency of the spindle assembly complex in oocytes and early preimplantation embryos, which rely on maternally supplied spindle assembly complex mRNAs and proteins until EGA. Evidence for this is provided by mouse embryo studies showing that the knockdown of the spindle assembly complex components, Bub3, BubR1, and Mad2, leads to improper chromosome segregation, aneuploidy generation, and the formation of micronuclei, or small nuclei that encapsulate chromosomes or pieces of chromosomes that failed to incorporate into the primary nucleus of one of the daughter cells during division (Wei et al. 2011). Furthermore, additional studies have demonstrated that chromosome lagging commonly arises from the formation of merotelic attachments in mitosis, whereby a single kinetochore on one side of the chromosome is attached to microtubules emanating from both spindle poles (for a review, see Gregan et al. 2011). Whereas lagging chromosomes arising from the formation of merotelic attachments have been shown to occur in oocytes during meiosis (Kitajima et al. 2011; Lane et al. 2012), the exact mechanism(s) of chromosomal mis-segregation during early mitotic divisions in mammalian embryos remains an enigma. This may be because, unlike other mitotic cell types, the cleavage-stage embryo undergoes mitosis to produce cells of decreasing cell size with each subsequent division; this can impact spindle length and symmetric versus asymmetric division, as discussed in a recent review (Howe and FitzHarris 2013).

Non-disjunction, on the other hand, is the failure of the sister chromatids to separate at the centromere during metaphase, resulting in the loss of a chromosome in one daughter cell and a reciprocal gain in the other. This event can be easily recognized as 3:1 chromosomal ratios between daughter cells and will give rise to an aneuploid zygote or mosaic aneuploid embryo depending on whether it occurs during meiosis or mitosis, respectively (Delhanty et al. 1993; Coonen et al. 2004; Daphnis et al. 2005, 2008; Katz-Jaffe et al. 2005). Although non-disjunction and anaphase lagging may have the same consequences, aneuploidy due to a single chromatid loss in MII oocytes far exceeds the prevalence of non-disjunction, at least during meiosis (Pellestor et al. 2002; Kuliev and Verlinsky 2004). Mitotic errors, however, appear to be equally distributed between chromosome lagging during anaphase and chromosomal non-disjunction (Chow et al. 2014).

Cell cycle drivers and checkpoints

To make matters worse, human pre-implantation embryos have also been shown to express both heightened cell cycle drivers and diminished cell cycle checkpoints, possibly making blastomeres more vulnerable to chromosome lagging during early mitotic divisions (Harrison et al. 2000; Kiessling et al. 2010) (Fig. 1). Only after EGA, from approximately the 4- to 8-cell stage onwards (Braude et al. 1988; Dobson et al. 2004), does cell cycle control become apparent in human embryos. Thus, permissive or lack of cycle checkpoints might allow aneuploid embryos to proceed through mitosis, even if chromosomes are not yet properly aligned on the mitotic spindle at the completion of metaphase (Harrison et al. 2000; Los et al. 2004). In turn, with robust expression of cell cycle drivers and shortened cell division intervals as compared with other cell types, the embryo enters anaphase and the lagging chromosomes become lost upon cytokinesis. Taken together, this suggests that embryos rely more on the circadian expression of genes, perhaps regulated by oscillators as previously described, rather than precise cell cycle control per se (Kiessling et al. 2009).

Aneuploidy tolerance and rescue

Cellular fragmentation and micronuclei formation

In light of the lack of cell cycle control, frequent findings of aneuploid diploid mosaicism (Kuo et al. 1998; Baart et al. 2006), and complex aneuploid mosaicism, whereby multiple chromosomes are affected in embryos without a single euploid blastomere, it is not surprising that the majority of chromosomal errors are unlikely to be corrected (Vanneste et al. 2009a, 2009b; Chavez et al. 2012). Nevertheless, some evidence suggests that “embryo self-corrective” mechanisms do indeed exist based on biopsy and PGS of an aneuploid blastomere(s) at the cleavage-stage followed by similar analysis of euploid trophoctoderm cells from the same embryo a few days later (Munne et al. 2005; Barbash-Hazan et al. 2009). Although thought to occur only occasionally, the so-called embryo self-correction phenomenon might be explained by other processes, such as multi-polar divisions, blastomere exclusion, and cellular fragmentation, all of which are more frequently observed in embryos (Fig. 1). As discussed above, cellular fragmentation is relatively common in humans, with approximately 50 % or more of human embryos exhibiting fragmentation to some degree (Alikani et al. 1999; Antczak and Van Blerkom 1999). Pre-implantation embryos from other mammalian species, including rhesus monkeys, cattle, and even mice undergo cellular fragmentation, although to a lesser extent than humans and non-human primates (Enders et al. 1982; Sugimura et al. 2010; Fig. 3). Notably, cellular fragments are also detected in embryos conceived in vivo (Pereda and Croxatto 1978; Buster et al. 1985), indicating that fragmentation is not an artifact of in vitro culture. It is also important to note that cellular fragmentation is distinct from the cell-death-induced DNA fragmentation that can occur late in mammalian pre-implantation development, most significantly at the morula and blastocyst stages (Hardy 1999; Hardy et al. 2001; Xu et al. 2001). Cellular fragments can vary in size, in temporal and spatial distribution, and in organelle content (Jurisicova et al. 1996; Yang et al. 1998; Van Blerkom et al. 2001), making them sometimes difficult to differentiate from small blastomeres. High levels of fragmentation are associated with an increased incidence of aneuploidy (Pellestor et al. 1994; Munne 2006) and, depending on the timing of fragmentation, can be used to distinguish embryos with meiotic and/or mitotic

errors (Chavez et al. 2012). Although extensive fragmentation may correlate with low implantation and clinical pregnancy potential (Giorgetti et al. 1995; Ziebe et al. 1997; Alikani et al. 1999; Hardy 1999; Ebner et al. 2001), by itself, it is not a clear indicator of poor embryo quality, as live births have resulted from the transfer of fragmented embryos (Edwards et al. 1984; Hoover et al. 1995; Alikani et al. 1999; Antczak and Van Blerkom 1999; Pelinck et al. 2010). Rather, it is the pattern and degree of cellular fragmentation, together with the stage at which it initially occurs, that are better predictors of embryo viability (Alikani et al. 1999; Antczak and Van Blerkom 1999).

Recent evidence presented by Chavez and colleagues (2012) suggests that cellular fragments can also encapsulate mis-segregated chromosomes following extrusion from the embryo, but exactly how this occurs remains unknown. Given that fragmentation was originally thought to represent membrane-bound extracellular cytoplasm, the idea that cellular fragments might contain nuclear DNA was intriguing, especially since microsurgical removal of fragments was once promoted (Eftekhar-Yazdi et al. 2006; Keltz et al. 2006). Nonetheless, the authors also reported frequent findings of micronuclei in cleavage-stage human embryos, suggesting that the formation of micronuclei provides a mechanism by which chromosomes are sequestered into fragments after mis-segregation (Chavez et al. 2012; Fig. 3). Since cellular fragmentation is a dynamic process, whereby chromosome-containing fragments can remain as either discrete units or be reabsorbed by the embryo (Hardarson et al. 2002; Lemmen et al. 2008; Chavez et al. 2012), it may also allow corrective means to overcome aneuploidy generation. More specifically, if a fragment fuses with the blastomere from which it originated or a neighboring blastomere that lacked the encapsulated chromosome(s), euploidy could potentially be restored in an embryo following the breakdown of the nuclear envelope (Fig. 4a). However, not all fragments necessarily contain DNA, and thus, fragment resorption may have no impact on ploidy status, or it may even increase genotypic complexity in embryos upon fusion with a euploid blastomere (Chavez et al. 2012). Regardless of the potential consequences, little is still known about what leads to cellular fragmentation and it will be important to determine the precise mechanism(s) by which it occurs and the way in which the timing, degree, and/or resorption of fragments contributes to the generation and potential correction of embryonic aneuploidy. As cancer cells also sequester mis-segregated chromosomes and chromatid fragments within micronuclei (Ford et al. 1988; Norppa and Falck 2003; Fenech et al. 2011; Hatch et al. 2013), findings during pre-implantation development may also be translatable to cancerous somatic tissues. In support of this, the blebbing of cytoplasmic membrane resembling cellular fragmentation in embryos has been shown to occur simultaneously with the extrusion of nuclear content in cancer cell lines during interphase, suggesting that micronuclei formation may be similarly regulated between somatic and embryonic cells (Shimizu et al. 2000; Utani et al. 2011).

Unlike human embryos, murine pre-implantation embryos not only have a low incidence of aneuploidy, but also rarely exhibit micronucleation during development (Bond and Chandley 1983; Lightfoot et al. 2006; Chavez et al. 2012). However, a recent study indicates that the formation of micro- and multiple-nuclei (multi-nuclei) can be induced in mouse embryos by disrupting the function of particular cell cycle proteins critical for developmental progression. Chavez and colleagues (2014) determined that translational block of *Rps6ka4/Msk2*, a mitogenic factor known to be involved in the G1 phase of the cell cycle

(Bettencourt-Dias et al. 2004), in mouse zygotes resulted in the appearance of multiple micro- or multi-nuclei at the cleavage stage. In addition, increased blastomere movement and lysis, resembling cellular events described during mitotic catastrophe (Vakifahmetoglu et al. 2008), was also observed in mouse embryos following mitotic arrest at the 3- to 8-cell stage. This suggests that, in contrast to human embryos, which appear to respond to aneuploidy generation by fragmenting, mouse embryos undergo blastomere lysis as a potential mechanism to avoid chromosomal instability (Fig. 1). It may also explain the low aneuploidy rates observed in mice, if micro- and multi-nuclei formation in blastomeres results in mitotic arrest rather than continued division as part of a selection process. Further support for this is provided by findings that deletion of BUB3, BUBR1, and MAD2 of the spindle assembly complex, which is essential for normal mitotic progression, leads to the formation of micronuclei, chromosome misalignment, aneuploidy, developmental delay, and decreased implantation rates. Overexpression of these spindle assembly complex components, on the other hand, prevents sister chromatid segregation (Wei et al. 2011). Disruption of α -thalassemia/mental retardation X-linked (ATR-X), a protein that binds to pericentric heterochromatin in MII and mediates chromosome alignment along the metaphase plate, also causes micronuclei formation and aneuploidy in mouse oocytes and zygotes, suggesting that similar selection mechanisms also exist during meiosis (Baumann et al. 2010).

Chromothripsis and sub-chromosomal rearrangements

In comparison with primary nuclei, micronuclei display altered nuclear functions, including aberrant DNA replication, transcription, and DNA-damage repair as shown by in vitro experiments with cultured cells. The results of such studies also indicate a direct link between micronuclei formation and the incidence of “chromosome pulverization” or chromothripsis (Hoffelder et al. 2004; Terradas et al. 2009; Xu et al. 2011; Crasta et al. 2012). First described in cancer genomes (Stephens et al. 2011), chromothripsis is a phenomenon through which one or a few chromosomal segments are “pulverized” into many pieces and randomly reassembled in one unique cellular event during a single cell division (Pellestor et al. 2014). Chromothripsis has also been suggested to occur during pre-implantation development (Pellestor 2014; Pellestor et al. 2014) based on frequent observations of micronuclei formation, cellular fragmentation, segmental rearrangements, and abnormal mitotic divisions in cleavage-stage embryos. This pulverization and random reassembly can lead to chromosomal duplications, deletions, translocations, and inversions, which are often detrimental, resulting in subfertility or congenital abnormalities depending upon when it occurs (Loup et al. 2010; Kloosterman et al. 2011).

In incredibly rare cases, however, chromothripsis may lead to the spontaneous elimination of a deleterious gene and effectively “cure” a disease if the event occurs in an adult stem cell that can repopulate the affected niche (McDermott et al. 2015). Nevertheless, chromothripsis during gametogenesis or early embryogenesis is thought to be a likely explanation for the de novo occurrence of complex chromosomal rearrangements in offspring from parents with otherwise normal karyotypes (Pellestor 2014; Pellestor et al. 2014). Given that sub-chromosomal aberrations have also been reported in embryos from women of advanced maternal age or with more than one implantation failure (Voullaire et al. 2002; Wilton et al.

2003; Rius et al. 2010, 2011; Mantikou et al. 2012), chromothripsis may also be involved in recurrent pregnancy loss. The vast majority of chromothripsis events are thought to be catastrophic and result in embryonic lethality rather than being passed onto the next generation via the germ cells. Moreover, the de novo germline complex rearrangements that are detected in the offspring, but not inherited from the parents, also exhibit fewer chromosomal breaks in comparison with those detected in cancer genomes (Giardino et al. 2009; Kloosterman and Cuppen 2013). On the other hand, in cases of complex genomic rearrangements that are inherited, chromothripsis has been found to be one of the major culprits (Kloosterman et al. 2011, 2012; Chiang et al. 2012; Macera et al. 2015). However, how DNA damage in micronuclei can lead to chromothripsis and the role that this phenomenon plays, if any, in the high rates of aneuploidy observed in human embryos remains unclear.

As an explanation for the means by which DNA damage-induced chromothripsis might arise, a model correlating micronuclei formation with chromosome pulverization was proposed by Crasta et al. (2012) and confirmed by Zhang and colleagues (2015). Based on previous work with both cancer cells and solid tumors, Crasta et al. and others revealed that DNA damage via double-strand breaks appears irrespective of whether the micronucleus is intact (Crasta et al. 2012; Huang et al. 2012) or compromised by nuclear envelope collapse (Cimini et al. 2001; Hatch et al. 2013). Consequently, DNA repair, replication, and transcription become altered, leading to genomic instability as documented by the accumulation of gamma-H2A histone family, member X (γ -H2AX) foci. Recently, Zhang et al. (2015) used “LookSeq”, a combination of live-cell imaging and single-cell whole-genome sequencing, to confirm that micronuclei can partition into a daughter cell and undergo nuclear envelope rupture and defective DNA replication, leading to chromosomal copy number asymmetry between daughter cells. After only one cell division, micronuclei were reincorporated into the primary nucleus, and by sequencing the daughter cells with nuclear fusion, this process was determined to be restricted to mis-segregated chromosomes. Moreover, the authors observed that micronuclei formation resulted in diverse genomic rearrangements, some of which recapitulated all known hallmarks of chromothripsis (Zhang et al. 2015). In another study that involved time-lapse imaging to assess micronuclei formation in a cancer cell line, DNA-containing micronuclei extruded extracellularly were shown to fuse back with a daughter cell after cell division (Rao et al. 2008). Thus, analogous to pre-implantation embryo development, if extracellular micronuclei are resorbed and fuse with primary nuclei following attempted repair by non-homologous end joining and nuclear envelope breakdown, then the incorporation of compromised chromosomes might result in additional genetic mutations (Fig. 1). Chromothripsis is only one of the proposed mechanisms for generating the complex chromosomal rearrangements observed in congenital disorders and pre-implantation embryo loss; other possible mechanisms include DNA replication errors (P. Liu et al. 2012), telomeric dysfunction (Soler et al. 2009), and translocation events during meiosis (Pellestor et al. 2011). Although such events can be viewed as deleterious, similar mechanisms are thought to influence accelerated karyotypes in species evolution (Carbone et al. 2014), and it will be important to determine whether the sub-chromosomal rearrangements that emerge following chromothripsis are tolerated, or if they impact human fecundity.

Multi-polar cell divisions and trisomic rescue

Another relatively frequent event observed during pre-implantation development is the multi-polar division of zygotes or blastomeres, whereby embryos divide into three or more daughter cells rather than the typical two (Fig. 4b). Through the formation of multiple centrosomal spindle poles that pull anaphase chromosomes apart, embryos can undergo tri-polar or higher order divisions at the zygote stage or during early cleavage divisions (for a review, see Kalatova et al. 2015). Whereas the potential impact of multi-polar divisions at the 2-cell stage and beyond may not be as detrimental and is still being investigated, TLM studies estimate that approximately 12 % of 1-cell human embryos are characterized by multi-polar divisions (Chamayou et al. 2013) and, contrary to previous notions, are often associated with triploidy or tetraploidy. Moreover, embryos that exhibit 1:3 divisions at the zygote stage are also much less likely to reach the blastocyst stage than zygotes that undergo a bi-polar division and have been shown to correlate with poor implantation potential (Hlinka et al. 2012).

As a consequence of multi-polar divisions, diverse asymmetrical chromosome distribution can result, and one of the leading hypotheses is that centrosome duplication is to blame. In support of this, genetic variants of maternal *PLK4*, which is involved in centriole duplication and alters mitotic fidelity upon dysregulation, has been shown to correlate with mitotic aneuploidy and failed pregnancies in women undergoing IVF (McCoy et al. 2015). Notably, Ganem et al. (2009) determined that multi-polar divisions are rare in cancer cells and often become non-viable within the first two cell divisions. In contrast, human zygotes can undergo 1:3 cell divisions and are still able to progress further in pre-implantation development as discussed above. Ganem et al. (2009) also observed that in cancer cells, extra centrosomes spend the majority of mitosis in transient multi-polar intermediates before undergoing centrosome clustering. This type of configuration leads to an increase in merotelic kinetochore-microtubule attachment errors, whereby microtubules from both poles attach to a single kinetochore on one side of a chromosome. Consequently, even though cancer cells with extra centrosomes are able to proceed through bipolar anaphase, merotelic attachments result in increased anaphase lagging chromosomes and subsequent chromosomal instability. Whether similar mechanisms regulate pre-implantation development is unknown, but multi-polar divisions may not necessarily be detrimental and can constitute a means for trisomic embryos to undergo rescue via loss of the extra chromosome(s) upon division (Kalousek 2000; Fig. 1). If both of the retained chromosomes are inherited from the same parent, then uniparental disomy (UPD) will result, as is discussed in further detail below.

Endoreduplication and UPD

A process that is almost the opposite of multi-polar divisions is called endoreduplication, or the replication of a chromosome without cell division. This can result in several different outcomes depending on the original state of the chromosomes involved. For instance, if one chromosome undergoes endoreduplication in a diploid blastomere, one of the daughter cells in the subsequent cell division will be trisomic and the other disomic. Another well-known example of endoreduplication is the parthenogenetic activation of a haploid oocyte, resulting in the formation of a diploid parthenote capable of further development (Lin et al. 2007;

Sritanaudomchai et al. 2010; Elling et al. 2011; Leeb et al. 2012; Paull et al. 2013; Daughtry and Mitalipov 2014).

Therefore, endoreduplication not only is a mechanism by which aneuploidy can be generated, but it can also be involved in the rescue of monosomic embryos (Fig. 4c). Analogous to embryos with trisomies, monosomic embryos could gain the missing chromosome(s) following endoreduplication to produce a diploid organism. However, unlike embryos that undergo trisomic rescue, monosomic rescue always results in UPD since the duplicated chromosome is either paternally or maternally inherited (Fig. 1). Thus, UPD can have clinical consequences such as the disruption of parent-specific genomic imprinting and other developmental disorders due to homozygosity. In a study by Conlin and colleagues (2010), two UPD patients exhibited homozygosity for all alleles genotyped on the afflicted chromosome, and the patient with UPD15 presented with Angelman syndrome, consistent with monosomic rescue by endoreduplication of paternal chromosome 15. The other patient with UPD14, however, displayed morphological and developmental abnormalities indicative of paternal UPD14 (Conlin et al. 2010). Given that, in both cases, the UPD was paternal, the most likely scenario was that a sperm fertilized a nullisomic oocyte produced from maternal meiotic non-disjunction. Subsequently, the haploid paternal chromatid in the fertilized oocyte underwent endoreduplication and led to the restoration of euploidy by UPD. While these patients exhibited obvious phenotypic manifestations due to the chromosome that was affected, certain UPD chromosomes can be tolerated, suggesting that endoreduplication and UPD can be an effective mechanism for monosomic rescue of embryos in some cases (Berend et al. 1999; Fig. 1).

Blastomere and fragment exclusion

Whereas the above-discussed corrective mechanisms might occur at the earliest stages of pre-implantation development, other means appear to exist for the resolution of aneuploidy later in embryogenesis during the transition from the cleavage to the blastocyst stage (Fig. 1). In particular, the complete exclusion of large fragments and blastomeres has been observed in both compact morulas and blastocysts from several mammalian species, including humans, rhesus macaques, cattle, and mice homozygous for the yellow allele (A^Y) of the agouti locus (Calarco and Pedersen 1976; Lindner and Wright 1983; Alikani et al. 1999, 2002; Hardy 1999; Van Soom et al. 2003; Prados et al. 2012; Chavez et al. 2014). Although the frequency of blastomere exclusion is unknown since it is not a well-documented process, pre-implantation embryos are generally thought to tolerate the loss of one or more blastomeres based on blastomere biopsy for PGS. Moreover, given that lower aneuploidy rates are observed in blastocysts as compared with cleavage-stage embryos (Magli et al. 2000), one possible explanation for this may be the exclusion of aneuploid blastomeres from the rest of the embryo (Fig. 4d). To our knowledge, however, the chromosomal and/or genetic analysis of extruded blastomeres or fragments is still lacking; this may be due to the limitations of aCGH and other microarray-based methods for the detection of complex or polyploid genotypes. Nevertheless, the extrusion rather than the reincorporation of aneuploid or micronuclei-containing blastomeres may be beneficial for embryo genomic integrity based on the original chromosomal content and increased propensity for DNA damage in the micronuclear environment (Thompson and Compton

2011; Crasta et al. 2012; Huang et al. 2012; Hatch et al. 2013). Future work should focus on the precise frequency of this process and how blastomere exclusion might relate to the “embryo self-correction” phenomenon as a whole.

Confined placental mosaicism

Alternatively, another mechanism termed confined placental mosaicism has also been proposed to explain embryo correction and the reduced incidence of aneuploidy at the blastocyst stage (Magli et al. 2000). In this scenario, aneuploid cells are deliberately confined to the trophoctoderm layer, whereas euploid cells are incorporated into the inner cell mass (ICM) and give rise to a chromosomally normal fetus (Kalousek and Dill 1983). Indeed, the presence of two or more cell populations with a different chromosomal make-up has been reported at the blastocyst stage, and this appears to be more prevalent in embryos from women of advanced maternal age (Fragouli et al. 2011; J. Liu et al. 2012). After careful separation of the ICM and trophoctoderm cells, however, several studies demonstrated that chromosomally abnormal cells detected at the 8-cell stage are not preferentially allocated to the trophoctoderm compartment but, instead evenly distributed throughout the blastocyst (Mottla et al. 1995; Evsikov and Verlinsky 1998; Magli et al. 2000; Derhaag et al. 2003; Fragouli et al. 2008; Johnson et al. 2010a; Capalbo et al. 2013). As further support, Johnson and colleagues (2010a) disaggregated over 50 blastocysts from mothers of average maternal age and observed discordance between trophoctoderm and ICM samples in only 4 % of the embryos; this occurred in cases of structural chromosome aberrations (Johnson et al. 2010a). It is important to note, however, that this study evaluated ploidy status in multiple cells of the same cell type at once, rather than single cells, which may mask discordance between the trophoctoderm and ICM. Nevertheless, other studies have reported the preferential allocation of aneuploid cells in chorionic villi samples as compared with fetal tissues, suggesting that confined placental mosaicism plays a role later in post-implantation development (Kalousek and Dill 1983; Ledbetter et al. 1992; Kalousek and Vekemans 1996; Phillips et al. 1996; Clouston et al. 1997; Goldberg and Wohlfert 1997; J. Liu et al. 2012). Taken together, this data indicates that the trophoctoderm layer of blastocysts might still preferentially contain chromosomally abnormal cells and it will be necessary to determine whether this can be observed using more comprehensive methods than DNA-FISH and at the single cell level in whole embryos.

Clinical aspects of pre-implantation aneuploidy

Over 38 years have now passed since Louise Brown, the first “test tube” baby, was born (Steptoe and Edwards 1978). After many failed attempts involving several different methodologies and donors, Robert Edwards and Patrick Steptoe successfully produced, via IVF, an embryo that was able to implant, progress in development, and result in the birth of a healthy child. That such a relatively straightforward experimental procedure could produce a perfectly healthy baby demonstrates the extremely remarkable, and yet tightly controlled, cellular programs that a pre-implantation embryo must undergo in the first few days of development. Despite this extraordinary accomplishment, one alarming statistic is that even with the progressive increase in the number of IVF cycles performed each year, live birth rates from transferred embryos have hovered around 30–35 % for decades. Moreover, the

implementation of more advanced IVF techniques such as intracytoplasmic sperm injection, PGS, and assisted hatching has not significantly changed the percentage of live births (Harper and Sengupta 2012; Kissin et al. 2014; Boulet et al. 2015). Thus, it will be essential to determine how more recently developed methods, including time-lapse imaging, DNA-Seq, and “-omic” profiling, either alone or in combination, impact IVF success rates over the next several years.

With over 5 million children born via IVF to date from hundreds of clinics worldwide, there are clear indications that the IVF procedure itself is not the only explanation for the plateau in live birth rates. Indeed, it is well recognized that the mammalian pre-implantation embryo does not develop cell-autonomously, and that the numerous extrinsic factors provided by the oviduct in vivo may not necessarily be emulated by culture media in vitro (Lee and Yeung 2006). Nevertheless, there is evidence that several of these developmental processes observed in IVF and described in this review do indeed occur naturally following in vivo conceptions (Pereda and Croxatto 1978; Buster et al. 1985). More specifically, a study from the 1970s, whereby a naturally conceived embryo was obtained from the oviduct following hysterectomy, demonstrated that in vivo pre-implantation embryos exhibit cellular fragmentation, blastomere asymmetry, and other morphological features, indicating that these events are not simply a consequence of in vitro embryo culture (Pereda and Croxatto 1978; Buster et al. 1985). Further evidence is provided by the high incidence of mosaicism detected in early spontaneous human abortions (Lebedev et al. 2004; Vorsanova et al. 2005) and the similar rates of mosaicism observed in chorionic villus samples (CVS) between IVF and natural pregnancies later in development (Huang et al. 2009; Mantikou et al. 2012).

Although much of the increase in the number of IVF cycles performed each year can be explained by couples continuing to delay child bearing for a variety of reasons, aneuploidy is not only associated with patients of an advanced maternal age. Mothers of all ages can produce a high percentage of aneuploid embryos as shown by a previous study, wherein 83 % and 70 % of embryos from both young and fertile women exhibited whole-chromosomal imbalances and sub-chromosomal abnormalities, respectively (Vanneste et al. 2009a, 2009b). Therefore, the argument that low IVF success rates are solely explained by advanced maternal age and infertility alone is slowly shifting since infertility affects both men and women equally and young fertile couples also produce aneuploid embryos (Mascarenhas et al. 2012; Taylor et al. 2014). Nevertheless, more healthy couples are using egg donors or surrogates for child bearing, and the cryogenic preservation of embryos for transfer later during a natural cycle is becoming increasingly popular (Fatemi et al. 2010; Evans et al. 2014). Lastly, some lesbian, gay, bisexual, and transgender couples are seeking artificial reproductive technologies for family planning, and yet, in all of these groups, no dramatic increase in live births has been observed when compared with more traditional IVF patients (Nordqvist et al. 2014). By subtracting factors such as advanced maternal age, infertility, and other clinical variables, healthy couples still produce embryos that arrest before the blastocyst stage and present abnormal karyotypes, indicating that aneuploidy is a universal phenomenon (Baart et al. 2006; Franasiak et al. 2014).

Summary

Infertility in humans is relatively common, and the use of IVF or other ARTs has more than doubled in the past decade and is expected to continue to increase as reproductive-age couples delay childbirth (Mascarenhas et al. 2012). Despite considerable advances in alleviating certain forms of this disorder, IVF success rates still hover around ~30–35 % (cdc.gov/art), and one of the main culprits is thought to be whole and sub-chromosomal abnormalities that arise during pre-implantation development (Munne et al. 1994). While it may occur at a higher frequency in human embryos, evidence from other closely related mammals indicates that aneuploidy is not unique to human pre-implantation development (Dupont et al. 2010). Moreover, given the complexity of early mammalian embryogenesis, it is likely that multiple factors such as parental contribution and inherent genomic vulnerability are associated with aneuploidy generation, multi/micronuclei formation, and cellular fragmentation during pre-implantation development. Although significant insight has been obtained from correlations with cancer cells and other mitotic cell types, certain aspects of genomic regulation and cell division are clearly unique to cleavage-stage embryos. As comprehensive chromosomal assessment techniques are further developed and optimized, especially in whole genomes and at the single cell level (Hou et al. 2013; Fiorentino et al. 2014; Wang et al. 2014), it is envisioned that the precise underlying cause(s) of embryonic chromosomal instability will be identified for potential therapeutic intervention. Ultimately, the overall goals of this research and future work are to select and transfer the embryo(s) with the highest probability of resulting in a normal term pregnancy and, preferably, single healthy baby. We also anticipate that the findings from these studies will enhance our understanding of natural human conception and the chromosomal requirements of early embryogenesis across diverse mammalian species.

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Fig. 1.

Chromosomal and genetic aspects of mammalian pre-implantation development. Recent advances in **a** mammalian pre-implantation embryology, including but not limited to, **b** time-lapse image analysis have greatly contributed to our knowledge of the key chromosomal and genetic characteristics impacting each major stage of pre-implantation development. **c** Approximate cell number of embryos at the morula and blastocyst stages. **d** Embryo chromosomal integrity may be influenced by the lack of and/or inheritance of aberrant parental mRNAs, proteins, or other factors such as paternal contribution of the centrosome, which mediates the first mitotic divisions. A select group of maternal mRNAs termed maternal-effect genes is recruited for translation following fertilization, whereas the remaining maternal mRNAs are degraded. In addition to individual maternal proteins, multi-protein complexes such as the subcortical maternal complex (SCMC) are important for embryonic progression beyond the 2-cell stage and for potential prevention of mitotic aneuploidy. **e** Additional mechanisms can contribute to the generation of chromosome

instability, particularly in human embryos, including cellular fragmentation, anaphase lagging, chromosomal non-disjunction, and the lack of cell cycle checkpoints and overexpression of cell cycle drivers. **f** Unlike human embryos, which respond to chromosomal aberrations by fragmenting and continuing to divide, mouse embryos appear to deal with multi- and micronuclei formation by inducing blastomere lysis. **g** Chromosomes that are lagging during mitotic anaphase are encapsulated by the nuclear envelope to form a micronucleus, where they tend to undergo frequent double-strand DNA breaks, resembling events described during chromothripsis. This induces repair by non-homologous end joining, an error prone mechanism that can result in sub-chromosomal arrangements. As micronuclei might merge back with the primary nucleus upon nuclear envelope breakdown, rearranged chromosomes are able to incorporate into the genome and produce de novo mutations. **h** Despite the high incidence of whole and sub-chromosomal instability, there is evidence that potential corrective mechanisms exist, including the restoration of euploidy upon fragment resorption, multi-polar divisions in cases of trisomic rescue, endoreduplication followed by uniparental disomy for monosomic errors, and the exclusion of aneuploid blastomeres during the morula-blastocyst transition (see Fig. 3)

Fig. 2.

Evolutionary timeline of time-lapse imaging for embryo assessment. A timeline from 1997 to the present day showing the various “eras” of time-lapse image analysis with relevant references *above* and some of the major milestones *below* in the evaluation of embryo viability. The first time-lapse publications **a** examined the implementation, utility, and safety of this technology to monitor embryo development. Subsequently, studies began identifying cellular parameters predictive of success in the blastocyst stage **b**, followed by the identification of parameters predictive of embryo ploidy status **c**. More recent reports **d** have focused on the development of computer-assisted algorithms for automatic embryo assessment and classification. Other studies **e** have clinically applied and validated these parameters or newly identified parameters in either retrospective or prospective registered clinical trials. The first randomized control trial evaluating time-lapse image analysis versus traditional IVF assessment was recently published, with others likely to follow

Fig. 3.

Incidence of cellular fragmentation and multi-/micronuclei formation in cleavage-stage mammalian embryos. *Top* Representative micrographs of **a** human embryo taken by bright field imaging, **b** rhesus macaque embryo using Hoffmann modulation contrast, **c** bovine embryo using differential interference contrast (DIC), and **d** mouse embryo taken by dark field illumination time-lapse imaging. Note the appearance of several cellular fragments (*black or white arrows*) in cleavage-stage human and non-human primate embryos, but not in mouse embryos, with a lesser extent being observed in bovine embryos. *Bottom* Incidence of cellular fragmentation (*arrows*) is highly associated with multi- and micro-nuclei formation as indicated by confocal microscopy of LAMIN-B1 (*green*) expression in DAPI-stained (*blue*) cleavage-stage embryos from the same mammalian species (**a'-d'**); *Scale bars* 50 μ m

Fig. 4.

Potential mechanisms of aneuploidy resolution during pre-implantation development. **a** Proposed model for the origins of complex embryonic aneuploidy based on fragmentation timing, fragment resorption, and underlying chromosomal abnormalities. Human embryos with meiotic errors (monosomies and trisomies) and those that appear to be triploid or tetraploid typically exhibit fragmentation at the 1-cell stage, suggesting that embryos respond to aneuploidy by fragmenting, perhaps as a survival mechanism. In contrast, fragmentation is most often detected at the 2-cell stage in embryos with mitotic errors. We also propose that the embryo probably divided before the lagging chromosomes were properly aligned on the mitotic spindle, resulting in the formation of micronuclei and the sequestering of the missing chromosomes into cellular fragments. As development proceeds, these fragments can either remain or be reabsorbed by the blastomere from which they originated to restore ploidy status or fuse with a neighboring blastomere to generate complex embryonic aneuploidies (adapted from Chavez et al. 2012). **b** Series of dark field time-lapse images, with the time in hours (*hr*) and minutes (*mins*) in the *lower left corner* showing of a rhesus macaque zygote with three cleavage furrows (*white arrows*) as it divides directly from 1-cell to 3-cells. **c** Similar bright field time-lapse image series demonstrating how one daughter cell divides twice (*black arrows* indicate cleavage furrows) before the other (*star*) to suggest a mechanism by which monosomic chromosomal errors may be resolved. **d** Single-

channel (*left*) and multichannel (*right*) confocal analysis of histone modifications in human morulas that are undergoing cavitation to form a blastocyst reveals the presence of a large and likely aneuploid blastomere (*arrows*) excluded from each embryo (adapted from Chavez et al. 2014)

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