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## Mammalian pre-implantation chromosomal instability: species comparison, evolutionary considerations, and pathological correlations

Lucia Carbone<sup>1,2,3,4</sup>, Shawn L. Chavez<sup>5,6,7,\*</sup>

<sup>1</sup>Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Portland, Oregon, USA

<sup>2</sup>Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, Oregon, USA

<sup>3</sup>Department of Molecular & Medical Genetics, Oregon Health & Science University, Portland, Oregon, USA

<sup>4</sup>Bioinformatics & Computational Biology, Oregon Health & Science University, Portland, Oregon, USA

<sup>5</sup>Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Science University, Portland, Oregon, USA

<sup>6</sup>Department of Obstetrics & Gynecology, Oregon Health & Science University, Portland, Oregon, USA

<sup>7</sup>Department of Physiology & Pharmacology, Oregon Health & Science University, Portland, Oregon, USA

### Abstract

Pre-implantation embryo development in mammals begins at fertilization with the migration and fusion of the maternal and paternal pro-nuclei, followed by the degradation of inherited factors involved in germ cell specification and the activation of embryonic genes required for subsequent cell divisions, compaction, and blastulation. The majority of studies on early embryogenesis have been conducted in the mouse or non-mammalian species, often requiring extrapolation of the findings to human development. Given both conserved similarities and species-specific differences, however, even comparison between closely related mammalian species may be challenging as certain aspects, including susceptibility to chromosomal aberrations, varies considerably across mammals. Moreover, most human embryo studies are limited to patient

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\*Address correspondence to Shawn L. Chavez, Ph.D., Oregon National Primate Research Center, Oregon Health & Science University, 505 NW 185th Avenue, Beaverton, OR 97006, USA. Tel: 503-629-4044. chavesh@ohsu.edu.

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Declaration of interest

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samples obtained from *in vitro* fertilization (IVF) clinics and donated for research, which are generally of poorer quality and produced with germ cells that may be suboptimal. Recent technical advances in genetic, epigenetic, chromosomal, and time-lapse imaging analyses of high quality whole human embryos have greatly improved our understanding of early human embryogenesis, particularly at the single embryo and cell level. This review summarizes the major characteristics of mammalian pre-implantation development from a chromosomal perspective, in addition to discussing the technological achievements that have recently been developed to obtain this data. We also discuss potential translation to clinical applications in reproductive medicine and conclude by examining the broader implications of these findings for the evolution of mammalian species and cancer pathology in somatic cells.

### Keywords

Aneuploidy; cancer; chromothripsis; embryo; evolution; fragmentation; IVF; micronuclei; preimplantation

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### Introduction

Mammalian pre-implantation development encompasses a series of events beginning with the fertilization of a mature oocyte and resulting in the formation of a totipotent embryo capable of implantation into the uterus. Much of our knowledge regarding this critical time in development has been derived from studies using model organisms, including rodents [Whitten and Biggers 1968; Whittingham 1968] and larger agricultural species such as cattle [Brackett et al. 1982]. As *in vitro* conditions for embryo culture have been expanded and adapted to other mammalian species, a closer examination of higher organisms has provided additional insight into the fundamental aspects of pre-implantation development [Bavister et al. 1984]. Moreover, early studies characterizing human embryos following natural conception [Hertig et al. 1954; Hertig et al. 1956] and more recent studies in the context of *in vitro* fertilization (IVF) have also significantly contributed to our understanding of human embryogenesis [Edwards et al. 1969; Edwards et al. 1970]. Although mammalian embryos appear morphologically similar at this stage of development, it is clear that while certain features are conserved across species, others are species-specific and may limit extrapolation between different mammals. The susceptibility to chromosomal instability during early embryogenesis is one of these features and the focus of this review.

It is important to note that the mammalian pre-implantation embryo does not develop cell-autonomously and there are numerous extrinsic factors provided by the oviduct *in vivo* that might not necessarily be emulated by culture media *in vitro* [Lee and Yeung 2006]. Nevertheless, there is evidence that several of these developmental processes do occur naturally [Buster et al. 1985; Pereda and Croxatto 1978] and recent technological advances in genetic, epigenetic, chromosomal, and time-lapse imaging analyses have greatly assisted in further investigation of the underlying molecules and mechanisms, especially at the single cell level [Chavez et al. 2012; Guo et al. 2014; Hou et al. 2013; Smith et al. 2014; Wong et al. 2010; Xue et al. 2013; Yan et al. 2013]. In this review, we summarize both shared similarities and distinct differences in the major characteristics of pre-implantation

development between different mammalian species from a chromosomal point of view as well as the technological achievements that have recently been developed to obtain these findings. We also discuss the potential consequences for the diagnosis and treatment of infertility via assisted reproduction technologies (ARTs), and the broader implications beyond the realm of Reproductive Medicine in species evolution and pathological conditions such as cancer.

## A Brief Overview of Pre-Implantation Development Across Mammalian Species

Once a single sperm successfully fertilizes an oocyte by penetrating the outer glycoprotein layer known as the zona pellucida and depositing its genetic material, the fertilized oocyte undergoes second polar body extrusion to remove its remaining set of extra chromosomes. Subsequently, the maternal and paternal pro-nuclei, each containing haploid genomes under normal conditions, appear and migrate towards one another and fuse during a process called syngamy [Payne et al. 1997]. At this stage of development, the 1-cell embryo is termed a zygote; as the parental chromosomes combine, duplicate, and line-up together on the metaphase plate in preparation for mitosis, the embryo remains largely transcriptionally silent. This occurs until embryonic genome activation (EGA), which begins at different times of preimplantation development depending on the mammalian species. The mouse exhibits the earliest transition from maternal to embryonic transcriptional control beginning at the 2-cell stage, but there is minor transcription of a few mRNAs earlier at the 1-cell stage and is often referred to as zygotic gene activation (ZGA) [Flach et al. 1982]. While rat EGA begins at the 2- to 4-cell stage [Zernicka-Goetz 1994] and the 4-cell stage in pigs [Hyttel et al. 2000], human embryos initiate EGA on day 3 at approximately the 4- to 8-cell stage [Braude et al. 1988], which is similar to non-human primates as EGA occurs at the 6- to 8-cell stage in rhesus monkeys [Schramm and Bavister 1999]. Analogous to the mouse, human embryos have also been shown to exhibit minor transcriptional activity of preferential mRNAs prior to day 3 in development [Vassena et al. 2011]. The cow, sheep, and rabbit, initiate EGA later in pre-implantation development at the 8- to 16-cell stage [Brunet-Simon et al. 2001; Crosby et al. 1988; Plante et al. 1994] to likely coincide with delayed implantation timing as compared to other mammalian species. Thus, while all mammalian embryos begin pre-implantation development under relative transcriptional silence, the duration of this quiescence greatly differs between species. It also implies that the mammalian pre-implantation embryo must initially rely on maternally-derived RNAs and proteins, the majority of which are rapidly degraded during the oocyte-to-embryo transition, a process that is largely complete by the 2-cell stage in the mouse and the 8-cell stage in human embryos [Dobson et al. 2004; Evsikov et al. 2006; Galan et al. 2010; Hamatani et al. 2004; Zhang et al. 2009]. However, there must be a certain amount of selectivity in the destruction of maternal mRNAs and proteins to facilitate the successful shift from maternal to embryonic control of development [Alizadeh et al. 2005; Wong et al. 2010]. Additional studies also suggest that a small population of paternal transcripts inherited from sperm is important for sustaining early embryogenesis beginning at the 3- to 4-cell stage in human embryos [Sendler et al. 2013; Taylor et al. 1997] (Figure 1).

Following syngamy, the 1-cell zygote undergoes a series of mitotic cell divisions to produce an increasing number of progressively smaller cells called blastomeres without changing the overall size of the embryo [Bell et al. 2008]. This continues until the embryo begins the process of intracellular adhesion known as compaction to form a morula, which is thought to be important for later morphogenetic events such as lineage specification [Kidder and McLachlin 1985; Levy et al. 1986]. Compaction occurs earlier in rodents at the 8-cell stage than in other mammalian species, including humans, non-human primates, cattle, pigs, and sheep, where it begins between the 16- and 32-cell stage [Edwards et al. 1981; Enders et al. 1990; Reima et al. 1993; Steptoe et al. 1971; Van Soom et al. 1997]. In conjunction with compaction, intracellular polarization is also thought to occur, whereby the chromosome-containing nucleus of each blastomere faces basolaterally (inward) and cytoskeletal components such as actin and microtubules accumulate apically (outward) in the embryo [Houliston and Maro 1989; Johnson and Maro 1984; Reeve and Kelly 1983]. Thus, subsequent cell divisions are either symmetric or asymmetric depending on the orientation of the cleavage plane so that this polarity is inherited in the daughter cells. These symmetric versus asymmetric cleavage divisions and/or other previously described factors such as blastomere position within the embryo that has recently regained recognition for their role in cell fate determination [Tarkowski and Wroblewska 1967; Watanabe et al. 2014], generating two distinct cell populations. While the cells on the inside of the embryo will become a part of the inner cell mass (ICM), the cells on the outside will contribute to the trophoctoderm (TE) layer [Johnson and Ziomek 1981; Sutherland et al. 1990] (Figure 1).

Regardless of the underlying mechanisms(s), the process of compaction eventually leads to the formation of a totipotent blastocyst that comprises a fluid-filled cavity called a blastocoel. Blastocoel formation is initiated almost immediately after compaction by the assembly of tight junctions and the establishment of high epithelial resistance in TE cells, beginning on day 3 in mouse embryos and later on day 4.5 during human pre-implantation development [Edwards et al. 1981; Sheth et al. 1997]. Because of this delay, human embryos are also likely to undergo at least one additional round of cell division to form an ~256-cell blastocyst, whereas mouse blastocysts typically comprise ~164 cells. However, morphological changes such as cavitation have been shown to be a function of developmental timing rather than cell number per se, at least in the human pre-implantation embryo [Hardy et al. 1989; Niakan et al. 2012]. Analogous to humans, the vast majority of other mammalian species, including non-human primates, sheep, pigs, rabbits, and the rat, form multi-cellular blastocysts on day 5 to 7 [Daniel 1965; Dobrinsky et al. 1996; Gardner et al. 1994; Seshagiri and Hearn 1993; Surani 1975]. Bovine embryos, in comparison, do not exhibit blastocyst formation until day 7 to 9 [Keskintepe et al. 1995] and may be due to differences in DNA damage, metabolic requirements, and/or the extent of placental invasion [Sturmey et al. 2009] (Figure 1). In preparation for implantation into the uterus, the blastocyst then ‘hatches’ from the zona pellucida to allow for increased embryo growth and development as well as TE adhesion to the lining of the uterine wall. Prior to implantation, which occurs on day 7 during human embryonic development, a second lineage decision is made, whereby the ICM of the blastocyst differentiates into either early epiblast or primitive endoderm to form the future fetus and parietal/visceral endoderm, respectively [Cockburn and Rossant 2010].

## Incidence and Detection of Aneuploidy During Mammalian Pre-Implantation Development

The formation of a blastocyst is one of first major landmarks in mammalian development and yet, only 30–50% of pre-implantation embryos from the majority of mammals will typically reach this stage when cultured *in vitro* [Alper et al. 2001]. In contrast, approximately 80% of mouse embryos will form blastocysts *in vitro* since developmental arrest prior to this stage is far less frequent in this species. Although the cause(s) of embryo arrest may vary 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]. Previously, the most frequently used method for diagnosing aneuploidy was pre-implantation genetic screening (PGS) of day 3 biopsied blastomeres via DNA florescent *in situ* hybridization (FISH) [Munne and Weier 1996], which suffers from mosaicism between cells and limited chromosomal analysis [Baart et al. 2006; Kuo et al. 1998; Mastenbroek et al. 2007]. As there is a limitation on the number of cells that can be biopsied from patient embryos, supernumerary cleavage-stage embryos subsequently consented for research have provided an alternative source for aneuploidy assessment, although they are generally of poorer quality.

More recent studies using array-based methods to evaluate all 24 chromosomes in high-quality whole human embryos, 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 or whether a fresh versus frozen/thawed IVF cycle, since a similar frequency was observed in embryos from fertile couples and following cryopreservation [Chavez et al. 2012; Johnson et al. 2010b; Vanneste et al. 2009]. Furthermore, the current view is that the incidence of aneuploidy *in vitro* likely reflects the situation *in vivo* considering that 20–30% of natural human conceptions are estimated to result in a live birth [Macklon et al. 2002; Slama et al. 2002; Zinaman et al. 1996] and chromosomal abnormalities have been reported in the majority of spontaneous miscarriage cases [Benkhalifa et al. 2005; Fritz et al. 2001]. 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 is important to note that microarrays for the detection of chromosomal copy number variants (CNVs) are far more utilized for humans than 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] as well as frequent observations of abnormal nuclear structure in blastomeres from rhesus embryos as compared to other mammalian species [Chavez et al. 2012] does suggest that the incidence of chromosomal abnormalities in non-human primates is similar to humans (Figure 2).

Given the high incidence of mosaicism in cleavage-stage human embryos [Baart et al. 2006; Kuo et al. 1998] and potential detriment of blastomere biopsy on embryonic development,

alternative approaches such as extended culture of embryos to the blastocyst stage and analysis of chromosomal status via trophoctoderm biopsy have recently been implemented to evaluate aneuploidy [Schoolcraft et al. 2010; Scott et al. 2013]. Technological advances in next-generation whole genome sequencing have also provided an additional platform besides array-based methods to comprehensively assess aneuploidy in human embryos, including at the single cell level [Fiorentino et al. 2014; Hou et al. 2013]. While 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 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; 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]. Moreover, 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 [Fernandez-Gonzalez et al. 2009; Kallen et al. 2010; Katari et al. 2009; Khosla et al. 2001; Peramo et al. 1999]. 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 [Alper et al. 2001; Munne et al. 1994; Schoolcraft et al. 2010].

Despite higher probability that aneuploid embryos will arrest at the cleavage-stage, embryos with chromosomal abnormalities can still form blastocysts and are often indistinguishable from chromosomally normal (euploid) embryos. Thus, while attempts have been made to correlate morphology with aneuploidy, it is well known that aneuploid embryos can appear normal and suitable for transfer under traditional IVF assessment techniques, especially when obtained by static observations [Baltaci et al. 2006]. More recently, the implementation of time-lapse imaging has provided a non-invasive approach to monitor embryos throughout development and potentially assess embryo viability. Once it was determined that time-lapse monitoring (TLM) was not detrimental to embryo development, several studies began investigating morphological, spatial, and/or temporal correlates between imaging behavior and embryo quality [Cruz et al. 2011; Nakahara et al. 2010; Wong et al. 2010]. To this end, Wong et al. [2010] demonstrated that TLM can be used to predict blastocyst fate prior to EGA by measuring the duration and time between the first three mitotic divisions. Whether the first three mitotic divisions are similarly predictive for other mammalian species besides 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 [Burrueal et al. 2014; Pribenszky et al. 2010; Sugimura et al. 2012].

In a follow-up study, Chavez and colleagues determined that the timing of the first three mitotic divisions, in conjunction with assessment of a dynamic process called cellular fragmentation, might also be used to distinguish euploid from aneuploid human embryos at the cleavage-stage [Chavez et al. 2012]. Since this initial report, additional imaging parameters such as the time to fifth cell, initiation of cavitation, and completion of blastulation have been identified that may differentiate between chromosomally normal and abnormal cleaving human embryos as well as blastocysts [Basile et al. 2014; Campbell et al.

2013]. Of course, clinical validation of these findings is required and recent prospective studies 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; Yang et al. 2014]. While there currently is some discrepancy as to whether TLM is actually beneficial for embryo selection [Kaser and Racowsky 2014], the publication of the first randomized control trial (RCT), which evaluated implantation rates, ongoing pregnancy, and early pregnancy loss, suggests that TLM is more effective than conventional IVF techniques [Rubio et al. 2014]. It remains to be determined, however, if TLM can also positively impact live birth rates, particularly in cases of single embryo transfers (SET). Nevertheless, it is clear from these studies that (i) standardized nomenclature for time-lapse markers is required as recently described [Kaser and Racowsky 2014], (ii) the method of parameter measurement is important as the use of a common start point such as the time of intra-cytoplasmic sperm injection (ICSI) may have confounding effects on other overlapping parameters, and (iii) additional RCTs investigating SET following TLM and PGS for aneuploidy detection are needed.

## Potential Mechanism(s) of Embryonic Aneuploidy Generation and Resolution

It is thought that most chromosomal errors occur during oogenesis as the maternal chromosomes congress and segregate from one another upon division [Nagaoka et al. 2012]. Other errors can occur on the mitotic spindle during embryonic cleavage divisions, which may be perpetuated due to the apparent lack of cell cycle checkpoints in cleaving human embryos [Harrison et al. 2000; Kiessling et al. 2010]. Anaphase lagging, or the failure of one or more chromosomes to connect to the spindle, has also been proposed to contribute to mitotic errors at the blastocyst stage [Coonen et al. 2004]. In comparison to other mammalian species such as the mouse, whereby only 0.05–1% of oocytes are typically aneuploid, estimates of meiotic error rates in women are relatively high at 5–20% of oocytes [Hassold and Hunt 2001]. Therefore, it is not unexpected that maternal meiotic errors are much more frequent than that of paternal origin in human embryos identified as aneuploid [Johnson et al. 2010b]. However, it is important to note that the centrosome, which consists of two centrioles for the generation of spindle microtubules during the first embryonic division(s), are paternally inherited in the majority of mammalian species [Palermo et al. 1994; Sathananthan et al. 1991] (Figure 1). In contrast, it appears that the centrosome is maternally inherited in the mouse embryo [Schatten et al. 1991], the only known exception to date. This may help explain the relatively low aneuploidy rates observed in mouse embryos [Lightfoot et al. 2006] and suggests that paternal contribution to human embryonic aneuploidy is likely greater than thought, thereby invoking consideration of paternal age as maternal age-related aneuploidy is considered [Nagaoka et al. 2012]. Additional studies have supported this idea by determining that mitotic errors are just as, if not more, frequent than meiotic errors in embryos from women of average maternal age [Chavez et al. 2012; Johnson et al. 2010b; Vanneste et al. 2009]. Further evidence is provided by observations of increased mitotic mosaicism in embryos fertilized by testicular sperm extraction (TESE) from men with non-obstructive azoospermia (NOA) to suggest that non-ejaculated spermatozoa may be less effective in organizing the first mitotic spindle [Silber et al. 2003].

Besides the transfer of defective centrosomal components, the lack of and/or inheritance of aberrant parental mRNAs, proteins, or other factors may also influence the chromosomal integrity of embryos [Burrue et al. 2014; Xanthopoulou et al. 2012]. 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]. More recent studies have shown, however, that sperm actually retain both specific coding as well as non-coding RNAs, some of which have known roles in mitotic progression and the prevention of aneuploidy generation [Dawlaty et al. 2008; Sandler et al. 2013; Taylor et al. 1997].

In contrast to inherited paternal transcripts, the importance of particular maternal RNAs and proteins during the oocyte-to-embryo transition has been more widely characterized. By definition, these factors are termed maternal effect genes as they are transcribed during oogenesis and persist in the embryo, but certain maternal effect genes are not expressed until after EGA to complicate the differentiation between maternal and embryonic effects [Li et al. 2010]. Numerous maternal effect genes have now been identified and include factors involved in maternal mRNA degradation, transcription, chromatin remodelling, and DNA methylation. In addition to individual maternal proteins, multi-protein complexes such as the subcortical 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]. One of the first members of this complex to be identified was maternal antigen that embryos require (Mater/Nlrp5) [Tong et al. 2000], and its depletion prevents SCMC formation and results in pre-implantation embryonic lethality in both mice [Li et al. 2008] and rhesus monkeys [Wu 2009]. A similar phenotype was observed in mouse embryos lacking factor located in oocytes permitting embryonic development (*Floped/Ooep*), another member of the SCMC complex [Tashiro et al. 2010]. The absence of other SCMC proteins such as *Filia* (*RIKEN*), in comparison, produces less pronounced phenotypic effects, but a high incidence of mitotic aneuploidy was detected in those embryos that failed to reach the blastocyst stage [Zheng and Dean 2009] (Figure 1). Although humans only possess a *Filia* ortholog named ES cell-associated transcript 1 (*ECAT1/KHDC3L*), which was lost in rodents [Pierre et al. 2007], the other SCMC members appear to be conserved across mammalian species [Li et al. 2010]. Thus, it will be important to determine whether these maternal effect proteins play an analogous role in regulating ploidy status during human pre-implantation development.

Even with the existence of parental euploidy-maintaining factors, frequent findings of complex mitotic aneuploid mosaicism, whereby multiple chromosomes are affected in embryos without a single euploid blastomere, indicate that it is unlikely that the majority of chromosomal errors can be corrected through development [Chavez et al. 2012; Vanneste et al. 2009]. Nevertheless, there is some evidence to suggest that chromosomal correction can occasionally occur during pre-implantation development based on PGS and biopsy of an aneuploid blastomere on day 3 followed by euploid TE cells on day 5 [Barbash-Hazan et al. 2009; Munne et al. 2005]. In support of this concept, it was proposed that euploid cells may exhibit advantageous growth over aneuploid cells in cases of diploid-aneuploid mosaicism or perhaps, there is preferential contribution of chromosomally normal cells to the ICM and chromosomally abnormal cells to the TE layer [Barbash-Hazan et al. 2009; Fragouli et al. 2008; Wells and Delhanty 2000]. Recent studies, however, have shown no preferential



distribution of chromosomally abnormal cells between the ICM and TE, suggesting that chromosomal asymmetry may not explain how an embryo is able to overcome aneuploidy generation [Capalbo et al. 2013; Johnson et al. 2010a]. Despite this apparent contradiction, the probability that aneuploidy can be resolved during pre-implantation development may instead depend on the type of mosaicism, the number of TE cells biopsied, and/or whether a single or several chromosomes have been impacted [Fragouli et al. 2011; Novik et al. 2014]. There also remains the possibility that alternative mechanisms such as blastomere exclusion or DNA replication in the absence of cell division known as endoreduplication, followed by multi-polar divisions may still play a role in the so-called 'embryo self-correction' phenomenon [Capalbo et al. 2013; Johnson et al. 2010a] (Figure 3).

As mentioned above, Chavez et al. [2012] demonstrated that dynamic assessment of cell cycle parameters in combination with cellular fragmentation analysis can assist in the differentiation between chromosomally normal and abnormal human embryos. Based on the timing of fragmentation, the authors also determined that embryos with meiotic errors typically exhibited fragmentation at the 1-cell stage, whereas fragmentation was most often detected at the 2-cell stage in embryos with mitotic errors to suggest that the human embryo responds to aneuploidy generation by fragmenting [Chavez et al. 2012]. While the cause(s) of cellular fragmentation has been disputed for several years, greater than 50% of human embryos at the cleavage stage fragment to some degree [Antczak and Van Blerkom 1999] and there is evidence to suggest that fragmentation occurs naturally *in vivo*, indicating that it is not only a consequence of *in vitro* embryo culture [Buster et al. 1985; Pereda and Croxatto 1978]. In addition, cellular fragmentation is distinct from the cell death-induced DNA fragmentation that can occur later in mammalian pre-implantation development, most significantly at the morula and blastocyst stages [Hardy 1999; Hardy et al. 2001; Xu et al. 2001]. Although originally thought to represent anucleate cytoplasmic components, Chavez and colleagues demonstrated that cellular fragments can contain nuclear DNA and that the high frequency of human embryonic aneuploidy may have contributions from chromosome-containing embryonic micronuclei, which are likely to be sequestered via fragmentation [Chavez et al. 2012]. It is important to note, however, that not all fragments necessarily enclose mis-segregated chromosomes, and may partially explain how certain fragmented embryos can still implant and result in a successful pregnancy outcome [Alikani et al. 1999; Edwards et al. 1984; Pelinck et al. 2010]. Given this evidence and that fragmentation, which usually arises at the 1- to 2-cell stage [Chavez et al. 2012], well precedes embryo arrest typically at the 8-cell stage, we would suggest that cellular fragmentation is also different from a form of mitotic cell death called chromosome fragmentation [Stevens et al. 2011; Stevens et al. 2007]. Notably, unbalanced partial chromosomal losses and gains have also been observed in fragmented aneuploid embryos and not euploid embryos, to suggest a relationship between sub-chromosomal instability and aneuploidy in the human embryo [Chavez et al. 2012; Vanneste et al. 2009]. Regardless of its origins, cellular fragmentation is observed in pre-implantation embryos from other mammalian species such as non-human primates, cattle, and even mice, although to a lesser extent [Enders et al. 1982; Sugimura et al. 2010] (Figure 2). Thus, it will be important to determine the precise mechanism(s) by which it occurs and how dynamic events such as the timing, degree, and/or resorption of chromosome-containing fragments contributes to the generation and potential correction of

embryonic aneuploidy [Chavez et al. 2012; Hardarson et al. 2002; Lemmen et al. 2008; Van Blerkom et al. 2001].

## Differential Response to Chromosomal Instability Between Mammalian Species

Unlike human embryos, which appear to respond to aneuploidy generation by fragmenting [Chavez et al. 2012], recent evidence suggests that mouse embryos deal with multi- and micronuclei formation much differently. By microinjecting mouse zygotes with morpholino oligonucleotides (MOs) to *Rps6ka4/Msk2*, a mitogenic factor known to be involved in the G1 phase of the cell cycle [Bettencourt-Dias et al. 2004], Chavez and colleagues demonstrated that reduced Msk2 expression results in mitotic arrest at the 3- to 8-cell stage [Chavez et al. 2014]. Upon assessment of embryo behavior by time-lapse imaging, the authors also determined that Msk2-MO-injected embryos exhibited an unusual phenotype of increased blastomere movement and lysis following arrest that resembled cellular events described during mitotic catastrophe [Vakifahmetoglu et al. 2008]. As mitotic catastrophe is thought to follow aberrant chromosome segregation, they investigated the nuclear structure of each blastomere and detected the formation of both multi- and micronuclei in Msk2-MO-injected, but not control-MO-injected embryos prior to lysis [Chavez et al. 2014]. This suggests that blastomere lysis may constitute a mechanism for mouse embryos to avoid chromosomal instability and that the low aneuploidy rates observed in mice are the result of a selection process [Lightfoot et al. 2006]. A correlation between *MSK2* and human aneuploidy generation was also demonstrated by findings of reduced *MSK2* expression, abnormal cell cycle parameter timing and micronuclei formation in aneuploid over euploid human embryos. Besides cell cycle regulators, the deletion of mitotic spindle assembly complex (SAC) components also leads to the formation of micronuclei, chromosome misalignment, aneuploidy, developmental delay, and decreased implantation rates in mice [Wei et al. 2011]. Taken together, this suggests that while human embryos continue to divide and undergo cellular fragmentation in spite of aneuploidy [Chavez et al. 2012], mouse embryos lyse rather than divide when faced with chromosomal instability [Chavez et al. 2014] (Figure 1). Future work will assist in determining if other mammalian species similarly respond to the generation of aneuploidy and whether *MSK2* prevents chromosomal aberrations directly or indirectly during preimplantation development.

## Clinical Consequences of Embryonic Aneuploidy During Pre-Implantation Development

Despite the progressive increase in the number of IVF cycles performed each year ([cdc.gov/art](https://www.cdc.gov/art/)), the percentage of live births has not significantly changed even with the implementation of more advanced IVF techniques, including intracytoplasmic sperm injection (ICSI), PGS, and cryopreservation [Boulet et al. 2015; Harper and Sengupta 2012; Kissin et al. 2014]. Previously, it was thought that low IVF success rates could be explained by infertility and/or advanced maternal age, but both fertile and young women produce embryos that are aneuploid with similar frequency [Vanneste et al. 2009]. By factoring out maternal age, fertility status, and other clinical variables such as whether donor materials were used, a

large number of human embryos still arrest before the blastocyst stage and are chromosomally abnormal to suggest that aneuploidy is a common phenomenon among reproductive-age couples [Baart et al. 2006; Franasiak et al. 2014]. It is important to note, however, that the function of sexual reproduction is to reduce the chromosomal instability generated in somatic cells over time. Thus, IVF could bypass this filter, which in turn may potentially increase chromosomal instability in embryos [Horne et al. 2013]. Nevertheless, there is evidence that several of the morphological features observed in embryos following ART procedures, including cellular fragmentation, blastomere asymmetry, and other developmental processes do occur naturally following *in vivo* conceptions [Buster et al. 1985; Pereda and Croxatto 1978]. Further support for this is provided by the high incidence of mosaicism detected in early spontaneous human abortions [Lebedev et al. 2004; Vorsanova et al. 2005] and similar rates of mosaicism in chorionic villus samples (CVS) between IVF and natural pregnancies later in development [Huang et al. 2009; Mantikou et al. 2012]. Therefore, it will be essential to determine how our increased understanding of chromosomal instability during pre-implantation development may be used to direct efforts toward improved fetal diagnosis and reduced spontaneous loss via cell free DNA or other methods. Lastly, it will also be critical to evaluate how additional recently developed approaches, including time-lapse imaging, DNA-Seq, natural-cycle frozen embryo transfer, either alone or in combination, impacts IVF success rates over the next several years [Evans et al. 2014; Fatemi et al. 2010].

## **Implications of Chromosomal Instability Beyond Reproductive Medicine: Cancer and Tumor Biology**

Chromosomal instability is not only commonly observed during pre-implantation embryo development, but also a quintessential hallmark of cancer cells. The majority of solid tumors contain aneuploid cells as the result of a high frequency of chromosome mis-segregation. In turn, this aneuploidy is often associated with poor prognosis and advanced tumor stage, leading to both metastatic potential and resistance to drugs [Thompson and Compton 2011]. Since its identification almost two decades ago [Lengauer et al. 1997], significant progress has been made to characterize the underlying mechanisms causing chromosomal instability in cancer cells. Among the main culprits, defects in chromatid cohesion, kinetochore-microtubule attachment, spindle assembly, and centrosome copy number appear to play a central role (reviewed in detail by [Thompson et al. 2010]). In addition, numerous proteins involved in cell cycle regulation, including Breast Cancer1 (BRCA1), BRCA2, and tumor protein 53 (TP53), have also been implicated, although their involvement in aneuploidy is less well defined. Merotelic kinetochore orientation, whereby a single kinetochore binds microtubules from both spindle poles, appears to be the most frequent cause for chromosomes to remain at the spindle equator after anaphase (anaphase lagging) in mammalian cells [Cimini et al. 2001]. Importantly, this type of error does not induce mitotic arrest, suggesting that it is not detected during the spindle assembly checkpoint, and most likely the mechanism by which aneuploidy arises in wild type (euploid) cells.

## Chromothripsis and Chromosomal Reassembly

Similar to what has been described for aneuploidy generation during pre-implantation development [Chavez et al. 2012], the formation of micronuclei (MN) is also associated with mis-segregation of whole or partial chromosomes in cancer cells and increases with culture and propagation. MN form because mis-segregated chromosomes are able to recruit a nuclear envelope whose structure appears comparable to that surrounding the primary nucleus [Hatch et al. 2013]. However, *in vitro* experiments with cultured cells have shown that MN display altered nuclear functions, including aberrant DNA replication, transcription and DNA-damage repair. Such studies also proposed a link between the recruitment of chromosomes in MN and a process initially termed genome or karyotype chaos, which is thought to represent a stress response, and is more recently known as chromothripsis [Crasta et al. 2012; Hoffelder et al. 2004; Horne and Heng 2014; Liu et al. 2014; Terradas et al. 2009; Xu et al. 2011]. First described in cancer genomes [Stephens et al. 2011], chromothripsis is a phenomenon through which one or a few chromosomal segments are ‘pulverized’ and randomly reassembled in one unique cellular event [Pellestor et al. 2014]. This is a complex process that includes chromosomal duplications, deletions, translocations, and inversions, and was identified via whole genome sequencing analysis of several cancer genome datasets. A model correlating MN formation with chromosome pulverization was proposed on the basis of *in vitro* studies in which lagging chromosomes and MN formation were experimentally induced by treating with nocodazole [Crasta et al. 2012] or monastrol [Janssen et al. 2011] and DNA damage was observed in phase G2 entry. DNA breaks appeared to occur in a ‘replication-dependent’ manner as blockage of DNA replication also prevented DNA damage. Interestingly, long-term live cell imaging showed that 97% of MN remain stable during interphase and are not degraded, nor extruded. After breakdown of the nuclear envelope, however, a significant portion (38%) of MN merges with the main nucleus (Figure 4). More detailed analyses of the structural changes occurring in the nuclear envelope of MN in comparison to the primary nucleus showed that DNA damage is a consequence of nuclear envelope collapse in both cultured cells and solid tumors [Hatch et al. 2013]. Destabilization of the nuclear envelope seems to be a consequence of structural defects in the lamina of MN, although the causes of such structural changes are unknown. As a consequence, basic nuclear functions, including DNA repair and replication are altered and genomic stability negatively affected as documented by the accumulation of gamma-H2A histone family, member X ( $\gamma$ -H2AX) foci. This appears to occur irrespective of whether the MN is intact or compromised [Cimini et al. 2001] and has been correlated with impaired DNA replication. However, the precise mechanism underlying DNA damage in MN and how this phenomenon might be linked to the massive accumulation of fragmented chromosomes leading to chromothripsis is still unclear.

Regardless of its origins, chromothripsis has also been suggested to occur during pre-implantation development [Pellestor 2014; Pellestor et al. 2014] based on frequent observations of MN formation, cellular fragmentation, and abnormal mitotic divisions in cleavage embryos [Chavez et al. 2012]. Whether aneuploid embryos undergo cellular fragmentation as a survival mechanism or to initiate their demise remains unknown, but we note that cancer cells undergo chromosomal rejoining following fragmentation as a survival

strategy in response to cellular stress [Horne and Heng 2014; Liu et al. 2014]. Although a lack of control over the maintenance of genome stability seems contradictory to normal embryo survival, aspects such as heightened cell cycle drivers and diminished checkpoints might make blastomeres more vulnerable to chromosome lagging during early mitotic divisions [Harrison et al. 2000; Kiessling et al. 2010]. Consequently, MN formation may occur and contribute to the high rate of embryonic and fetal aneuploidy observed *in vitro* and *in vivo* [Benkhalifa et al. 2005; Chavez et al. 2012; Fritz et al. 2001; Johnson et al. 2010b; Vanneste et al. 2009]. Thus, identification of the genes linked to these functions and the genetic mutations that lead to abnormal cell divisions, chromosome mis-segregation, and MN formation is required for potential therapeutic intervention in both embryology and cancer pathology. In addition, an examination of MN chromosomal content and cellular fate in detail may also assist in determining the similarities and/or differences between embryonic micronuclei [Chavez et al. 2012] and the MN that are prevalent in cancer cells [Crasta et al. 2012; Huang et al. 2012].

## Chromosomal Rearrangements and Species Evolution

Although chromosome instability has been extensively examined in the context of cancer biology and reproductive medicine, recent studies have shown that analogous mechanisms might also be behind species evolution. The importance of chromosomal rearrangements in species differentiation was recognized early on by Dobzhansky and Sturtevant in *Drosophilid* species [Dobzhansky and Sturtevant 1938]. This was later expanded upon by King [1993] and White [1969], who demonstrated that after inversions become fixed in a subpopulation, the hybridization between neighbor populations produced sterile hybrids. As part of the chromosomal speciation theory, it is thought that meiotic recombination between rearranged chromosomes results in unbalanced gametes causing infertility and reproductive isolation within the population. Although cytogenetic studies suggest that most placental mammals have karyotypes similar to the inferred 'ancestral karyotype' [Wienberg 2004], comparative genomics has shown that some mutations, including simple point substitutions are relatively frequent. However, other genetic changes such as large-scale chromosomal rearrangements are far less common in the majority of mammalian species. Given that these events are likely to be deleterious, it is not surprising that they tend to be eliminated by natural selection. It is important to note, however, that not all mammals maintain a slow rate of karyotypic evolution since the mouse, dog, and horse are exceptions to this rule. Within primates, gibbons exhibit the most accelerated rate of chromosome evolution, roughly 20 fold higher than in other mammalian species [Carbone et al. 2006]. There are four gibbon genera that separated from each other in only 5 million years and they carry highly diverged [Capozzi et al. 2012] karyotypes, with chromosome numbers ranging from 38 to 52. Interestingly, with 19 recognized species, gibbons are also the most species-rich lineage among the hominoids. Population genetics demonstrated that the radiation of the four gibbon species was almost instantaneous, possibly as the result of a high number of chromosomal rearrangements, in combination with extensive bio-geographical changes occurring in the Sunda shelf around the time of the split [Veeramah et al. 2015]. This rapid divergence, along with extensive lineage sorting experienced by these species, are the main reasons why it has

not been possible to reconstruct a consistent phylogenetic tree for the four gibbon genera thus far.

Through sequencing analysis of the gibbon genome reference, a novel gibbon-specific retrotransposon called LAVA [Carbone et al. 2012] was found to preferentially insert into genes whose products are involved in chromosome segregation processes. Retrotransposons, or so-called jumping genes, have been colonizing mammalian genomes for millions of years by creating new genetic copies via production of an RNA intermediate and a copy-and-paste mechanism. The LAVA element is a composite retrotransposon containing portions of repeats commonly found in primate genomes, including, L1, *AluS*, VNTR, and *Alu*<sub>LIKE</sub>. Further analysis of the gibbon genome revealed the presence of more than 1,200 full-length LAVA insertions in the Nleu1.0 reference assembly with a preference for intragenic locations [Carbone et al. 2014]. Gene Ontology (GO) term and pathway analyses of genes carrying LAVA insertions also showed functional enrichment for genes whose protein products are involved in chromosome segregation processes, including mitotic spindle integrity/architecture (MAP4 [Samora et al. 2011], CEP164 [Leber et al. 2010], and BUB1B [Baker et al. 2009]), cell division checkpoints, as well as kinetochore assembly and attachment to the spindle (MAD1L1 [Schuyler et al. 2012] and CLASP2 [Maia et al. 2012]). The importance of these genes in several chromosome segregation processes is substantiated by the fact that some are mutated in cancer cells with chromosomal instability [Borum-Auensen et al. 2008]. Given that Carbone et al. [2014] showed that LAVA insertions have the potential to prematurely terminate transcription, it is plausible that LAVA-induced protein truncation in this group of chromosome segregation genes directly influences the elevated frequency of chromosome mis-segregation during meiosis and/or mitosis. This may help explain the high rate of evolutionary chromosomal rearrangements observed in gibbons. Since they are an endangered species, it would be difficult if not impossible to test this hypothesis in gibbons. However, it is possible to extrapolate results from studies in assisted reproduction and cancer biology to the gibbon and potentially uncover the precise mechanisms of increased chromosomal instability that drive the evolution of particular species.

## Summary and Conclusions

Human infertility is relatively common and the use of IVF or other ARTs is likely to increase as reproductive-age couples continue to postpone having children [Mascarenhas et al. 2012]. Despite significant advances in treating certain forms of this disorder, IVF success rates still remain only ~30% and one of the main culprits is thought to be chromosomal abnormalities that arise during pre-implantation development ([cdc.gov/art](https://www.cdc.gov/art/)). As the tools and technologies necessary for comprehensive chromosomal analysis are further developed and optimized, especially at the single cell level, the direct cause(s) and potential consequence(s) of embryonic chromosomal instability may finally be realized for possible therapeutic intervention. While multiple factors are likely to be involved in aneuploidy generation, micronuclei formation, and cellular fragmentation during early embryogenesis, the findings from these studies may easily translate to cancer risk as well as the shared chromosomal aspects that drive species evolution. Collectively, the ultimate goals of this research and future work is to select and transfer the embryo(s) with the greatest likelihood to result in a

normal term pregnancy and enhance our knowledge of the chromosomal requirements of pre-implantation development across different species and the implications for somatic health.

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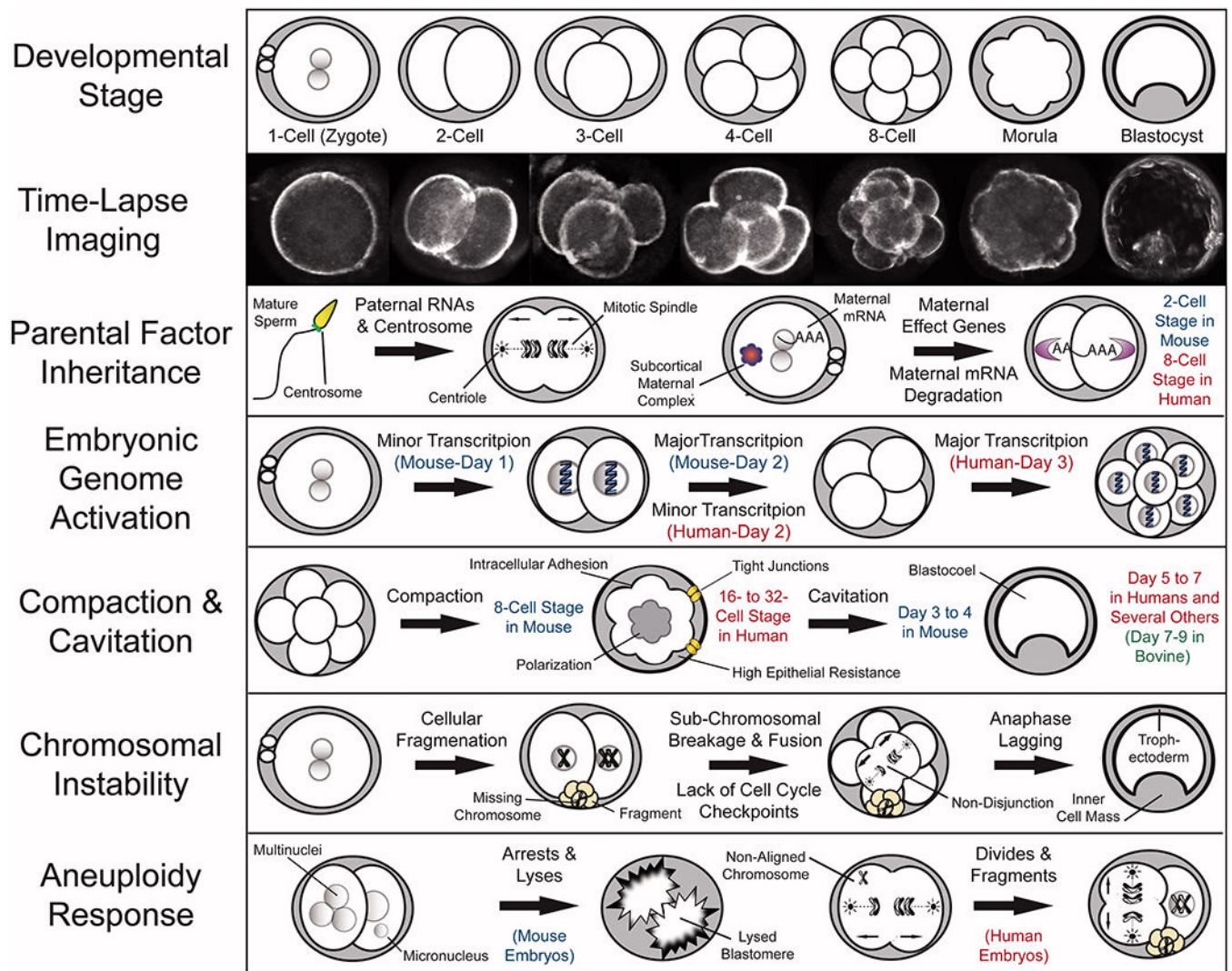
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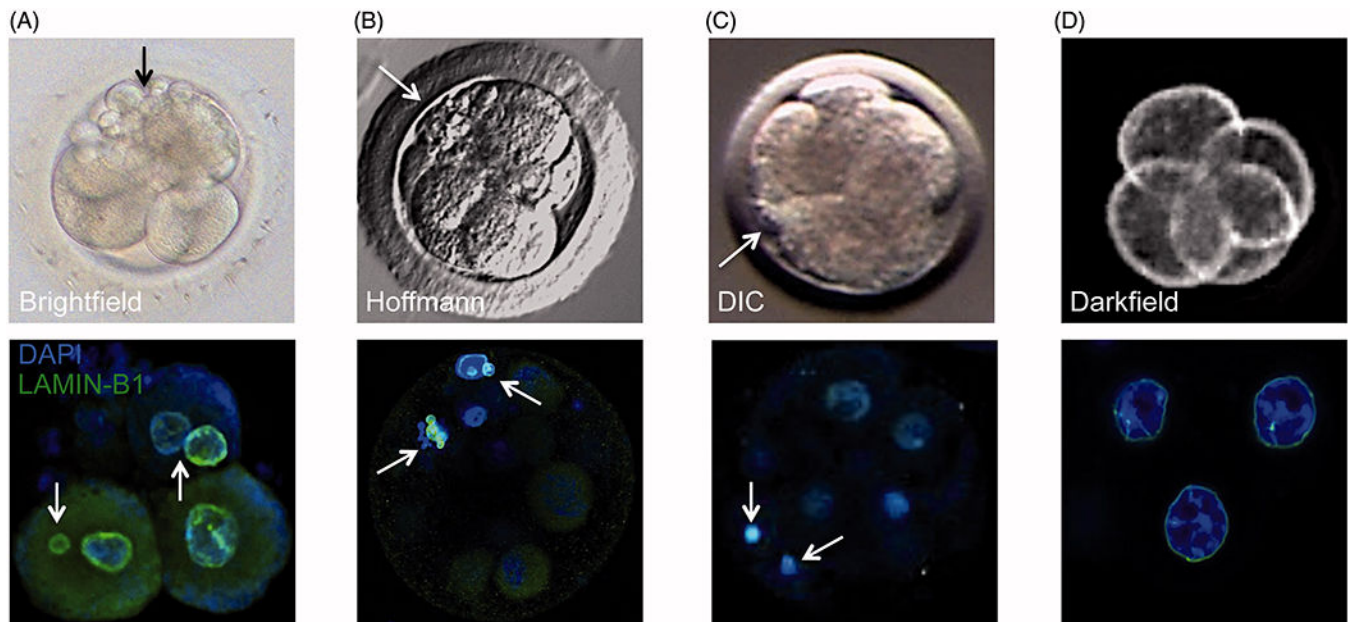
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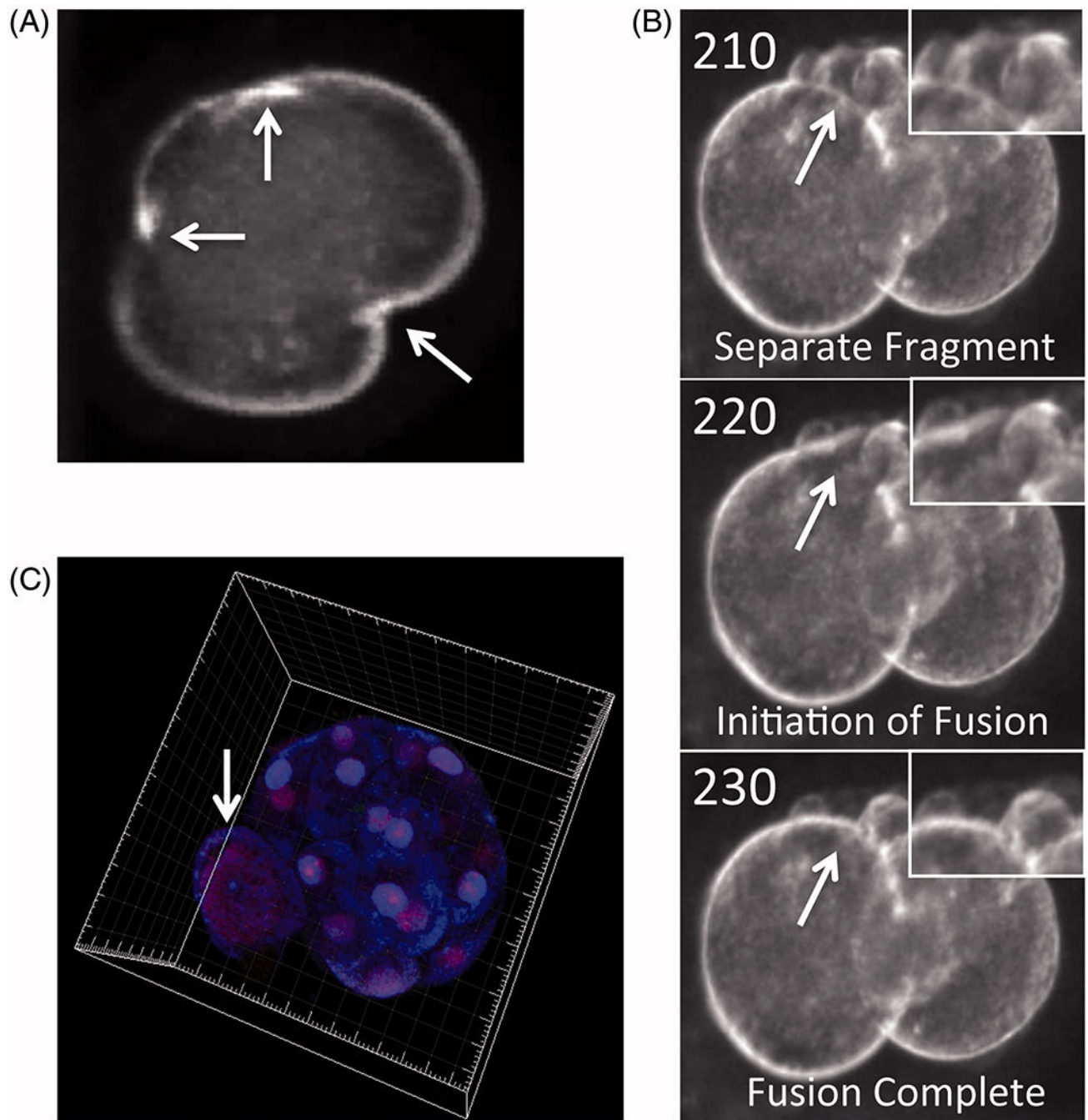


**Figure 1.** Fundamental aspects of mammalian pre-implantation development from a chromosomal perspective. Recent advances in mammalian embryology, including single cell whole genome/transcriptome analyses and time-lapse imaging, have greatly contributed to our understanding of the key characteristics in each major pre-implantation developmental stage. Time-lapse imaging has provided a non-invasive approach to monitor embryo development as well as the means to identify imaging parameters predictive of developmental success and embryo ploidy status. Embryo chromosomal integrity may be impacted 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 are recruited for translation following fertilization, whereas the remaining maternal mRNAs are degraded, a process that is essentially complete by the 2-cell stage in mouse embryos and the 8-cell stage in human embryos. Besides individual maternal proteins, multi-protein complexes, including the subcortical maternal complex (SCMC) is important for embryonic progression beyond the 2-cell stage and potential prevention of mitotic aneuploidy. This oocyte-to-embryo transition is

largely dependent upon embryonic genome activation (EGA), the major wave of which occurs in the mouse at the 2-cell stage and begins in humans on day 3 at approximately the 8-cell stage. Following EGA, an embryo undergoes the processes of compaction, intracellular adhesion, and polarization to result in the formation of a morula at the 8-cell and 16- to 32-cell stage in mouse and human embryos, respectively. The majority of mammalian species, including humans, undergo cavitation to form a fluid-filled cavity called a blastocoel between days 5 and 6, whereas mouse embryos begin blastulation earlier between day 3 and 4 and bovine embryos later between day 7 and 8. There are several factors that can contribute to the generation of chromosome instability, particularly in human embryos, including cellular fragmentation, sub-chromosomal breakage and fusion, a lack of cell cycle checkpoints, and chromosomal lagging during anaphase. It appears that unlike human embryos, which respond to chromosomal aberrations by fragmenting and continuing to divide, mouse embryos deal with multi- and micronuclei formation by inducing blastomere lysis.



**Figure 2.** Correlation between cellular fragmentation and multi-/micronuclei formation in cleavage-stage embryos from different mammalian species. Top row: a representative photo of (A) human embryo taken by brightfield imaging, (B) rhesus macaque embryo using Hoffmann modulation contrast, (C) bovine embryo using differential interference contrast (DIC), and (D) mouse embryo taken by darkfield 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 observed in bovine embryos. Bottom row: the incidence of cellular fragmentation is highly associated with multi- and micronuclei formation as indicated by confocal microscopy of LAMIN-B1 (green) expression in DAPI-stained (blue) cleavage-stage embryos from the different mammalian species.



**Figure 3.** Potential mechanisms of aneuploidy resolution during embryo pre-implantation development. (A) Time-lapse image showing a human zygote with three cleavage furrows as it divides directly from 1-cell to 3-cells. (B) Individual imaging frames of a human embryo with cellular fragmentation demonstrating blastomeric resorption of a fragment. Adapted from Chavez et al. [2012]. (C) Multi-channel confocal analysis of histone modifications in a human morula that is undergoing cavitation to form a blastocyst reveals the presence of a

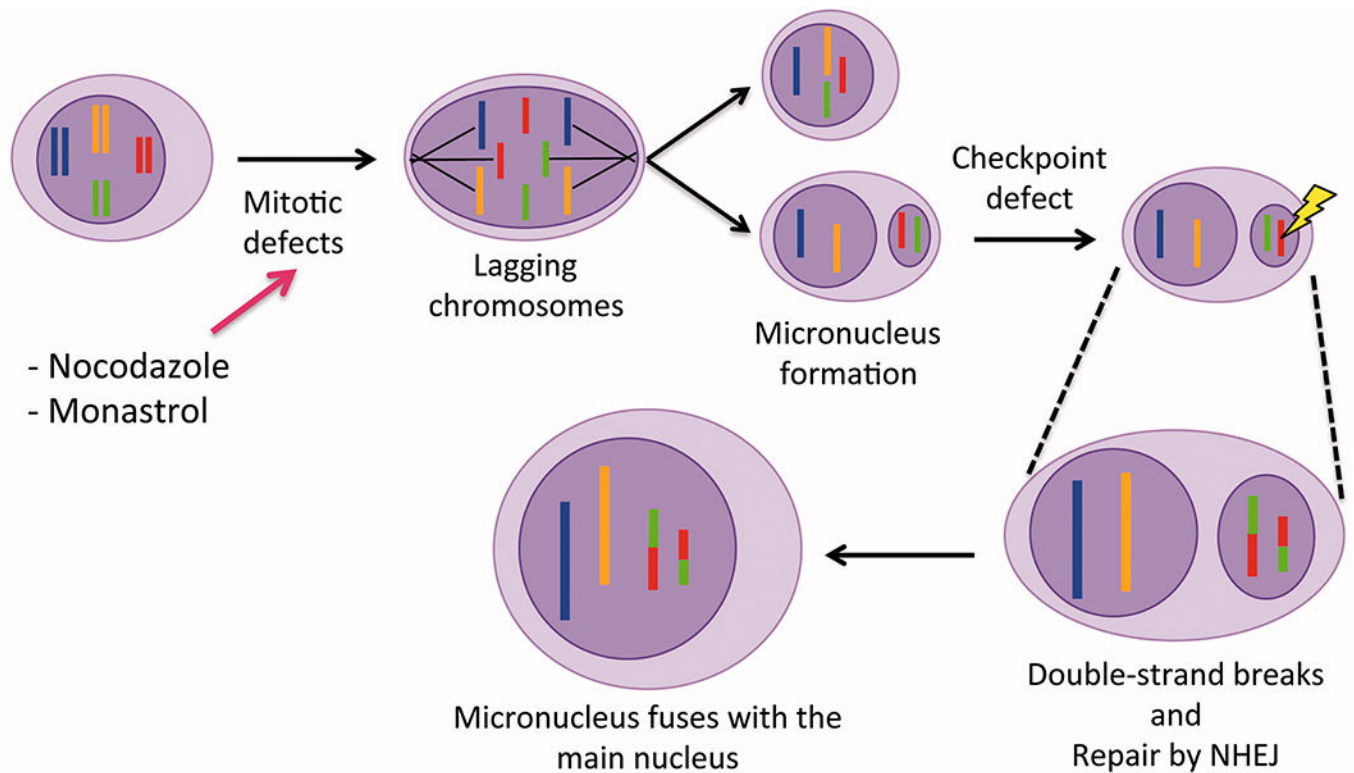
large and likely polyploid blastomere excluded from the embryo. Adapted from Chavez et al. [2014].

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**Figure 4.**

Model describing the association between chromosome mis-segregation, micronuclei, and chromosomal rearrangements. Chromosomes that are lagging during anaphase as a consequence of mitotic defects are encapsulated by nuclear envelope to form a micronucleus (MN). Within the MN, chromosomes tend to sustain frequent double-strand breaks most likely due to changes in chromatin conformation. This induces repair by non-homologous end joining (NHEJ), an error prone mechanism that can produce chromosomal aberrations. As MN may merge back with the main nucleus, this will result in the rearranged chromosomes becoming a part of the genome as described by Janssen et al. [2011].