Mechanisms and Consequences of Mitotic Mis-Segregation During Early Embryogenesis

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Abstract

Investigations into the relatively high frequency of spontaneous miscarriages have revealed that **chromosomal instability** (CIN) is common in human preimplantation embryos. This instability is characterised by an elevated rate of missegregation of whole chromosomes or parts of chromosomes during mitosis, often resulting in aneuploidy. Aneuploidy is a condition marked by an aberrant chromosome number in a cell and is particularly evident in the initial three cleavages following fertilization. Interestingly, these observations provide valuable insight into the challenges associated with successful fertility rates. However, the underlying mechanisms driving early-stage embryonic aneuploidy and the fate of aneuploid embryos remain unclear. Suggestions of protective mechanisms that act against aneuploid cells, including apoptosis, preferential allocation, and trisomic rescue, among others, have been proposed. Here again, a comprehensive understanding of these mechanisms is still lacking. This review therefore aims to elucidate the established and strongly indicated causal mechanisms of preimplantation embryo aneuploidy known thus far and discuss the potential consequences and outcomes of impacted embryos. Exploring these mechanisms is crucial for advancing our understanding of human fertility and is instrumental in improving the success rates of human IVF by unveiling potential targetable mechanisms that are capable of mitigating aneuploidy.

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Plain Language Summary

Early-stage embryonic aneuploidy, an abnormal number of chromosomes in a cell, is the primary factor contributing to human reproduction inefficiency. It is caused by chromosomal instability (CIN), which arises from errors during cell division. Our current understanding of aneuploidy is owed to technological advancements that allow for the analysis of chromosomes, despite some inherent limitations.

Understanding the underlying mechanisms behind early embryo aneuploidy is crucial. Maternal and paternal factors play important roles in maintaining euploidy (the correct number of chromosomes). Key developmental maternal proteins are stored on structures within the ovaries, and detrimental changes in their composition can disrupt key checkpoints and the orientation of chromosomes, leading to chromosome missegregation during cell division. Paternally inherited abnormal centrosomes (key components of mitosis that organise structural fibres) may also disrupt chromosome segregation, and issues like underdeveloped sperm and DNA damage could introduce mistakes in the genome. The exposure of the genome to stresses during replication (replication stress) and DNA damage, on the other hand, may cause a specific type of aneuploidy called segmental aneuploidy: gains or losses of pieces of chromosomes. Moreover, the integrity of the genome is dependent upon the efficient response of DNA to damage (DNA damage response - DDR) and repair mechanisms that prevent the transmission of damaged DNA during division. However, in embryos, where the stress levels are typically moderate, DDR activation is low, and the need for DNA repair is unrecognised. This sends under-replicated DNA into mitosis, potentially leading to aneuploidy.

Another contributing factor is the spindle assembly checkpoint (SAC), which pauses mitosis in the event of errors, but is weakened in embryos. This allows mitosis to proceed despite the presence of faults, increasing error risk. A particular protein called Aurora C kinase is predominant at early embryo prometaphase and contributes to rectifying these errors. Aurora C kinase may be linked with high error rates during cell division in early embryos. Moreover, early embryos lack a specific mechanism to trigger cell death following excessively prolonged mitosis, called the mitotic timer, further increasing aneuploidy risk. Additionally,

the absence or inactivation of checkpoint proteins that function to maintain error-free cell division also contributes to aneuploidy.

The fate of the embryo as a result of the aforementioned mechanistic aberrations has been an area of great interest. Mosaic embryos, composed of both normal and aneuploid cells, have shown a decline in the proportion of aneuploid cells as development progresses, often termed "aneuploid self-correction". The main proposed mechanisms for this phenomenon include apoptosis, where aneuploid cells undergo programmed cell death to allow euploid cells to dominate, preferential allocation, which proposes that aneuploid cells will locate themselves away from the cells destined to become the fetus, and trisomic rescue, which suggests albeit with no supporting data, that cells with three rather than two chromosomes (trisomies) can lose their extra chromosome. Nonetheless, "self-correction" mechanisms are still under debate, and further research is needed to understand the fate of aneuploid early embryos.

To conclude, it's important to consider that most of the data discussed is provided from embryos produced in a lab (*in vitro* fertilisation), a process that itself has been suspected to induce mitotic errors. Moreover, improved standardization within the field is needed to enhance reliability and comparability. This will also aid in addressing current IVF shortcomings and encourage progress.

Introduction

The introduction of *in vitro* fertilization (IVF) has revealed that only around 30-50% of most mammalian pre-implantation embryos, including human embryos, will reach the blastocyst stage. This stage occurs approximately five days after fertilization and marks the first key milestone in early development. Several defining features characterize the blastocyst stage, including the formation of a fluid-filled cavity called the blastocoele, the grouping of cells to one side of the **zona pellucida**'s interior to form the **inner cell mas**s (ICM), and the development of the **trophectoderm (TE)**. TE cells surround and safeguard the ICM while also lining the inner surface of the zona pellucida (Alper et al., 2001; Daughtry & Chavez, 2016; Khan & Ackerman, 2024). Contrastingly, mouse embryos have an 80% success rate in forming blastocysts *in vitro*, attributed to the infrequent occurrence of arrest in earlier stages of development in this species (Daughtry & Chavez, 2016). Thus, the inefficient nature of human reproduction has been recognized and questioned, and in recent years, the contributing factors have been increasingly brought to the forefront. High rates of embryonic **aneuploidy**, a consequence of **CIN** characterized by the deviation of chromosome numbers from a multiple of the haploid set, have been shown to pose a significant challenge to successful pregnancies (E. B. Baart & Van Opstal, 2014; Orr et al., 2015). Aneuploidy has been observed in various states of human preimplantation embryos, including arrested, developing, fresh, frozen-thawed, and fragmented embryos (E. B. Baart et al., 2006; Iwarsson et al., 1999; Mantikou et al., 2012). This suggests that aneuploidy is an intrinsic characteristic of human conception, and it has been identified as the primary factor contributing to developmental arrest in early-stage embryos (Mantikou et al., 2012).

Aberrant **chromosome segregation** during gametogenesis, generally resulting in whole-embryo aneuploidy of meiotic origin, has been extensively described (Chiang et al., 2012). However, research has shown that the majority of preimplantation embryos consist of cells with various chromosomal constitutions, an observation that is associated with mitotic errors (E. B. Baart & Van Opstal, 2014; Mantikou et al., 2012; McCoy, 2017). Such a phenomenon, termed chromosomal **mosaicism**, can be classified into two main groups. The most frequently observed classification involves the presence of both diploid and aneuploid cells within the same embryo, resulting in diploid-aneuploid mosaics. Alternatively, embryos could consist of cells with different aneuploidies across various chromosomes, giving rise to

aneuploid mosaics (Mantikou et al., 2012). Embryonic mosaicism has garnered increased attention fairly recently, and further work needs to be done to understand the underlying causal mechanisms of such embryos and their ultimate developmental outcomes. This process will assist in the improvement of IVF techniques and deepen our understanding of human reproductive capabilities.

Mosaic aneuploidy of mitotic origin is associated with errors such as anaphase lag, mitotic nondisjunction, multipolar spindle, and premature cell division. Additionally, the process of endoreplication, while distinct from mitotic errors, may also contribute to mosaic aneuploidy. (Ivanova & Semenova, 2023; Mantikou et al., 2012; Taylor et al., 2014). Cleavage-stage embryo mosaicism is most frequently caused by anaphase lag and mitotic nondisjunction. Anaphase lag, characterised by delayed chromosome movement following the separation of the other sister chromatids towards the poles (Ivanova & Semenova, 2023), is commonly caused by **merotelic attachments**. It may lead to various outcomes including the loss of the retained chromosome(s), their inclusion into **micronuclei**, or the missegregation of the lagging chromosome into the primary nuclei, resulting in a chromosome gain in one daughter cell, and a chromosome loss in the other (Cimini et al., 2001). Mitotic nondisjunction, on the other hand, is caused by kinetochore anomalies (E. B. Baart & Van Opstal, 2014; Ivanova & Semenova, 2023), and describes an unbalanced distribution of chromatids between two daughter cells. As a result, one cell acquires extra chromosomes while the other experiences a loss. Other processes, such as endoreplication, less frequently contribute to preimplantation embryo mosaicism. Endoreplication is the repeated replication of the nuclear genome without mitosis, giving rise to tetraploid cells. (Ivanova & Semenova, 2023; Mantikou et al., 2012). Chromosome loss, on the other hand, may be a consequence of chromosome breakage and premature cell division prior to the completion of DNA replication. Additionally, the presence of multipolar spindles, such as **tripolar spindles**, may also cause chromosome loss through inadequate chromosome attachment and unequal chromosome segregation (Ivanova & Semenova, 2023).

The continuously improving knowledge of aneuploidy and mosaicism in early preimplantation embryos can be largely attributed to the technological advancements in the field. Most of the pioneering studies in the early embryonic aneuploidy field made use of karyotype analysis (Clouston et al., 1997; Jamieson et al., 1994; Pellestor et al., 1994), which although is useful for chromosomal analysis, requires dividing and metaphase-stage cells. It

has been revealed that only 24-36% of embryo metaphases are of sufficient quality for accurate analysis (Clouston et al., 1997). This, together with other factors such as the challenges in obtaining optimal chromosomal banding and the risk of chromosome loss during nuclei fixation, led to the discontinuation of karyotype analysis for early embryo aneuploidy (Clouston et al., 1997; Mantikou et al., 2012; Pellestor et al., 1994). Following karyotype analysis, fluorescence in situ hybridization (FISH) emerged as the technique used most frequently for aneuploidy analysis in early embryos (Mantikou et al., 2012). Advantageously, single cells may be analysed using FISH, and chromosome numbers can be investigated in metaphase and interphase nuclei (Mantikou et al., 2012). However, a limitation is posed by the number of probes that may be used concurrently, which was addressed by the use multiple sequential FISH rounds. Nonetheless, hybridization efficiency is reduced with each round, prompting a restriction to a maximum of 3 rounds, unless advanced FISH techniques like spectral karyotyping or multiplex-FISH are used, allowing the analysis of up to 24 chromosomes (Liu et al., 1998; Schröck et al., 1996; Speicher et al., 1996). Caution is still needed since FISH exhibits an estimated accuracy of 92-99% per probe (Ruangvutilert et al., 2000), and moreover, fixation and spreading of the nucleus on slides, as required by the methodology, may lead to chromosome damage, breaks, and loss, amongst other artifacts (Ruangvutilert et al., 2000). It’s also important to note that such a technique cannot detect partial or segmental aneuploidy, characterised by the gain of loss of a small piece of a chromosome during cell division. (Babariya et al., 2017; Wilton et al., 2003). Recently, there has been an increasing preference for the use of comparative genomic hybridization (CGH), CGH-microarrays (aCGH), and single-nucleotide polymorphism-based (SNP) microarrays following whole genome amplification (WGA) which make it possible to analyse all chromosomes (Mantikou et al., 2012). The rise in popularity is also attributed to the lack of fixation and spreading in their methodologies, automation, the possibility to study the copy number variation (CNV) of all chromosomes, and increased resolution, with the flexibility to use cells at any stage of the cell cycle further enhancing their utility (Coughlin et al., 2012; Wilton et al., 2003). In contrast to FISH, segmental aneuploidy can also be detected (Wilton et al., 2003). Nonetheless, one must keep in mind that complete accuracy is still not guaranteed with such technologies. The potential for bias remains, especially if amplification of one parental allele fails or excess amplification is carried out, (Handyside et al., 2004; Iwamoto et al., 2007; Piyamongkol et al., 2003; Renwick et al., 2006) and thus, continuous caution is necessary.

By analysing data accumulated through years of research using both traditional methodologies, like karyotype analysis, and contemporary approaches, such as CGH and microarray technologies, this review explores the evolving understanding of embryonic aneuploidy within the field. The discourse will encompass the underlying mechanisms of pre-implantation aneuploidy, the possible fates of affected embryos, and the implications for advancing **assisted reproductive techniques (ART)**.

1. Mechanisms Underlying Preimplantation Aneuploidy

1.1 The Impact of Maternal and Paternal Factors

1.1.1 Maternal Factors

Genome activation of early embryos, termed the **maternal-to-embryonic transition (MET)**, has been shown to initiate at the 4- to 8-cell stage, prior to which the embryo lacks autonomous protein synthesis (Braude et al., 1988). Thus, it relies on proteins and stored maternal factors accumulated by the oocyte during **folliculogenesis** and **oogenesis** to facilitate the initial stages of embryonic development (Zhang & Smith, 2015). A recent study has shown that key developmental maternal proteins are stored by the oocyte **on cytoplasmic lattices** to result in protein compartmentalization which is necessary for correct functionality (Jentoft et al., 2023). Jentoft et al. present female infertility as the main consequence of mutations in cytoplasmic-lattice-related genes and proteins like *Padi6* and the **subcortical maternal complex (SCMC)**, a multiprotein complex that plays key roles in the oocyte-to-embryo transition such as meiotic spindle formation and positioning, and translation regulation. (Li et al., 2008; Zheng & Dean, 2009). They show that individual mutations lead to the failed development of both mice and human embryos (Jentoft et al., 2023). Although the cause of development arrest was not explored, one may notice that the protein FILIA is part of SCMC, and is crucial for embryonic mitosis, as demonstrated by delayed preimplantation development and reduced fecundity in *Filia* null phenotype mice (Zheng & Dean, 2009). In these mice, increased **spindle assembly checkpoint** (SAC) dysregulation and abnormal spindle assembly also ensued, giving rise to aneuploidy. This leads to the conclusion that maternal Filia plays a key role in the maintenance of **euploidy** in cleavage-stage embryogenesis by ensuring proper mitotic checkpoint functionality and successful spindle assembly (Zheng & Dean, 2009).

Moreover, Jentoft et al. demonstrated that the oocyte cytoplasmic lattices contain proteins involved in **embryo epigenetic reprogramming**, an important process for the successful transition of a single **totipotent cell** to a complex multicellular organism. Although not specifically mentioned, we can speculate the inclusion of another important maternal protein, H3.3, necessary for **chromatin remodelling** during zygotic development (Jentoft et al., 2023;

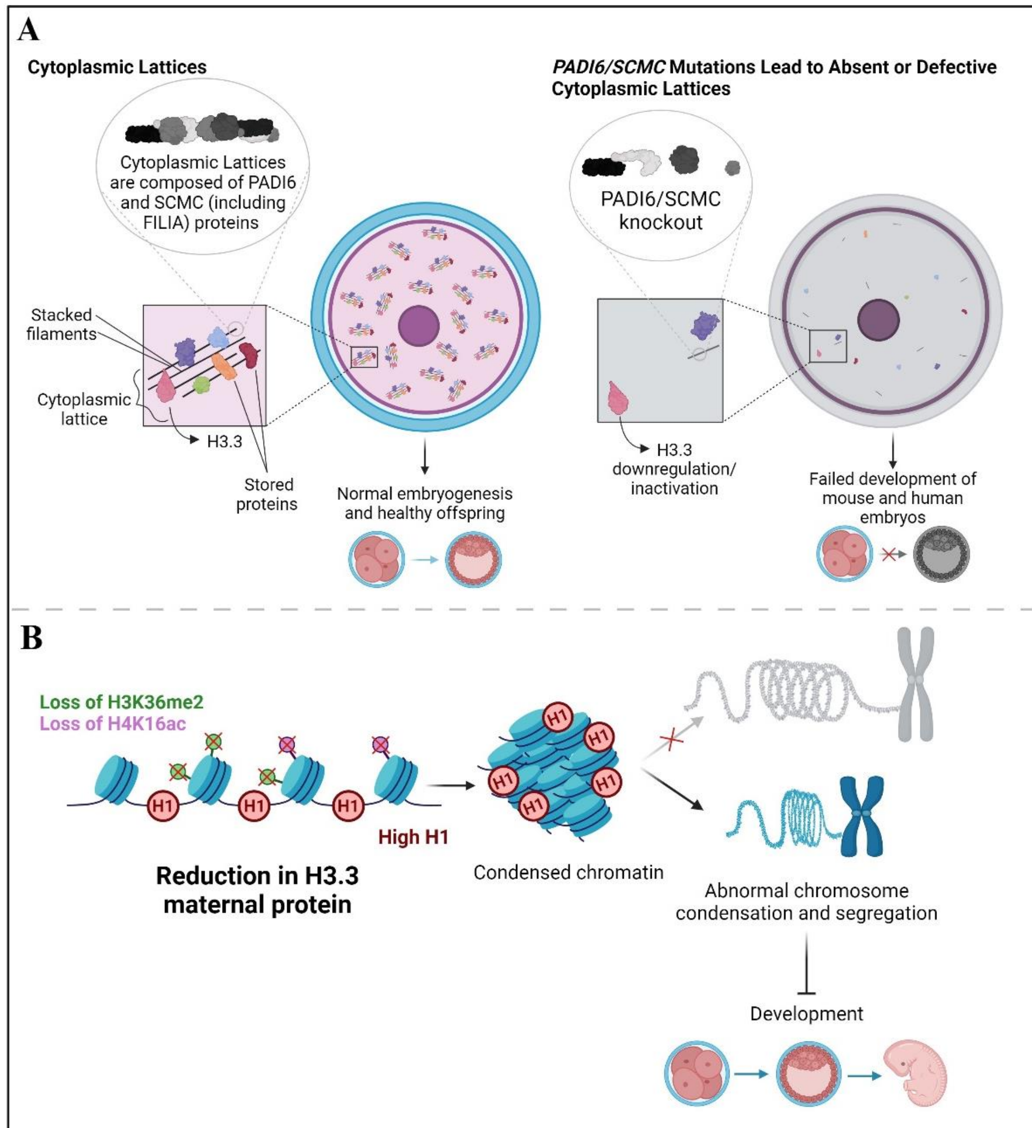
C.-J. Lin et al., 2013). **H3.3** contributes to a vital epigenetic landscape supporting mouse embryo development. Its knockdown results in significant epigenetic and cytogenetic changes, such as the loss of **K36me2** and **H4K16Ac**, increased levels of **H1**, and abnormal chromosome condensation and segregation, factors which are incompatible with continued development (C.-J. Lin et al., 2013). Thus, a causal relationship between epigenetic changes involving the maternal protein H3.3 and chromosome missegregation is observed. This data shows that the occurrence of aneuploidy is dependent upon the effective functioning of maternal proteins that play a pivotal role in embryogenesis and mitosis (Figure 1.1).

1.1.2 Paternal Factors

While the oocyte makes a substantial contribution to embryogenesis as described above, oocyte maturation results in the degeneration of its two centrioles (Simerly et al., 2018). These subcellular organelles are contrastingly found in the spermatozoan neck, and as a result, the centrosomes are paternally inherited (Avidor-Reiss et al., 2022). The centrosomes, composed of two centrioles, act as the **microtubule-organizing centers** of the cell (MTOCs), maintaining an equal distribution of chromosomes in daughter cells. Anomalies in centrosome functionality or positioning may result in aneuploidy, as well as irregular chromosome segregation, formation of micronuclei, and developmental problems (Avidor-Reiss et al., 2022). This was demonstrated in a study carried out to test the centrosomal function of cat testicular spermatozoa in which the significance of **centriole maturation** for a proper first cleavage and successful embryonic development was shown. Testicular **spermatozoa immaturity** gives rise to reduced centrosomal function, leading to a slower first cleavage, and developmental arrest as a result of faulty **aster** formation (Comizzoli et al., 2006). A link to aneuploidy may be made due to the key mitotic role of centrosomes. A higher incidence of mosaicism has also been seen in patients with **non-obstructive azoospermia** undergoing **testicular sperm extraction (TESE)**, with the cause likely to be sperm centrosome irregularities (Magli et al., 2009; Silber et al., 2003). Separately, mosaicism and aneuploidy were shown to be a result of **dispermic penetration** during fertilization, due to the presence of a tripolar spindle as opposed to a bipolar spindle (Palermo et al., 1994). Profound DNA damage in sperm, caused by **protamine** imbalances, advanced male age, storage temperatures, and infections, amongst others, may also lead to increased mitotic whole chromosome and chromosome segment aneuploidy and genomic instability. This can be seen by disordered mosaic patterns and aneuploid bovine blastomeres as a

consequence of fertilization with DNA-damaged sperm induced by γ-radiation (Middelkamp et al., 2020).

Figure 1.1 **– The Impact of Maternal Factors on Preimplantation Aneuploidy**



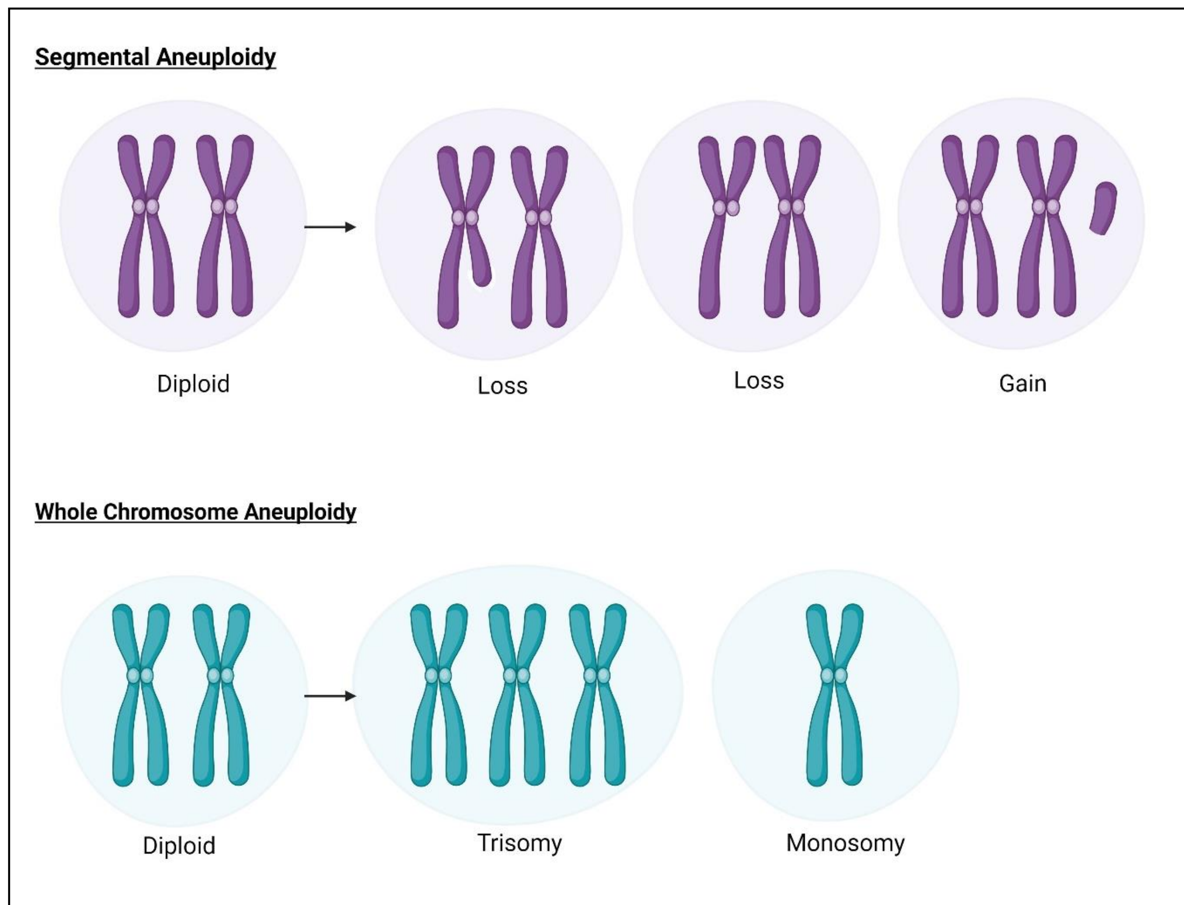
(A) Key maternal proteins needed for embryogenesis are stored by the oocyte on cytoplasmic lattices. Mutations in genes and proteins like *PADI6* and the subcortical maternal complex (SCMC), which are essential components of the cytoplasmic lattices, lead to a reduction in maternal proteins, and thus, failed development of mice and human embryos. The SCMC protein FILIA, for example, is crucial for embryonic mitosis, and thus, erroneous production may contribute to aneuploidy. (B) Reduction in the maternal protein H3.3 is incompatible with life as a result of K36me2 and H4K16Ac loss, increased levels of H1, and abnormal chromosome condensation and segregation.

1.2 Replication Stress and DNA Damage: Sources of Segmental Aneuploidy

1.2.1 Segmental Aneuploidy

Segmental aneuploidy, which is the gain or loss of chromosomal fragments during cell division (Figure 1.2), represents a notable but often overlooked form of aneuploidy in early embryonic cells (Babariya et al., 2017). A study carried out by Babariya et al. illustrated the frequent nature of segmental aneuploidies in early development which occur independently from aneuploidy affecting whole chromosomes, but are often seen alongside one another in a common sample. Predominance was seen at the cleavage stage, possibly due to the dormant embryonic genome and accelerated cell cycles which lend themselves to error risk. The location of chromosome breakpoints often occurred at “hotspots”, shown to correlate with fragile sites in the genome, both known and novel (Babariya et al., 2017). Numerous studies have suggested that while breakpoints may be observed in **subtelomeric regions**, regions abundant in repeat elements, and gene-dense areas, “hotspots” tend to be enriched in or near **peri-centromeric heterochromatin** when associated with structural variations and chromosomal rearrangements (Barra & Fachinetti, 2018; Kolesnikova et al., 2022). This enrichment is likely attributed to the repetitive sequences and low-copy repeat elements characterizing peri-centromeric heterochromatin (Barra & Fachinetti, 2018). In such instances, the proximity of the breakpoint to the centromere suggests that arm-level segmental aneuploidies will be observed more frequently than small segmental aneuploidies. Moreover, segmental aneuploidies tend to be a result of DNA double strand breaks (DSBs), which may be caused by various endogenous and exogeneous factors including **oxidative stress** and **replication stress** (Mehta & Haber, 2014). While the clinical implications of segmental abnormalities in embryos remain uncertain, it is reasonable to hypothesize a level of embryo lethality (Babariya et al., 2017). Therefore, understanding the root of occurrence is of utmost importance.

Figure 1.2 **– Segmental Aneuploidy and Whole Chromosome Aneuploidy**



Segmental aneuploidy is the gain or loss of chromosomal fragments whereas whole chromosome aneuploidy is the gain or loss of entire chromosomes as seen in trisomies (gain of one chromosome) and monosomies (loss of one chromosome).

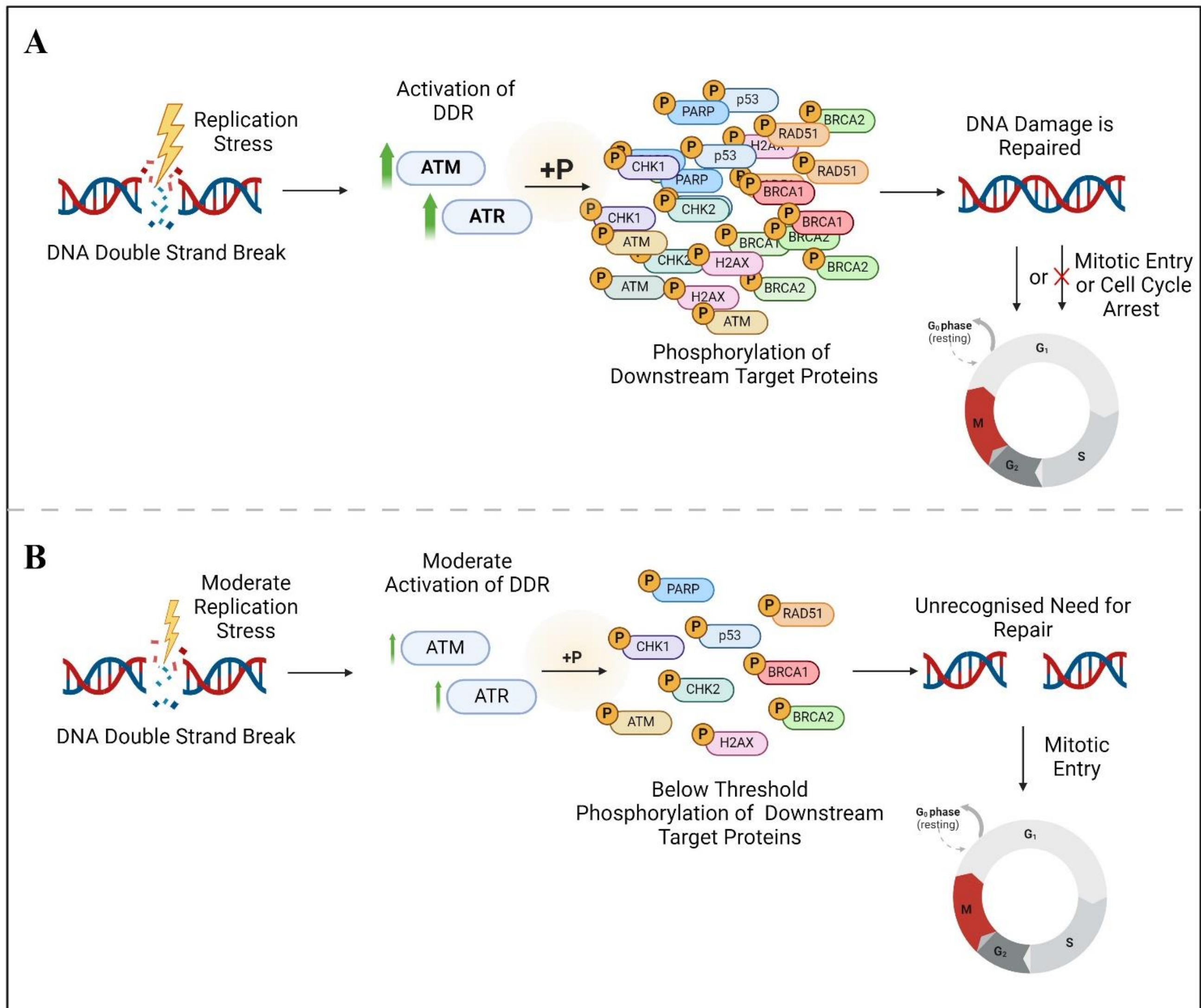
1.2.2 DNA Replication Stress and Repair Proteins

Replication stress encompasses any situation that results in the stalling or slowing down of DNA replication forks, disrupting the timely and accurate completion of the S phase, and thus compromising chromosome segregation (Gelot et al., 2015; Wilhelm et al., 2014). In somatic cells and specifically in the context of cancer, factors that may disturb replication have been extensively identified, namely DNA lesions, chemical compound adducts, UV or ionizing radiation, and reactive oxygen species (ROS), amongst others (Mazouzi et al., 2014; Zeman & Cimprich, 2014). Moreover, fragile sites that are inherently challenging to replicate, such as telomeres or ribosomal DNA (rDNA), also have the potential to disturb replication

(Gadaleta & Noguchi, 2017; Maestroni et al., 2017; Warmerdam & Wolthuis, 2019). As a result of replication stress, DNA forks may break or collapse, giving rise to replication-associated DNA double-strand breaks (DSBs) (Cortez, 2019), and triggering the **DNA damage response** (DDR) (Ciccia & Elledge, 2010). The resultant activation of the core DDR transducer kinases, ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR), results in the initiation of repair processes and mitotic re-entry through the phosphorylation of specific key proteins, including p53, CHK1, and itself (ATM) (Burrell et al., 2013; Koundrioukoff et al., 2013). However, in the event that replication stress is only moderate, lower levels of protein phosphorylation may ensue which are below detectable level. As a result, DDR activation and the need for repair is unrecognised, allowing the cell to enter mitosis in the presence of under-replicated DNA, and potentially leading to breakage in these regions. This may give rise to incomplete or incorrect chromosome segregation, resulting in aneuploidy (Figure 1.3) (Burrell et al., 2013; Koundrioukoff et al., 2013). In other cases, the formation of **anaphase bridges** have been recorded as a result of mitotic progression in cells with incompletely replicated, tangled DNA regions (Gelot et al., 2015). This consequence of replication stress may also lead to particular aneuploidies such as whole-arm deletions and translocations (Finardi et al., 2020).

In light of the knowledge derived from somatic cells, the contribution of replications stress and resultant DNA damage in early embryonic aneuploidy was challenged. Indeed, we see the upregulation of the DNA damage marker **GADD45** in arrested aneuploid human IVF embryos, indicating an elevated level of DNA damage in these embryos, and suggesting that the DNA damage itself caused the arrest (Vera-Rodriguez et al., 2015). A recent study carried out by Palmerola et al. demonstrated the occurrence of spontaneous DNA breaks (DSBs or a combination of DSBs and ssDNA gaps) during the first cell cycle in human embryos as a result of DNA replication stress, with observable DNA damage and repair foci indicating *de novo* damage emerging after the first S phase. Such results were correlated with the generation of micronuclei and chromatin bridges, which could contribute to the formation of aneuploidies and impair developmental potential. Importantly, this reflects what was previously described in somatic cells. (Palmerola et al., 2022). A possible source of DNA replication stress is aberrant DNA demethylation in the embryo, which gives rise to abasic sites and ssDNA breaks, leading to replication fork stalling (Guo et al., 2014; Tolmacheva et al., 2020). Paternal DNA damage may also contribute here and has been significantly recognised in the spermatozoa of infertile men (Simon et al., 2011; Xavier et al., 2019).

Figure 1.3 – Replication Stress and Mitosis in the Presence of Under-replicated DNA,



(A) Replication stress may result in DNA double-strand breaks, necessitating DNA damage repair via activation of DNA damage repair (DDR) and downstream repair proteins. (B) Under conditions of moderate stress, the downstream repair pathway is not sufficiently activated in the embryo. Thus, the need for repair is unrecognised, allowing the cell to enter mitosis with under-replicated DNA.

Notably, the differences in how mouse and human embryos respond to replicated DNA were also highlighted here. Mouse embryos demonstrated a greater ability to induce DNA damage foci in G2, arrest zygote progression in case of improper DNA replication, and expressed higher levels of the cell cycle regulator WEE1 kinase, enabling more effective DNA repair before mitotic entry. These findings offer insights into the infrequency of mitotic chromosome segregation errors in mice and raise questions about the suitability of mice as an experimental model for human development (Palmerola et al., 2022).

The susceptibility of early embryos to DNA damage and its association with aneuploidy presents the need to understand the activation of DNA repair mechanisms throughout embryonic development. In a study carried out by Jaroudi et al. expression of DNA repair genes for all types of DNA repair was detected in human blastocytes, suggesting the ability for all pathways to be carried out: **base excision repair (BER)**, **double-strand break repair (DSBR)**- especially **homologous recombination**, **mismatch repair (MMR)**, and **nucleotide excision repair (NER)** (Jaroudi et al., 2009). However, one may note the lower levels of some DNA repair genes in the blastocyst as compared to the oocyte, probably due to the oocyte's role in maintaining genome integrity prior to EGA (Jaroudi et al., 2009; Khokhlova et al., 2020). Indeed, a study involving rhesus monkey embryos revealed the limited expression of a number of DNA repair proteins in the initial stages of embryo development, such as *CHECK2* and *OGG1* potentially hindering DSBR and BER activity, respectively. (Zheng et al., 2005). The significance of DNA repair proteins in development and the impact of their expression at lower levels in blastocysts can be evaluated by mutating repair genes. For example, mutating Flap endonuclease *Fen1 and Atr,* results in impaired blastocyst or inner cell mass (ICM) formation. Additionally, mutating genes like Poly(ADP-ribose)polymerase 1 (*Parp1*) combined with the downregulation of *Xrcc5 (Ku80)* or *Atm* leads to increased apoptosis in blastocysts (Zheng et al., 2005). Thus, we can conclude that the lower expression of certain DNA repair genes in the blastocyst, along with potential mutations in the expressed genes, may lead to inefficient DNA damage repair. Consequently, this may contribute to developmental defects throughout embryonic development, in which it is tempting to speculate the inclusion of aneuploidy (Khokhlova et al., 2020).

1.3 Cell Cycle Checkpoint Dysregulation as a Driver of Aneuploidy

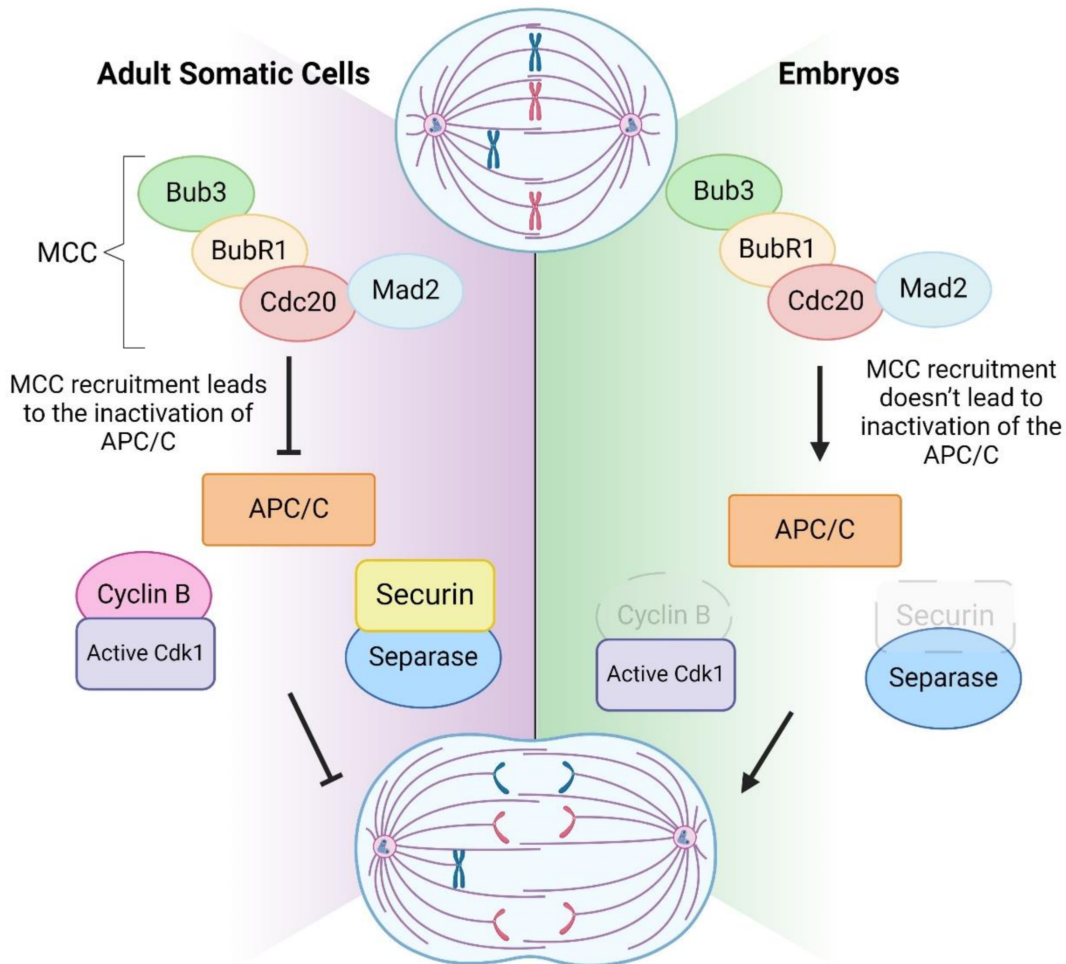
1.3.1 Weakened SAC

Successful cell division relies on a number of checkpoints, one such checkpoint being the mitotic or spindle assembly checkpoint (SAC). The SAC senses unattached chromosome kinetochores to spindle microtubules and halts mitotic progression, preventing entry into anaphase until thesei kinetochores are attached. (Chenevert et al., 2020; McAinsh & Kops, 2023). Advancement into anaphase is inhibited through the recruitment of SAC components which give rise to the formation of a complex called the **mitotic checkpoint complex (MCC)**, inhibiting a key regulator of the cell cycle, the **anaphase-promoting complex/cyclosome (APC/C)**. Following kinetochore attachment and thus error correction, the SAC is satisfied, and the APC/C is activated, marking the initiation of various processes which lead to mitotic exit (Chenevert et al., 2020).

Similar to somatic cells, the SAC mechanism is crucial for accurate mitosis in embryonic development, preventing premature anaphase initiation and varying chromosome numbers in daughter cells. The significance of the SAC in guiding embryos into anaphase is evident in its localization to kinetochores and coordinated 'on' to 'off' state transition (Wei et al., 2011). This is substantiated by experiments in which key components of the SAC, such as Monopolar spindle 1-like 1 kinase, Mad 2, Bub1 and BubR1 are perturbed, resulting in high chromosome segregation errors, reduced cell numbers, and/or apoptosis (Bolton et al., 2016; ncElowe, 2011; Kaplan et al., 2001; Schmid et al., 2014; Tilston et al., 2009; Wei et al., 2011). Interestingly, the SAC has been shown to exhibit weaker signalling in the early embryonic development of various species than in adult somatic cells, where the unattachment of one kinetochore is sufficient to activate the SAC and induce a mitotic pause (Figure 1.4). This may potentially play a role in the elevated incidence of aneuploidy, as was first demonstrated in *Xenopus laevis* blastomeres and zebrafish embryos which were treated with the microtubule depolymerizing drug nocodazole. This treatment gives rise to increased misaligned chromosomes at the metaphase plate due to the induced absence of a functional spindle apparatus (Clute & Masui, 1995; Ikegami et al., 1997). When *Xenopus laevis* blastomeres were treated with nocodazole, mitotic timing of the first 12 embryonic cycles was not delayed, as shown by analysis of division synchrony, mitotic index, and sensitivity (cellular response) of chromosome cycles to nocodazole (Clute & Masui, 1995). Likewise, treated zebrafish embryos displayed total destruction of all nuclei in the embryo’s deep cell layer before the midblastula transition (MBT), demonstrating that a metaphase arrest only occurs at this point (Ikegami et al., 1997). In both cases, the results suggest that the SAC was not immediately activated despite the presence of unattached kinetochores, giving rise to a

highly error prone mitosis (Clute & Masui, 1995; Ikegami et al., 1997). Such results contrast the application of nocodazole to somatic cells, such as HeLA cells, in which the SAC is activated, allowing a sufficient delay for error correction before anaphase is initiated (Douglas et al., 2020; Musacchio & Salmon, 2007). A similar experimental procedure was carried out by Chenevert et al. who demonstrated through mitotic progression monitoring that unattached kinetochores do not impede mitosis in fish, frog, amphioxus, and ascidian embryos, suggesting the absence of a fully functional, activated SAC. On the other hand, during the first cleavage division, sea urchin, mussel, jellyfish, nematode and insect embryos exhibited a prolonged delay in mitosis when spindle microtubules were absent mediated by a working SAC. This shows that a weakened SAC is a species-specific characteristic and is not an inherent feature of cleavage-stage fast division rates (Chenevert et al., 2020). Additionally, the examination of cell divisions in mouse embryos, confirmed by live imaging, revealed a notable portion advancing into the anaphase stage within the normal time frame despite the presence of misaligned chromosomes and the recruitment of Mad2/MCC by the chromosomes (Vázquez-Diez et al., 2019). Similarly, developmental arrest was not observed in response to the administration of the CENP-E inhibitor GSK923295 to murine embryos, which was surprising since CENP-E inhibition in somatic cells effectively activates the SAC, leading to mitotic arrest (Bennett et al., 2015; Ohashi et al., 2015). Such an inhibitor leads to multiple misaligned chromosomes while maintaining an intact spindle architecture, illustrating the limited ability of the SAC to prevent mitotic progression following chromosome misalignment in early mouse embryos (Vázquez-Diez et al., 2019). Overall, the above research demonstrate that embryos have a weakened SAC when compared to adult somatic cells, a factor likely to contribute significantly to the elevated rates of early aneuploidy observed in embryonic development.

Figure 1.4 – The SAC Exhibits Weak Signaling in Early Embryos



The SAC involves cyclin B1 and CDK1 which form a crucial complex essential for activating the anaphase-promoting complex/cyclosome (APC/C), and securin which plays a key role by inhibiting separase, an enzyme responsible for maintaining the cohesion between sister chromatids. In adult somatic cells, one unattached kinetochore is sufficient to activate the SAC, efficiently forming the mitotic checkpoint complex (MCC), inhibiting a key regulator of the cell cycle the APC/C, and stopping separase activation. This prevents entry into anaphase, allowing time for error correction. In embryos, kinetochore unattachment results in a much weaker SAC activation, and anaphase entry is not prevented. Here, the APC/C is activated despite MCC formation, cyclin B1 and securin are proteolytically cleaved, activated separase cleaves cohesin, and cyclin dependent kinase (CDK) 1 is inactivated.

1.3.2 The Importance of the CPC and Aurora C

The **chromosomal passenger complex (CPC)** is another important component of cell division, and it functions to correct chromosome-microtubule attachment errors. The CPC dynamically positions itself throughout mitosis, aligning with its involvement in other key processes including chromosome condensation, spindle assembly, communication with the SAC, and cytokinesis execution. The CPC is composed of four subunits: the inner centromere protein (INCENP), survivin, borealin, and Aurora B kinase. In mammals, the Aurora family kinases include Aurora A and Aurora C, with the latter arising from an Aurora B gene duplication event (Brown et al., 2004). In somatic cells, Aurora B kinase has been extensively explored and identified as the predominant player among the three kinases. It serves various roles, including stability of the bipolar mitotic spindle, promotion of chromosome bi-orientation via misattachment correction, cohesion between sister chromatids and at the centromere, and cytokinesis (Carmena et al., 2009). Although it was initially thought that early embryos followed a similar pattern, Aurora C has recently been a subject of focus (Kimmins et al., 2007). According to Kimmins et al., 2007, Aurora C not only induces aneuploidy when inhibited in HeLa cells but also restores the functions of Aurora B in mitosis after its inhibition. This suggests a possible mitotic role for Aurora C independent of Aurora B (Kimmins et al., 2007). Supporting data showed that Aurora C is the predominant Aurora kinase at prometaphase in zygotes, 2-cell, and 4-cell embryos. Conversely, Aurora B expression is either absent or present at notably lower levels until the 8-cell stage (Avo Santos et al., 2011). Based on the observed larger coverage area of Aurora C on zygotic prometaphase chromosomes versus the limitation of Aurora B to the centromeric regions, a complementary role between the two has been noticed, in which there is a gradual switch in the Aurora C to Aurora B ratio on day 4 of development (Avo Santos et al., 2011). Embryo IVF aneuploidy rates decline at a timepoint corresponding to the depletion of Aurora C mRNA and protein at the inner centromere, alluring the conjecture that high chromosomal segregation error rate is linked to Aurora C (Avo Santos et al., 2011; Santos et al., 2010). Alternatively, variations in the Aurora C/Aurora B ratio have been hypothesised to play a role in the high incidence of early embryo aneuploidy, substantiated by overexpression experiments of Aurora B and Aurora C which result in primary tumour development, polyploidy, and are observed in cancer cell lines (Avo Santos et al., 2011; Chen et al., 2005; Kimura et al., 1999; Tatsuka et al., 1998), Further research is required to understand the

accuracy of such speculations, and to confirm the potential causative links within these associations.

1.3.3 The Absence of a Mitotic Timer

We have previously seen that a prolonged mitosis, for example as mediated by the SAC, can be advantageous as it allows more time for error correction. However, studies have shown that an extended mitosis may also elevate the risk of errors, including erroneous chromosomal missegregation (Ghelli Luserna di Rorà et al., 2019; Shindo et al., 2021; Uetake & Sluder, 2010). To ensure precise mitotic timing for maximizing error correction while minimizing the risk of errors, somatic cells have shown to contain a 'mitotic timer' mechanism. This timer facilitates mitotic arrest in the subsequent G1 phase following a prolonged prometaphase of >1.5 hours (Dalton & Yang, 2009; Uetake & Sluder, 2010; Wong et al., 2015). Initial investigations demonstrated that an above-threshold prolonged mitosis triggers an irreversible p38- and p53-dependent block to daughter cell proliferation (Uetake & Sluder, 2010). However, further research has revealed that G1 phase arrest follows the activation of a signalling pathway involving USP28, 53BP1, p53 and p21 (Allais & FitzHarris, 2022). This mitotic timer is able to detect mitotic extensions lasting tens of minutes, and its presence has been proposed to be protective in nature, ensuring that erroneously divided cells do not continue to develop. Therefore, exploring the situation in early embryos and its contribution to aneuploidy becomes intriguing. A recent study has shown that by periodically arresting mitosis and subsequently releasing the arrest, mouse embryos at the two- to four-cell stage continue to divide normally following treatment for 6 hours (Allais & FitzHarris, 2022; Maliga et al., 2002; Sackton et al., 2014). Thus, an induced prolonged mitosis fails to arrest at the G1 phase in murine embryos. Moreover, the observed typical formation of the morula and then blastocyst together with comparable cell numbers and mitotic indices to those of control embryos, strongly indicate the absence of a robust mitotic timer mechanism in early embryos. Notably, cohesion fatigue is also observed here. During extended periods of mitosis, the spindle exerts sustained tension on the chromosomes, causing 'cohesion fatigue'. This occurrence is characterised by the loss of cohesion between sister chromatids, causing precocious chromatid separation (de Lange et al., 2015; Lara-Gonzalez & Taylor, 2012; Sapkota et al., 2018; Stevens et al., 2011; Worrall et al., 2018). This phenomenon, in conjunction with the lack of a mitotic timer mechanism, may contribute largely to the occurrence of aneuploidy in early embryos. (Allais & FitzHarris, 2022).

1.3.4 The Absence of RB and WEE1

Key proteins of the G1 and G2 cell cycle checkpoints, retinoblastoma protein (RB) and WEE1 respectively, have also been theorised to play a role in mitotic errors when erroneously synthesized. The G1 checkpoint is positioned towards the end of the G1 phase and just before S phase, and evaluates the favourability of environmental conditions for cell division. In the presence of unpropitious conditions, such as DNA damage or lack of growth factors, cells will arrest at this point. On the other hand, the G2 checkpoint stops cells from progressing to mitosis upon detecting DNA damage, which can arise either as a result of unrepaired DNA from the previous S or G1 phase, or from incomplete replication during the S phase (V. W. Yang, 2018). The G1 checkpoint is maintained by RB via the inhibition of E2F transcription factors. Cdk activity at the end of G1 inactivates RB, enabling the expression of cyclins E and A and the transition into S phase. Conversely, during the G2 phase, WEE1 kinase phosphorylates and in turn inactivates cyclin B-bound Cdk1. As cells approach the G2/M transition, WEE1's inhibitory influence is alleviated, allowing for the activation of Cdk1 and the progression into mitosis (Kiessling et al., 2009; Zhao et al., 2014). A 2009 study reported the silencing of RB and the suppression WEE1 on microarrays of two pools in 8-cell stage human embryos, and it was inferred that the canonical G1 and G2 cell cycle checkpoints may be inactive in totipotent human blastomeres (Kiessling et al., 2009). Because the roles of both checkpoints involve preventing the progression of highly error-prone cell divisions, the potential absence of these proteins in early development may be correlated with aneuploidy. Interestingly, the absence of RB and WEE1 in human embryos is mirrored by the lack of cell cycle checkpoints in early frog embryos, particularly in response to unreplicated DNA, DNA damage, or mitotic defects. These checkpoint are only later activated at the blastocyst stage in frogs (Carter et al., 2006; Pomerening, 2013). If this pattern extends to human embryos, RB and WEE1 could also become activated at the blastocyst stage, triggering programmed cell death in cells exhibiting karyotype abnormalities or DNA replication errors. Thus, it may be concluded that RB and WEE1 could be responsible for early cell aneuploidy prior to the blastocyst stage, cause the arrest of aneuploid cells at the blastocyst stage, and/or are simply not responsible for causing aneuploidy after the blastocyst stage (Hardy et al., 2003; Kiessling et al., 2010).

1.4 Supernumary Centrosome as a Result of Whole Genome Duplication

Aneuploidy can also occur as a consequence of multipolar mitosis which stems from supernumerary centrosomes. Centrosomes ensure accurate chromosome segregation during cell division, and their duplication, monitored by a regulatory checkpoint mechanism, takes place to provide each daughter cell with a single centrosome or MTOC. Molecular pathway aberrations, like mutations in centrosome duplication regulatory genes, may disrupt this process, leading to supernumerary centrosomes and irregular spindles, ultimately resulting in mitotic arrest or aneuploidy (Brinkley, 2001; Ganem et al., 2007). Other pathways may also contribute to the presence of **supernumary centrosomes**, including failure of cytokinesis or the fusion of two or more cells (blastomere fusion), contributing to over 50% of aneuploid embryos (Chavez et al., 2012; Hardy et al., 1993; Tšuiko et al., 2019).

1.4.1 Blastomere Fusion

Blastomere fusion, the less commonly described mechanism, is the merging of two or more blastomeres to form a single cell and has been observed in frozen-thawed embryos exhibiting a polyploid or diploid-polyploid mosaic phenotype (Balakier et al., 2000). Such a phenomenon has been thought to be caused by cell membrane changes, changes in pH, temperature, or osmotic pressure, or directly as a consequence of freeze/thawing. Nonetheless, it has been associated with treatment effects and currently, there is a lack of evidence to show that it could occur naturally in embryos (Mantikou et al., 2012).

1.4.2 Failed Cytokinesis

Failed cytokinesis, on the other hand, describes the incomplete division of the cytoplasm following division of the nucleus, resulting in the failure to make two separate daughter cells (Normand & King, 2010). A 1993 study, which examined the nuclei of blastomeres from 200 human cleavage stage preimplantation embryos, noted the frequent occurrence of binucleate blastomeres featuring two nuclei of equal size and concluded that the underlying cause was a failure of cytokinesis during the second, third, or fourth cleavage divisions, related to cleavage stage arrest (Hardy et al., 1993). Although the exact role or impact of binucleate blastomeres is still unclear, a clear link with low pregnancy success rates has emerged. Developmental arrest is evident from the coexistence of binucleate blastomeres at early

stages (four- and eight-cell) alongside mononucleate blastomeres at later stages (16- and 32-cell) within the same embryo. This observation suggests that these cells have maintained this state without progression for at least 48 hours (Hardy et al., 1993).

1.4.3 The Role of PLK4

It is interesting to mention Polo-like kinase 4 (PLK4), a protein kinase that plays a key role in regulating centriole formation during mitosis (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). It was found in mouse embryos that depletion of maternal Plk4 via an anti-Plk4 antibodies results in monopolar spindle formation and cytokinesis failure (Coelho et al., 2013). Moreover, there is evidence of a link between mitotic aneuploidy and genetic variants within the chromosome 4 region harbouring PLK4, implying that genetic or protein-level abnormalities of this gene could influence the accuracy of mitosis (McCoy et al., 2015). Additionally, Hudson et al. also demonstrated the need for Plk4 in late mitosis, specifically at telophase, as Plk4 null embryos on day 7.5 went into arrest and exhibited an increase in anaphase bridges (Hudson et al., 2001). Thus, the multifaceted role of PLK4 highlights its significance in ensuring an accurate mitosis, and thus its potential contribution to early embryo aneuploidy when its activity is aberrant.

2. The Fate of Embryos with Mosaicism and Aneuploidy

2.1 Self-Correction in Aneuploid Embryos

2.1.1 Reduction in Aneuploid Blastomeres as Embryonic Development Progresses

While we have discussed embryonic aneuploidies independently, this phenomenon is most commonly observed in the form of diploid-aneuploid mosaicism which affects 30%-70% of cleavage-stage embryos (Mertzanidou et al., 2013) and 5%-15% of blastocyst-stage embryos (Capalbo et al., 2013; E. Chavli et al., 2022; Johnson et al., 2010; Santos et al., 2010; Vanneste et al., 2009). Interestingly, it has been shown that the percentage of aneuploid and normal cells within a mosaic embryo varies-throughout embryonic development (Bielanska et al., 2002; McCoy, 2017). Analysis of the frequency of chromosomal mosaicism throughout embryonic development revealed almost all morula stage embryos to be mosaic, with a significant decrease in mosaicism by day 5, and further reduction by day 8 (E. B. Baart et al., 2007; Bielanska et al., 2002; Santos et al., 2010). Moreover, first-trimester diagnoses in **chorionic villi** also reveal mosaicism in only 1-2% of cases (Fritz et al., 2001; Hassold, 1982; Los et al., 2004; Warburton et al., 1978). At birth, the percentage of aneuploidy is remarkably low, approximately 0.3%, suggesting a gradual reduction of aneuploid cells over time (Hassold et al., 1996). However, this low percentage may also be due to the incompatibility of such aneuploidies with life. Aneuploidies perturb gene expression, result in cellular dysfunction, and have increased metabolic demands and oxidative stress, potentially leading to abnormal post-fertilization first or second cell divisions, which may contribute to the reduced viability of aneuploid embryos (McCoy et al., 2023). Nonetheless, mosaic preimplantation embryos show a decrease in the proportion of aneuploid cells as they progress towards the blastocyst stage, with almost total disappearance before the first trimester; this event is often referred to as aneuploid "self-correction" (Orvieto et al., 2020; Santos et al., 2010). However, this term is vaguely defined, with some authors referring to self-correction as the elimination of aneuploid cells via programmed cell death (Bolton et al., 2016; Regin et al., 2023; Singla et al., 2020; Yang et al., 2021), while others suggest the active correction of aneuploidy via a second mis-segregation event as suggested by the existence/generation of **monoparental diploidy** (Barbash-Hazan et al., 2009; Gueye et al.,

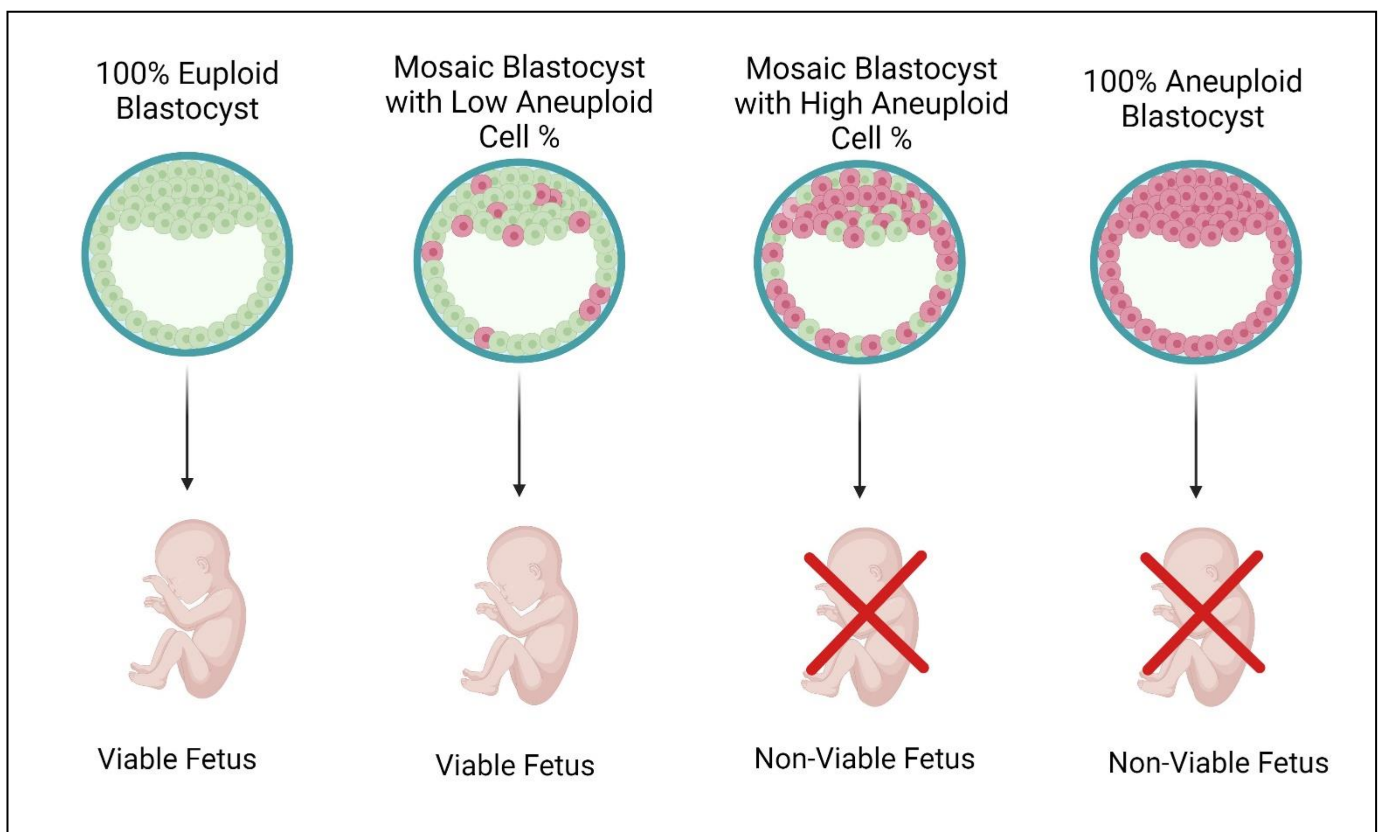
2014; Ivanova & Semenova, 2023; Los et al., 1998; Munné et al., 2005; Rubio et al., 2007). Other less frequently described mechanisms have also been mentioned (Amano et al., 2015; Orvieto et al., 2020).

2.1.2 The Diploid-Aneuploid Cell Ratio May Determine Viability

Interestingly, the diploid-aneuploid cell ratio of a mosaic embryo may be a crucial factor influencing normal foetal development and may offer valuable insights into the fate of mosaic embryos. Indeed, it has been observed that the success of a full-term pregnancy depends on the percentage of diploid blastomeres in mosaically aneuploid embryos (Figure 2.1). In a 2016 experiment, Bolton et al. generated murine aneuploid cells using the spindle assembly checkpoint inhibitor, Reversine. Interested in exploring early post-implantation rescue, they transferred 1:1 reversine-treated chimeras (aggregation chimeras created at the eight-cell stage that contained a 1:1 ratio of reversine-treated and control blastomeres) and control embryos into foster mothers, either recovering the embryos at E13.5 or allowing full-term development. They found that a similar portion of 1:1 chimera and control embryos developed to E13.5 (52.3% vs 58.8%). Similarly, 13 live pups were born out of the 26 implanted 1:1 chimera. Interestingly, coat fur analysis revealed that reversine-treated aneuploid cells were either reduced or completely depleted in 66% of the embryos collected at E13.5, and among the fully developed mice, 7 out of 13 showed no evidence of the reversine-treated clone. This demonstrates early post-implantation rescue, indicated by the observed depletion of aneuploid cells at E13.5, which potentially correlates with successful further development, as evidenced by the absence of the reversine-treated clone in the fully developed mice. Moreover, the possibility of aneuploidy rescue is illustrated, indicating the presence of protective mechanisms, and suggesting euploid-aneuploid mosaic embryo viability (Bolton et al., 2016). To analyse whether such observations can also be seen in human embryos, Yang et al. constructed a mosaic gastruloid model using RUES2 human embryonic stem cells (ESCs) with Reversine. Three different ratios of reversine-treated gastruloid to control cells were used (1:3, 1:1, and 1:3) to assess whether euploid cells are able to rescue the lineage formation and spatial patterning of partly aneuploid gastruloids. They found that gastruloids with normal self-organisation of embryonic and extraembryonic germ layers could develop from all three ratios, including mosaics containing 50% or 75% aneuploid cells. Thus, even a small proportion of euploid cells are able to give rise to normal self-organizing radial patterns, a result that is consistent with the previously mentioned

murine studies (M. Yang et al., 2021). Furthermore, in 2015, the transfer of mosaic IVF embryos resulted in eight clinical pregnancies, from which six healthy babies were born. Sampling of the chorionic villi confirmed that all embryos had a normal karyotype. This best exemplifies the fact that the ratio of diploid to aneuploid cells plays a pivotal role in determining embryo viability (Greco et al., 2015). Although a definite threshold hasn't been established due to the influence of various factors on viability, M. Yang et al. showed that gastruloids can develop even with 75% of starting aneuploid cells, indicating that embryo models with over 50% aneuploid cells can still be viable (M. Yang et al., 2021)

Figure 2.1 – The Diploid-Aneuploid Cell Ratio May Determine Viability



The diploid to aneuploid cell ratio in mosaic embryos is a crucial determinant of viability and may influence the success of pregnancy. Whilst a low ratio of aneuploid cells (red) may not influence the pregnancy outcome, a high ratio of over 75% aneuploid cells may give rise to a non-viable fetus. A completely aneuploid blastocyst is generally incompatible with life. Exceptions to this case include trisomy of chromosomes 13, 18, and 21, which give rise to Patau syndrome, Edwards syndrome, and Down syndrome, respectively, and sex chromosomes.

2.2 Mechanisms of Self-Correction

While the existence of a certain level of selection against aneuploidy has been observed by the reduction in aneuploid blastomeres as embryonic development progresses (Bolton et al., 2016; M. Yang et al., 2021), it is necessary to understand the potential mechanisms by which this is occurring. In this regard, we will discuss three proposed underlying mechanisms: apoptosis of aneuploid cells, preferential allocation at the trophectoderm (TE) vs inner cell mass (ICM), and trisomic rescue (Figure 2.2), followed by less frequently mentioned mechanisms of correction.

2.2.1 Apoptosis

Apoptosis, defined as programmed cell death, has been suggested as a mechanism enabling the selection against aneuploid cells. The generation of euploid and aneuploid murine chimeric embryos revealed that 30.9% of all ICM cells display distinctive apoptotic morphological features characterised by apoptotic cell disintegration followed by cellular debris engulfment by neighbouring cells (Bolton et al., 2016). Notably, the ICM is one of the cell lineages that arise at the blastocyst stage and is the only lineage that gives rise to the development of the **embryo proper**. The TE lineage, on the other hand, gives rise to the extra-embryonic parts, including most of the placenta and the embryonic membranes (Xenopoulos et al., 2012). Importantly, upon comparison of apoptosis rates between abnormal and control clones, a significantly higher rate of apoptosis was observed in aneuploid clones (41.4% vs 19.5%), with a notable increase in ICM apoptosis when compared to TE apoptosis. This indicates a high rate of apoptosis in aneuploid cells specifically, as well as preferential apoptotic elimination of aneuploid cells in the fetal lineage as opposed to the placental lineage in murine embryos. (Bolton et al., 2016). The identification of aneuploid cells within the ICM was possible by generating chimeras using embryos that had been injected with Tomato-RFP messenger RNA, thus making it possible to distinguish the abnormal clone (red) from the control clone. Similar results were also demonstrated in human gastruloids, in which aneuploidy is tolerated in the TE-like cells, but aneuploid cells in the post-gastrulation embryonic germ layers are eliminated by apoptosis,(M. Yang et al., 2021). This phenomenon, termed preferential allocation, will be discussed in detail below. Singla et al. employed a similar methodology to Bolton et al. and achieved consistent results, but went further to propose that the observed apoptosis in both

the ICM and TE lineages occurs in a p53-dependent manner, involving autophagy. Although there is evidence suggesting that p53 is inactive in early blastocysts (Jaiswal et al., 2020, 2021), the transcriptional upregulation of the p53 pathway members, specifically *p53*, *p21* and *cyclin G1* in reversine-treated aneuploid blastocysts was observed. Such a finding coincides with a significant accumulation of the autophagic marker LC3B in the **epiblast** following treatment with the proteasome inhibitor MG132, indicating autophagy upregulation (Singla et al., 2020). Similarly, in human preimplantation embryos, lower cell numbers and increased apoptosis were seen in aneuploid TE cells when compared to those in euploid TE cells. Although Oct4-positive ICM/Epiblast (EPI) aneuploid cells were also seen in lower numbers that in euploid cells, they did not exhibit signs of apoptosis, as indicated by CASP3/7 negativity (Regin et al., 2023). This is in contrast with results from previous mouse and gastruloid models, possibly due to species-related differences in ICM/EPI cell properties. It is also speculated that the absence of apoptosis in human ICM/EPI cells is a result of inefficient communication between aneuploidy-induced stress signals and apoptosis, similar to what is seen in human embryonic stem cells (ESCs) where the SAC does not initiate apoptosis as it typically does in somatic cells. (Mantel et al., 2007). Additionally, the lower cell numbers seen in the ICM/EPI of aneuploid embryos may be due to apoptosis-independent processes, including a p53-mediated decrease in cell proliferation, and the p53-mediated downregulation of *OCT4* and *NANOG* expression which drives cell differentiation (T. Lin et al., 2005; Regin et al., 2023).

2.2.2 Preferential Allocation of ICM vs TE

Therefore, we have established the high probability that apoptosis plays a role, at least to some extent, in eliminating aneuploid cells during the early stages of embryo development. However, it is interesting to note the lineage-specific response to aneuploidy, which underlies the second mechanism of aneuploid cell self-correction: preferential allocation of aneuploid cells at the ICM vs at the TE. The progenitor cells for both these compartments are blastomeres, which in their early stages have begun to commit to a specific lineage, maintaining limited plasticity (Wigger et al., 2017). Nonetheless, blastomeres committed to the TE lineage are more likely to contribute to the extraembryonic tissues, whereas those committed to the ICM lineage are more predisposed to form the embryo proper (Marikawa & Alarcón, 2009; Zernicka-Goetz et al., 2009). It has been suggested aneuploid cells may receive different signalling cues or environmental influences that bias their allocation towards

one lineage over the other. This theory implies that euploid cells are preferentially allocated to the ICM during the cleavage stage of development while aneuploid cells are oriented towards the extra-embryonic compartments, the TE, which is less crucial for implantation or post-implantation development. In this way, any negative effects of aneuploidy to the foetus may potentially be mitigated (Ivanova & Semenova, 2023; Kalousek, 2000). However, limited evidence supports this hypothesis. In one of a few supporting studies, Kalousek & Dill presented pioneering experimental data. They cultured and cytogenetically analysed 46 placental and foetal tissues, concluding that chromosomal mosaicism is confined to placental chorionic tissue in humans. This means that aneuploid cells were exclusively observed in the placenta, an occurrence that may be attributed to preferential allocation. Despite such observations, it is important to recognise that they may not be the result of a preferential allocation in response to aneuploidy. Firstly, the embryo proper is developed from only three or four mammalian blastomeres which could happen to be diploid by chance in a mosaic embryo. Secondly, the placenta has a greater number of cell progenitors compared to the foetus, making the occurrence of a mosaic placenta more likely than that of a mosaic foetus (E. A. Chavli et al., 2024; Kalousek & Dill, 1983). Moreover, the pioneering study has since been significantly challenged and has been reevaluated with additional context in light of a condition called confined placental mosaicism (CPM) which exists in only ±2% of pregnancies. Observations in contrast to the theory of preferential allocation have been made by Evsikov & Verlinsky who observed through blastocyst immunosurgery and FISH analysis for chromosomes 13, 18, and 21, that the degree of aneuploidy within the ICM is comparable to the degree of mosaicism in the blastocyst as a whole, and is not depleted. This suggests that euploid cells are not being selected for in the ICM, and aneuploid cells are not being selected against. However, this study specifically analysed three chromosomes that, although are associated with human developmental disorders when trisomic, are the only autosomal trisomies compatible with life (Patau syndrome, Edwards syndrome, and Down syndrome, respectively) Thus, we may question whether similar results would be obtained when analysing chromosomes that are associated with non-viable aneuploidy (Evsikov & Verlinsky, 1998). Similarly, Derhaag et al., employed fluorescent in-situ hybridization (FISH) to analyse the karyotype of the chromosomes X, Y and 18 which are, again, compatible with life, in the ICM and TE. This followed *in vitro* ICM/TE differentiation via *in situ* nuclei labelling with polynucleotide-specific fluorochromes (Derhaag et al., 2003; Handyside & Hunter, 1984). In both cell type populations, normal and abnormal (monosomic and trisomic) nuclei were detected, revealing no significant differences between the mean

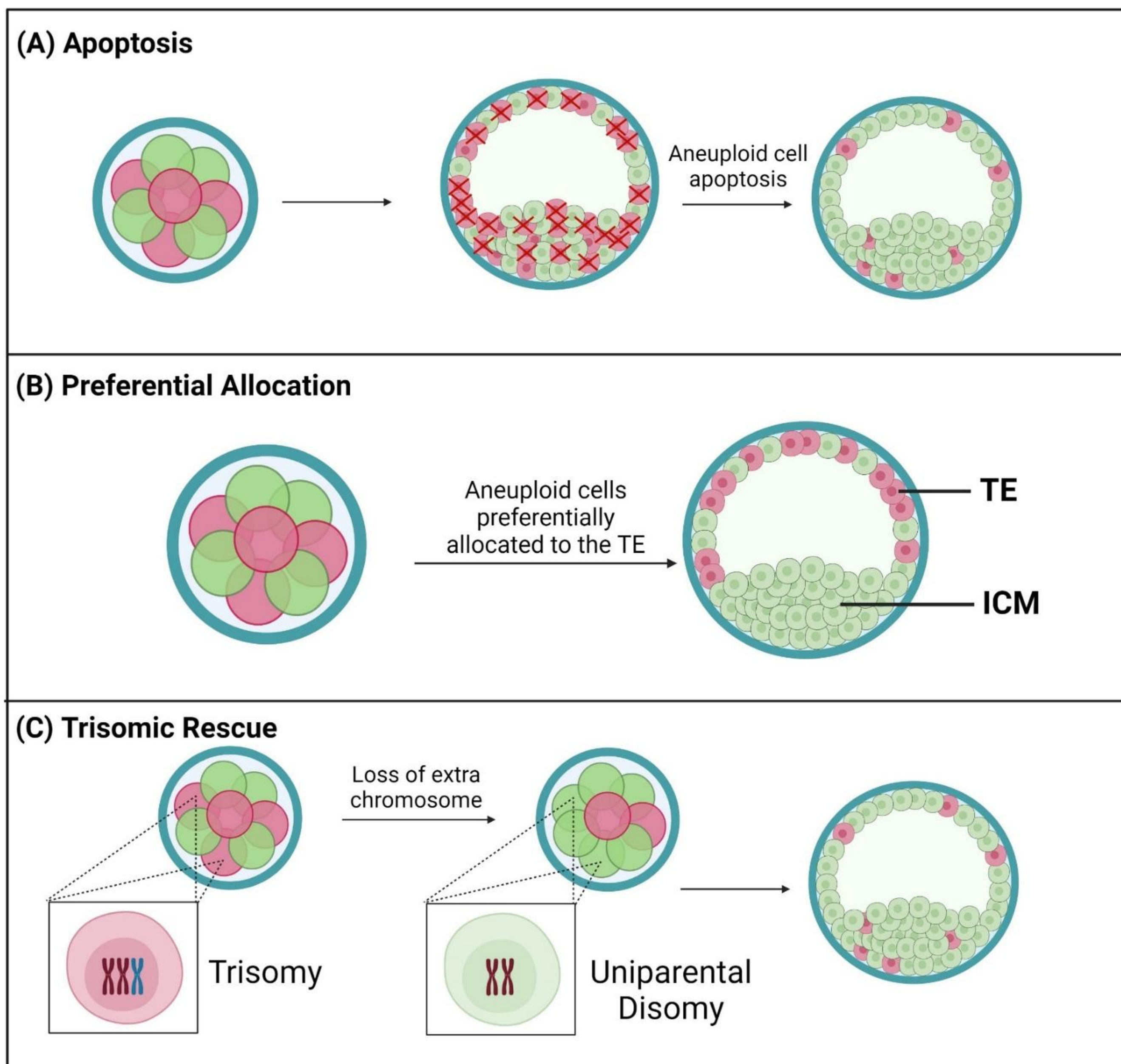
percentages of normal cells in the ICM and TE, or the distribution of aneuploid cells between the two compartments. Such results show that the ICM and the TE contain comparable numbers of cells with chromosomal abnormalities, thus challenging the idea of a preferential allocation mechanism (Derhaag et al., 2003). This is in contrast with findings by E. A. Chavli et al., who applied single-cell whole genome sequencing to the TE and ICM of good quality blastocysts. They found that although there was no demonstration of preferential allocation of abnormal cells to either the TE or ICM, an increase in complex aneuploidy in the TE was observed (E. A. Chavli et al., 2024). This discrepancy could potentially be explained by variations in mitotic error timing, chromosome-specific differences involving segregation error bias, and selective pressures on certain chromosomal abnormalities, amongst others. Therefore, the theory of preferential allocation is still up for debate. Although further research is required to reach a definitive conclusion, my proposition is that any sort of observed aneuploid cell enrichment away from the ICM is most likely a result of apoptosis and not an active allocation mechanism, given the limited evidence supporting the latter.

2.2.3 Trisomic Rescue

Trisomic rescue has also been suggested as a method of aneuploid self-correction and describes the loss or removal of one chromosome from a trisomy, allowing the cell to return to a normal diploid state. Such a process may occur in the cells making up the whole blastocyst, but must at least be carried out in the cells that will form the foetus proper (Los et al., 1998). Trisomic rescue is suggested to be evidenced by **uniparental disomy (UPD)**, a genetic phenomenon in which both copies of a chromosome are inherited from one parent (Shaffer et al., 2001). In such a situation, it can be speculated that a third chromosome inherited from the other parent was lost or inactivated during development in an attempt to achieve diploidy. Having said that, uniparental disomy (UPD) typically arises from a meiotic error from both the egg and the sperm (Robinson, 2000), which contrasts with the concept of trisomic rescue. Moreover, it has been argued that if such a corrective mechanism was in place, the prevalence of uniparental disomies should be high enough to reflect this. It has been shown that they occur very rarely, constituting a mere 0.06% in the human blastocyst (Gueye et al., 2014). Yet, the fact that the rate of UPDs continues to increase as embryonic development progress, leaves room for possibility, and thus we cannot exclude this hypothesis. In support of the trisomic rescue mechanism, Barbash-Hazan et al. carried out a self-correction investigation by reanalysing aneuploid or mosaic day 3 embryos on day 5.

They found that 41% of all trisomic embryos underwent self-correction by day 5, indicated by the presence of >50% normal cells via FISH, likely due to a mechanism of trisomic rescue. However, it is important to keep in mind that this observation may also be explained by aneuploid cell apoptosis, aneuploid cell expulsion, and potential takeover by euploid cells, amongst other mechanisms. Therefore, to reach a more accurate and detailed conclusion, apoptosis could also be examined, particularly at advanced stages, by investigating apoptotic markers. The correction of 41% of trisomic cells was a marked contrast to the 9.7% of other aneuploid cells that underwent self-correction, including monosomies, diploid-aneuploid mosaics, and multiple aneuploid blastomeres (MABs). Such results may indicate the existence of trisomic rescue by showing that trisomic embryos are able to self-correct at a higher rate than other aneuploidies, although other explanations, such as the ability of the embryo to eliminate trisomic cells more efficiently than other aneuploid cells, should also be considered (Barbash-Hazan et al., 2009). The theory of trisomic rescue is further substantiated by the fact that trisomic embryos produce a higher number of blastocysts than any other aneuploid embryos like monosomies, combined monosomies and trisomies, and mosaics, an observation that is in line with what is seen in products from spontaneous abortions. Here, single trisomies are amongst the most successful abnormalities in reaching the blastocyst stage, as opposed to monosomies, haploidies, and polyploidies. However, it is well known that trisomies are generally tolerated better than monosomies, adding to the complexity of the discussion (Torres et al., 2008). Nonetheless, an ongoing process of trisomic rescue may still be suggested in which some trisomic cells are actively correcting themselves in order to reach the blastocyst stage (Rubio et al., 2007). The mechanism by which trisomic rescue could be occurring is not yet known (Barbash-Hazan et al., 2009). Some have proposed the idea that a second mitotic error is taking place during the initial postzygotic cell divisions, possibly in the form of anaphase lagging or nondisjunction, in order to correct the initial mistake. However, there is no evidence to support this (Munné et al., 2005). In such a scenario, it is thought that the trisomal aneuploid cell would undergo another round of mitosis, and whilst two of the chromosomes would proceed through mitosis normally, the third would mis-segregate into a micronucleus and thus not integrate into the nuclei of the daughter cells. In any case, all ideas are poorly supported, and there's no evidence showing higher mis-segregation rates for a trisomic chromosome to validate this as an active correcting mechanism. Thus, I harbour scepticism regarding the existence of this mechanism. Nonetheless, there is need for further research to explore the various mechanisms by which a potential trisomic rescue could be occurring (Ivanova & Semenova, 2023).

Figure 2.2 – Mechanisms of Self-Correction



The reduction in aneuploid blastomeres as embryonic development progresses may be explained by one of three proposed mechanisms. (A) Apoptosis of aneuploid cells (red), allowing for the dominance and proliferation of euploid cells (green). (B) Preferential allocation of aneuploid cells to the extra-embryonic compartments, the TE, which doesn't contribute to the formation of the embryo proper. (C) Trisomic rescue, which describes the loss of one chromosome in a trisomic cell, is a phenomenon suggested to be substantiated by the existence of uniparental disomy. The proposed mechanism underlying trisomic rescue involves a second missegregation event, such as anaphase lagging or nondisjunction. As of now, there is no available data confirming the occurrence of such a mechanism.

2.2.4 Other Mechanisms of Correction

Above, the most commonly proposed mechanisms of aneuploid self-correction have been described. However, alternative mechanisms have also been suggested. In a 2020 study, Orvieto et al. observed the ability of human embryos to eliminate and expel aneuploid blastomeres as cell debris/ fragments, which were analysed using whole genome amplification and array-based CGH. Some cell blastomeres and their expelled debris had corresponding karyotype statuses, being either both euploid or both aneuploid. However, it's important to highlight that among 11 blastocyst pairs, 7 expelled debris with additional chromosomal rearrangements, and out of 9 euploid blastomeres, 5 demonstrated aneuploid cell debris. Thus, many euploid cells expel karyotypically abnormal debris, suggesting that they are expelling the aneuploid cell out. This indicates the capability for self-correction via aneuploid cell expulsion, a mechanisms distinct from apoptosis. (Orvieto et al., 2020). Separately, Zscan4 has been a gene of interest in the correction of aneuploid embryos. It has shown to be crucial for embryo preimplantation, particularly during its brief expression window in the late 2-cell stage and the early/mid 4-cell stage. During this period, Zscan4 is important for genomic stability in ES cells where it binds telomeres and regulates telomere elongation (Falco et al., 2007). Aneuploidy correction has emerged as a speculative function of the ZSCAN4 protein based on a study demonstrating an increase in euploid cells among cultured aneuploid cells after overexpression of the protein through synthetic mRNAs and Sendai viral vectors that encode human ZSCAN4. This study also demonstrated diploidy in 24% of Down syndrome (trisomy 21) human fibroblast cells following overexpression of ZSCAN4 in the cultured cells, with similar results obtained for trisomy 18 cells of Edwards syndrome (Amano et al., 2015). The mechanisms underlying ZSCAN4's suggested corrective properties remain purely speculative. One possibility is that ZSCAN4 is able to directly correct karyotype anomalies during cell replication. Alternatively, ZSCAN4 might eliminate aneuploid cells or suppress their proliferation, enabling the dominance of euploid cells. Molecularly, a correlation was made between telomere elongations and ZSCAN4. This association was made due to observed telomere elongation in human fibroblast cells following treatment with ZSCAN4, indicating that the protein may be involved in regulating telomeres. Importantly, telomeres play a role in maintaining chromosome integrity during cell divisions and thus may ensure genomic stability (Zalzman et al., 2010). ZSCAN4 has also been proposed to be able to identify unpaired chromosomes during meiosis or mitosis and disconnect them from the replication apparatus (Amano et al., 2015).

Despite the speculative nature of ZSCAN4's functions, such experimental results indicate that it may be of value to conduct further research involving ZSCAN4 in early human aneuploid embryos. This could involve determining the presence of the protein and, if so, identifying the developmental stage, placing particular emphasis on the 2-cell (2C) stage. This is because Zscan4 is known to be present at this stage of mouse embryonic development (Falco et al., 2007). Interestingly, this is also the stage when mitosis is most erroneous (Mantikou et al., 2012) Moreover, such research could unveil whether ZSCAN4 plays a functioning role in early embryos, specifically in aneuploid self-correction.

Conclusion

This review explores the prevalence of aneuploidy in early-stage embryos, unravelling the interconnected underlying causal mechanisms while also describing the potential fates that these embryos may encounter. However, to accurately interpret the provided data, it is crucial to consider the potential impacts of *in vitro* procedures on the incidence of embryonic aneuploidy. Indeed, the possibility to obtain such data on embryos has been facilitated by IVF procedures, but such a technique has been suspected to induce observed mitotic errors (Munne et al., 1997). Comparative data has revealed varying rates of embryonic aneuploidy and mosaicism between different IVF centers, and it has been shown that changes in culture protocols including temperature fluctuations, oxygen levels, culture medium, and hormonal stimulation regimes may influence spindle assembly and chromosome segregation (Mantikou et al., 2012; Munne et al., 1997). Additionally, the use of bulk DNA sequencing in embryonic aneuploidy-related research has been shown to overlook aneuploidy due its high detection threshold, resulting in inaccurate data and faulty conclusions. However, the introduction and rising popularity of single-cell DNA sequencing has started to successfully address such shortcomings (E. A. Chavli et al., 2024). Separately, the impact of ovarian stimulation has also been widely recognised and it is thought to induce chromosomal abnormalities while decreasing embryo quality (E. Baart et al., 2007). However, there are multiple approaches to ovarian stimulation, each having a different risk of inducing aneuploidy. For example, a comparison between two ovarian stimulation protocols, the GnRH agonist long protocol against the GnRH antagonist protocol, revealed that the latter is associated with a higher rate of aneuploidy in aborted material and blastocysts. This could potentially give additional insight to the observed varying rates of aneuploidy in IVF embryos (J. Wang et al., 2022). Moreover, such observations prompt one to question the reliability and comparability of the data, a concern reinforced by the variation in technology being used and the different model systems being employed. Therefore, there is a need for increased standardisation within the field to truly propel progress.

In an attempt to increase the success rates of IVF, preimplantation genetic screening (PGS) has been introduced, a procedure that involves FISH analysis of one of two blastomeres obtained from a biopsy of a day 3 embryo, which would consist of around 6-10 cells (N. Wang et al., 2021). However, the ability of PGS to provide valuable insights has recently been questioned (E. B. Baart & Van Opstal, 2014; Fragouli & Wells, 2012). Throughout this

review, we have demonstrated that aneuploidy predominantly manifests as diploid-aneuploid mosaicism in embryos. This implies that if by chance, a diploid cell is biopsied from a mosaic embryo with a high proportion of aneuploid cells, such an embryo may be transferred for implantation despite the high likelihood of unviability or congenital disorders (E. B. Baart & Van Opstal, 2014; van Echten-Arends et al., 2011). PGS also allows for the opposite scenario – if an aneuploid cell is biopsied from an embryo with a high proportion of diploid cells, it may result in the discarding of an embryo that would have otherwise been viable. It has thus been proposed that in order to enhance accuracy, blastocysts should be analysed at a later developmental stage, at which point aneuploid proportions may be lower and a more reliable assessment can be made (E. B. Baart & Van Opstal, 2014; van Echten-Arends et al., 2011).

Therefore, it becomes apparent that the complete depiction of embryonic aneuploidy is quite complex, with multiple factors contributing to our understanding of how embryos cope with aneuploidy at various stages of development and the outcomes of affected embryos. Further research with increased standardisation will continue to uncover current shortcomings in IVF procedures and encourage continued advancements.

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Glossary

Anaphase bridges: DNA threads that stretch between separating sister chromatids during mitotic anaphase.

Anaphase-promoting complex (APC/C): An E3 ubiquitin ligase essential for the initiation of anaphase.

Aneuploidy: The deviation of chromosome numbers from a multiple of the haploid set.

Assisted reproductive techniques (ART): All fertility treatments that involve eggs, sperm or embryos.

Aster: A star-shaped radial array of microtubules radiating from a centrosome during mitosis and meiosis. The functions of the aster include organizing and positioning the mitotic spindle apparatus.

Base excision repair (BER): A DNA repair mechanism that corrects damaged DNA bases or single-strand DNA breaks, typically arising from spontaneous DNA damage or exposure to environmental alkylating agents.

Centriole maturation: The process by which daughter centrioles undergo structural and functional changes at the end of S phase, specifically the removal of CEP120 and centrobin, and the recruitment of daughter centriole maturation proteins (DCMPs) TALPID3 and C2CD3, to become fully functional during cell division.

Chorionic villi: Tiny projections of placental tissue that are considered the functional units of the placenta, and contain a capillary network derived from fetal circulation.

Chromatin remodelling: The modification of chromatin architecture from a condensed state to a transcriptionally accessible state, thereby facilitating gene expression.

Chromosomal Instability (CIN): An elevated rate of chromosome segregation error during cell division.

Chromosomal passenger complex (CPC): A multi-protein complex whose primary function is to correct chromosome-microtubule attachment errors by localizing to the centromeres and kinetochores of chromosomes during mitosis and meiosis. Additionally, the CPC is also involved in other key cell division processes including chromosome condensation, spindle assembly, communication with the SAC, and cytokinesis execution.

Chromosome segregation: The separation of chromosomes into daughter cells during cell division.

Cytoplasmic lattices: Twisted fibers made up of individually stacked filaments, composed of PADI6 and SCMC proteins.

Dispermic penetration: The penetration of the oocyte by two spermatozoa.

DNA damage response (DDR): A series of signalling pathways that involve a network of genes that are activated in response to various types of DNA damage. This response encompasses machinery facilitating DNA repair, regulating the cell cycle, addressing replication stress, and initiating apoptosis.

Double-strand break repair (DSBR): The repair of DNA double-strand breaks using two major pathways: homologous recombination and nonhomologous DNA end joining (NHEJ).

Embryo epigenetic reprogramming: The erasure of gametic epigenetic patterns, essential for the embryo to establish a new epigenetic profile crucial for early development and the developing conceptus.

Embryo proper: The parts of the conceptus that will develop into the fetus. This term excludes the extraembryonic tissue.

Epiblast: One of two distinct cell lineages that arises from the ICM and is the pluripotent primary lineage that will form the three primary germ layers (ectoderm, definitive endoderm, and mesoderm) and the extraembryonic mesoderm of the visceral yolk sac, the allantois, and the amnion during gastrulation.

Euploidy: A karyotype with a chromosome number that is an exact multiple of the basic chromosome sets.

Folliculogenesis: The maturation of the ovarian follicles within the ovarian somatic cells, culminating in the formation of a viable and fertilizable egg.

GADD45: The growth-arrest- and DNA-damage-inducible 45 gene family is composed of three highly homologous small, acidic, nuclear proteins: GADD45α, GADD45β, and GADD45γ. These proteins play key roles in DNA repair regulation, cell cycle control, and apoptosis.

H1: A linker histone and one of the five main histone protein families. It functions in establishing the compaction state of nucleosomes and organizing them into higher-order chromatin structures.

Homologous recombination: A DNA repair pathway that acts on DNA double-strand breaks and interstrand cross-links (ICL). This pathway involves the invasion of

undamaged DNA molecules by damaged ones with nearly identical sequences, resulting in the restoration of the damaged region using the undamaged molecule as a template for resynthesis.

Inner cell mass (ICM): The other cell lineage that arises at the blastocyst stage and the only lineage that gives rise to the development of the embryo proper.

K36me2 and H4K16Ac: Specific histone modifications that play roles in chromatin structure and gene regulation.

Maternal-to-embryonic transition (MET): An early developmental phase during which the embryo gradually lessens its dependence on inherited maternal factors. This process involves embryonic genome activation (EGA) and maternal gene transcript degradation.

Merotelic attachments: A mitotic error that involves the attachment of a single kinetochore to microtubules emanating from both spindle poles.

Micronuclei: Small, extra-nuclear membrane-bounded compartments that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the primary nucleus during cell division.

Microtubule-organizing center (MTOC): A morphologically diverse structure, often located near the centrosome, where microtubules organise and undergo nucleation following depolymerisation.

Mismatch repair (MMR): A DNA repair system that corrects spontaneous base-base mismatches and small insertion-deletion loops (indels) that tend to arise during DNA replication as a result of misincorporation errors.

Mitotic checkpoint complex (MCC): A protein complex composed of the mitotic spindle assembly checkpoint proteins Mad2, Mad3 and APC/C co-activator protein Cdc20. The assembly of the MCC is catalyzed by unattached kinetochores, and it serves to pause mitosis until errors are corrected. It is a highly potent inhibitor of the Anaphase Promoting Complex/Cyclosome (APC/C).

Monoparental diploidy/ uniparental disomy: A genetic phenomenon in which both copies of a chromosome are inherited from one parent.

Mosaicism: The phenomenon in which both normal and aneuploid cells coexist within the same embryo.

Non-obstructive azoospermia: A complete lack of sperm in the ejaculate as a result of spermatogenesis failure. It is the most severe form of male infertility.

Nucleotide excision repair (NER): A major excision repair pathway that functions to remove major helix-distorting DNA lesions, such as UV-induced damage and bulky chemical adducts in a multistep 'cut and patch'-type reaction.

Oogenesis: Differentiation of the ovum and formation of the female gametes.

Oxidative stress: Imbalances between the cellular production of reactive oxygen species (ROS) and the organism’s capacity to detoxify them.

Peri-centromeric heterochromatin: A specific form of constitutive heterochromatin found on both sides of the centromere, creating silent compartments abundant in repressive marks.

Protamines: Specialized structural proteins found in sperm cells, characterized by their small size and high content of the amino acid arginine. These proteins play a crucial role in ensuring spermatozoa quality.

Replication stress: The presence of external and internal stress sources that can lead to disruptions in replication fork progression, diminished replication accuracy, and the occurrence of DNA breaks.

Spermatozoa immaturity: Testicular spermatozoa that have not fully developed, and may lack motility, normal morphology, or the ability to penetrate and fertilize the egg.

Spindle Assembly Checkpoint (SAC): A mitotic checkpoint that senses unattached chromosome kinetochores to spindle microtubules and halts mitotic progression, preventing entry into anaphase until errors are corrected.

Subcortical maternal complex (SCMC): A multiprotein complex expressed in mammalian oocytes and early embryos that is composed of a minimum of four proteins: oocyte expressed protein [OOEP; also known as FLOPED]; NLR family, pyrin domain containing 5 [NLRP5; also known as MATER]; transducin-like enhancer of split 6 (TLE6); and KH domain-containing protein 3 [KHDC3; also known as FILIA]. It plays key roles in the maternal-to-embryo transition including meiotic spindle formation and positioning, translation regulation, organelle redistribution, and epigenetic reprogramming.

Subtelomeric regions: Repeated elements typically composed of highly variable DNA sequences immediately adjacent to the telomeres, which are found at the ends of chromosomes.

Supernumary centrosomes: Extra centrosomes beyond the normal complement found in a cell (one pair of centrosomes each containing a pair of centrioles) caused by centrosome amplification, potentially disrupting orderly cell division.

Testicular sperm extraction (TESE): A procedure used to diagnose the cause of azoospermia (lack of sperm in the ejaculate) which involves taking a testicular biopsy from which sperm may be retrieved for assisted reproductive techniques.

Totipotent cell: A stem cell that can give rise to an entire organism, including extra-embryonic tissue.

Tripolar spindle: The formation of three spindle poles instead of the typical two, generally as a result of erroneous spindle pole assembly or positioning.

Trophectoderm (TE): One out of the two cell lineages that arise at the blastocyst stage. The TE gives rise to the extra-embryonic parts, including most of the placenta and the embryonic membranes.

Zona pellucida: A thick specialized area/coat that surrounds all mammalian oocytes.