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Abnormal cleavage and developmental arrest of human preimplantation embryos in vitro

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A B S T R A C T

Despite improvements in culture conditions and laboratory techniques still only about 50% of human embryos reach the blastocyst stage of development in vitro. While many factors influence embryo development, aberrant cleavage divisions have only recently been shown to directly affect the genome in individual cells of human embryos resulting in chromosome loss, mosaicism and cell arrest. In this article we review the current literature in the area of aberrant cleavage in human embryos and its effect on blastocyst development. Further to this, we propose a series of common abnormal cleavage events, with particular attention to timing and frequency, and illustrate how these might influence a number of different embryo fates.

1. Introduction

The introduction of various assisted reproductive technologies (ART) has been instrumental in improving pregnancy rates and live births in patients presenting with infertility. Current live birth rates per IVF treatment cycle are considerably higher when compared with the early years of IVF, but the fact remains that overall, success rates still remain low (Rødgaard et al., 2015), with live birth rates around 21% in 2016 (HFEA, 2016). High rates of developmental arrest partly account for the observed low success rates as only about 50% of embryos develop to the blastocyst stage in spite of improvements in culture media, laboratory equipment and techniques (Summers and Biggers, 2003; Biggers and Summers, 2008; Sfontouris et al., 2016). Chromosome aneuploidy, or incorrect chromosome copy number, in the early preimplantation embryo is a critical factor in determining IVF success as it results in either implantation failure, early pregnancy loss or chromosomally abnormal ongoing pregnancies. The main challenge for clinical embryologists is thus the selection of the "best" euploid embryo with the highest potential for implantation and successful post-implantation development. The difficulty lies in the fact that currently, there is no means of visualising the embryos' chromosomes non-invasively, and so embryologists must rely on educated guesses based on multiple scoring systems for identifying the embryos with the likely highest potential. The relationship between aneuploidy and embryonic arrest has been well documented, and aneuploidy *per* se does not preclude embryo development (Sandalinas et al., 2001). Moreover, chromosome copy number errors of meiotic origin in the parental gametes, mainly maternal, have been observed in blastocysts that display normal morphokinetics and morphology, and are indistinguishable from euploid blastocysts (Fragouli et al., 2014). The persistence of meiotic aneuploidies to the blastocyst stage suggests that other factors are involved, including, but not limited to ovarian stimulation protocols, culture media, and molecular and intracellular processes. Genetic variants may also contribute to developmental arrest of early embryos and in particular post-zygotic errors (McCoy et al., 2015a,b; Mantikou et al., 2012).

Normal development of the human embryo in vitro begins after syngamy and consists of a series of mitotic events that doubles the cell number after each round. The undifferentiated daughter cells (blastomeres) are expected to be genetically identical and are under the primary control of maternal factors stored within the oocyte (Vassena et al., 2011; Assou et al., 2011; Ambartsumyan and Clark, 2008; Fragouli et al., 2014). At the 8-cell stage zygotic genome activation occurs, triggered by the degradation of the maternal transcripts, and the embryo begins to develop autonomously (Braude et al., 1988). The blastomeres first begin to develop gap junctions and undergo compaction to form a morula (Assou et al., 2011), then differentiate to the cells of the inner cell mass and the trophectoderm of the blastocyst (Assou et al., 2011; Braude et al., 1988). Traditionally, selection of embryos for transfer has been primarily based on embryo morphology using various grading schemes (Balaban et al., 2011). For cleavage stage transfers, the

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emphasis is on blastomere counts and symmetry, as well as degree of fragmentation. Embryos with 8 cells on day 3 post insemination have been shown to exhibit the highest viability in comparison to those with significantly more or fewer cells (Racowsky et al., 2003; Alikani et al., 2000). As canonical embryo cleavage effectively doubles the cell count after each round of mitosis (1-2-4-8 cells), embryos exhibiting this pattern of cleavage would be more likely to have undergone normal mitosis and faithfully replicated the chromosome complement inherited at fertilisation. Therefore, these cell counts have some predictive value with respect to developmental potential, particularly when combined with other factors, such as multinucleation and fragmentation (Alikani et al., 2000).

The introduction of time lapse monitoring (TLM) to IVF allowing the continuous observation of early embryo development provides more informative data than cell counts at static time points. It not only allows the undisturbed visualisation of the cells resulting from cleavage and embryo development, but also the observation of the cleavage patterns from which the daughter cells originated. Most time lapse studies have focused on morphokinetics for the prediction of the developmental potential of early embryos (Meseguer et al., 2011). Many have shown that early cleavage stage kinetics are useful in predicting blastocyst formation (Desai et al., 2018; Cruz et al., 2012; Dal Canto et al., 2012; Kirkegaard et al., 2013). Although the use of morphokinetics has proved useful for selecting better quality embryos for implantation, this method nevertheless provides only little insight behind the causes of early embryonic arrest.

Ottolini et al. (2017) have recently described the relationship between abnormal patterns of early cleavage and developmental arrest. Their studies suggested that cleavage errors result in genome partitioning of the embryo leading to embryonic arrest. We herein explore the hypothesis that the majority of cell arrest is due to genome loss related to aberrant (non-canonical) cleavage patterns. We further examine the current knowledge of human embryo development in vitro, with special attention to the issue of chromosome mosaicism, and relate aberrant cleavage patterns to the development of several or more clonal mosaic cell lines that contribute to either embryonic arrest or poor blastocyst development and extruded cells.

2. Tripolar division and genome loss

Abnormal or multipolar mitosis in different cell types has previously been shown to distribute chromosomes between 3 or more cells at anaphase, resulting in aneuploidy (Duensing and Münger, 2001; Saunders, 2005). However, we are only just beginning to understand how this relates to and affects early embryo development. The association between the timing of abnormal cleavage patterns and embryonic arrest has been previously described (Zhan et al., 2016; Yang et al., 2015). It has also been shown that cells from arrested embryos exhibit higher rates of aneuploidy, particularly mosaic aneuploidy (McCoy et al., 2015). Recently, Ottolini et al. (2017) were able to reconstruct abnormal mitotic events in early embryonic development by utilising SNP genotyping in combination with TLM. In order to distinguish between meiotic and post-zygotic aneuploidies, polar body 1 and 2 testing by meiomapping (Ottolini et al., 2015, 2016) was performed to identify maternal meiotic errors that would be inherited by all cells in the embryos, followed by karyomapping (Handyside et al., 2010) of single cells from disaggregated arrested embryos to identify mitotic errors that would be inherited through specific cell lineages. Because embryos inherit chromosomes with a unique pattern of meiotic recombination, a fingerprint can be identified for each chromosome in each embryo permitting the determination of the distribution of parental chromosomes in the constituent single cells of each embryo. Fitting together the genetic information from disaggregated single cells was found to reconstitute the complete set of parental chromosomes expected for each of the embryos, given the pattern predicted by meiomapping. While genome loss (nullisomy) was characteristic of the single cell genomes, the ability to reconstruct the parental chromosome complement suggested that the genomic losses observed did not result from chromosomal degradation or random mitotic errors, but instead that abnormal chromosomal segregation into multiple cells (> 2) during mitosis resulted in redistribution of a “partial” genome throughout the dividing embryo.

Karyomapping revealed clear examples of arrested embryos with multiple cell lines (2 or more cells) of identical or closely related subdiploid karyotypes. These observations were suggestive of tripolar mitosis followed by normal faithful segregation of the reduced chromosome set in subsequent cell divisions. Retrospective time lapse analysis demonstrated the predicted patterns of abnormal cleavage, from 1 to 3 cells, followed by normal bipolar division; thus, providing strong evidence that developmental arrest frequently follows an aberrant cleavage event. Moreover, the evidence from trophectoderm biopsy samples for PGT-A suggests that nullisomy is vanishingly rare in blastocysts (data not shown), whereas the majority of the arrested single cells analysed (90%) exhibited high levels of genome loss. This further supports the view that genome loss prevents blastocyst development and is the leading cause of cellular and embryo arrest at the cleavage stage.

It was hypothesised that as a result of tripolar mitosis and other abnormal cleavage events in the developing embryos, the segregation and distribution of the embryonic chromosomes results in a lack of genetic information necessary to participate in normal embryo development and progress post zygotic genome activation. Cells within an embryo that undergo aberrant divisions and resultant genome partitioning would therefore arrest prior to compaction or blastocyst formation as observed by Ottolini et al. (2017).

These observations represent a novel category of mosaicism characterised by genome loss as a result of aberrant mitoses. This establishes the first solid link between abnormal cleavage patterns, embryo arrest and embryo ploidy throughout early development.

3. Abnormal cleavage is associated with embryo arrest

Zhan et al. (2016) in a study involving $> 21,000$ embryos, examined the occurrence and outcomes of direct uneven cleavages (DUC) (often referred to as multipolar or direct cleavage) during embryo development. The abnormal cleavage events were classified according to the cleavage division at which they occurred; DUC-1, DUC-2 or DUC-3 for the first, second and third divisions, respectively. DUC-1 was detected more frequently than late DUC (DUC-2, 3) and was associated with decreased blastocyst development compared to later abnormal cleavage events, with rates of blastulation decreasing from 40.2% (DUC-3), to 18.8% (DUC-2) to its lowest at 8.2% (DUC-1). A smaller study of 791 embryos by Lagalla et al. (2017) examined further the relation between irregular cleavages and embryo arrest; recognising and accounting for the difference between DUC and ‘rapid’ division, and other irregular patterns of cytokinesis. While both DUC and rapid division result in > 2 daughter cells per cleavage event, the embryos undergoing rapid or precocious division first exhibit normal 1–2 cell cleavage, followed by a premature division of daughter cells to produce 3 or more blastomeres. Out of 111 irregularly developed embryos, 78.4% arrested or were discarded due to poor blastocyst quality, with the greatest frequency of arrest at the cleavage stage (48.6%) compared to the morula stage (19.8%). Normally dividing embryos also experienced high rates of arrest (63%). However, the difference in arrest rates and thus, blastulation rates between the normally and abnormally dividing embryos was statistically significant ($p < 0.01$). These findings support our observations with abnormally cleaving embryos whereby rapid or precocious cleavage appeared to be less disruptive to blastocyst formation than DUC (unpublished data). Moreover, both these studies and Ottolini et al. (2017) noted the exclusion of some daughter cells resulting from errant divisions. Both Lagalla et al. (2017) and Zhan et al. (2016) hypothesised that these exclusions might provide the

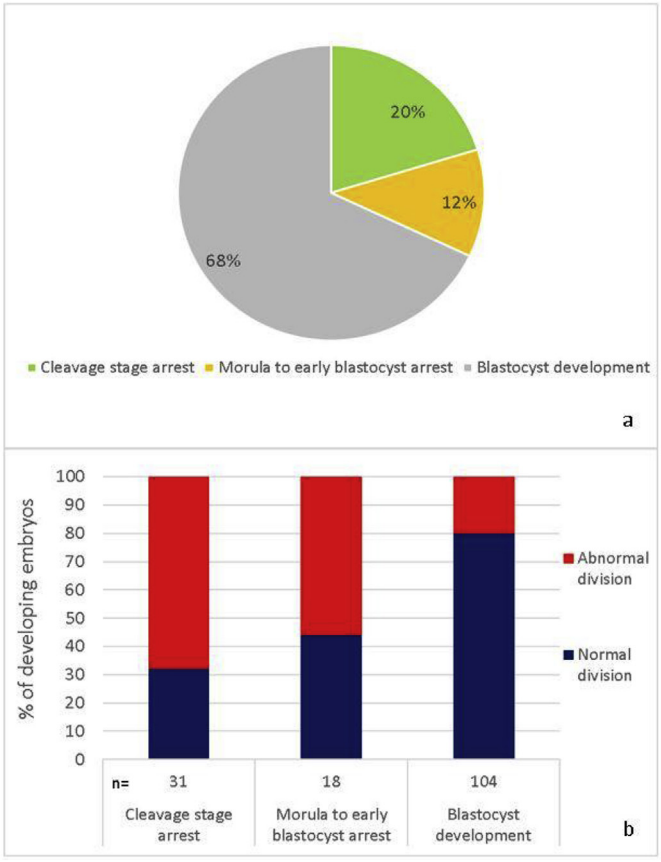

Fig. 1. Embryo development data from 153 embryos from a single IVF unit undergoing continuous culture with time lapse monitoring. a) Proportion of embryos at final stage of development in vitro. b) Cleavage patterns, assessed by time lapse monitoring, associated with embryo development.

mechanism for aneuploidy rescue in abnormally dividing embryos, providing the means of embryo "self-correction" to attain or maintain euploidy. However, we are of the view that it does not represent an active means of aneuploidy rescue by the embryo to allow for successful development but is rather a passive occurrence that demonstrates the inability of sub-diploid cells to incorporate during compaction, and subsequent blastocyst formation. Our findings are consistent with those of Zhan et al. (2016) and Lagalla et al. (2017) with reduced blastocyst development the earlier the aberrant mitotic event. Sixty eight per cent of embryos reached the blastocyst stage in culture, as shown in Fig. 1.a. of which 80% demonstrated normal canonical cleavage, as shown in Fig. 1. b. Conversely, for the 20% of embryos that arrested at the cleavage stage (Fig. 1. a.), only 33% demonstrated canonical cleavage (Fig. 1b). Importantly, 20% of embryos that developed to the blastocyst stage showed abnormal cleavage (Fig. 1b). It can be inferred that in comparison to the embryos that arrested at the cleavage and morula stages, these embryos experienced either abnormal mitosis later in their development or rapid first precocious division, which are both less detrimental to blastocyst formation.

4. Abnormal cleavage results in mosaicism that perturbs embryo development

Zhan et al. (2016) and Lagalla et al. (2017) performed PGT-A on all blastocysts and observed reduced euploidy rates with abnormal cleavage. Zhan linked the timing of the abnormal division to the ploidy status, noting that the rate of euploidy increased the later the timing of the aberrant division. In contrast, complex aneuploidy was highest in embryos with early cytokinesis errors and decreased significantly through DUC-2 and DUC-3. An early DUC would affect all the cells of the developing embryo increasing the likelihood of developmental arrest before compaction and blastocyst formation. By contrast, late DUC results in fewer cells that would be affected, potentially leaving a higher proportion of diploid cells capable of resulting in a competent blastocyst. Both Ottolini et al. (2017) and Lagalla et al. (2017) analysed the cells not involved in blastocyst formation, and in cases where the DNA was amplified found them to be either complementary to or possessing a larger number of aneuploidies than the blastocyst samples from which they were derived.

Abnormal cleavage patterns appear to give rise to mosaicism - clonal cell lines of different chromosome copy number. Mosaicism was first described using fluorescent *in-situ* hybridisation (FISH) studies of cleavage stage embryos (Delhanty et al., 1993; Munné et al., 1994) and was suggested to be of mitotic origin, notwithstanding the limitations of FISH when using a restricted number of chromosome specific probes. Classically, mosaicism was proposed to occur by several mechanisms of non-disjunction, anaphase lag, endoreplication and chromosome degradation (Taylor et al., 2014; Mantikou et al., 2012), the majority of which are related to mitotic spindle and centrosome errors (Chatzimeletiou et al., 2005). However, this type of mosaicism was generally not associated with embryonic or cellular arrest and only affects a single or limited number of chromosomes. Mosaicism can be broadly classified into 2 groups: (1) all aneuploid - all cells within the embryo are chromosomally abnormal; and (2) diploid-aneuploid - a fraction of the cells within the embryo are abnormal, whilst the remaining portion is diploid. Thus, the evidence suggests that the type of mosaicism is influenced by abnormal cleavage, the timing at which it occurs and the type of abnormal cleavage.

The impact of abnormal cleavage on cell arrest is evident, but not absolute. While it is apparent in the published literature that abnormal first mitotic divisions impact blastocyst development rates, not all embryos undergoing a 1- > 2 cell first mitotic event arrest prior to blastocyst formation. This broadens the scope of understanding of early embryo arrest presented by Ottolini et al. (2017), suggesting affected cells may have varying chromosome complements, including cells that have genome loss, are near-diploid and diploid.

We propose a series of common cleavage events (normal and abnormal) which could account for a number of different embryo fates following abnormal cellular cleavage, directly related to the hypothesis of genome loss and cellular arrest (Fig 2.0).

As per Ottolini et al. (2017), multipolar cleavage will result in sub-diploid cell lines in the embryo. If the cleavage division is equal and the replicated set of 92 chromosomes is segregated evenly between the multiple daughter cells (> 2) (Fig. 2. b.2), the resulting cells will all contain nullisomy. Therefore, as hypothesised, these cells would arrest prior to blastocyst formation. The ability of embryos which undergo multipolar cleavage to develop to the blastocyst stage is dependent upon the stage at which the errors occur. If the first mitotic event is multipolar and all the cells are affected, the embryo would be an all aneuploid mosaic (all cells sub-diploid) and would therefore arrest prior to blastocyst formation. Whereas, if a later mitotic event is multipolar only a fraction of the embryo is affected, thus rendering the embryo sub-diploid-aneuploid mosaic with part of the embryo capable of forming a blastocyst, and the affected cells arresting.

Embryos with clear multipolar, first mitotic divisions can result in blastocyst formation. It has been documented that a proportion of apparently normally fertilised (2 PN) embryos undergoing PGT-A are in fact triploid, containing an extra set of inherited chromosomes (McCoy et al., 2018). Triploid embryos with an extra maternal (digynic) or paternal (diandric) set of chromosomes frequently undergo tripolar mitosis and later develop to morulae (Kalatova et al., 2015). A triploid embryo that undergoes a tripolar mitosis in the first division would presumably result in three cells with distinctly different karyotypes, but quasi-diploid and unlikely to contain any nullisomy, which would explain their capacity to develop normally to the blastocyst stage.

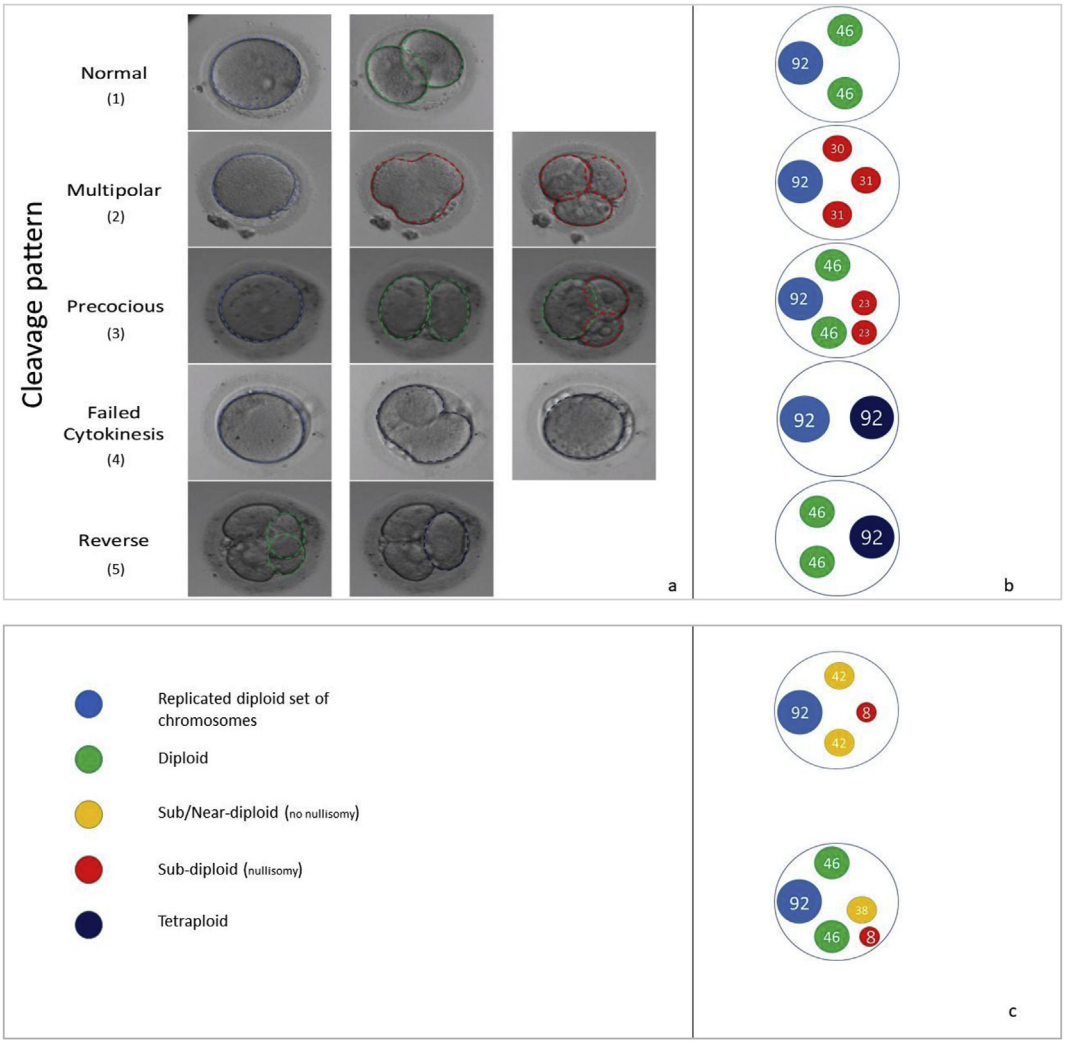

Fig. 2. Abnormal cleavage patterns are observable via use of timelapse monitoring during embryo culture, and can be associated with ploidy status of the developing embryo. a) Timelapse images showing five common cleavage patterns. Blastomeres are represented by the coloured rings. B) Chromosomal distribution across the range of cleavage patterns determines the ability of the embryo to undergo blastulation. C) Alternative cleavage patterns for "Multipolar" and "Precocious" divisions resulting in differing segregation of chromosomes to section B. Sub-diploid blastomere complements are associated with failure of blastocyst formation. Cells coloured green, yellow and dark blue may be capable of blastocyst formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Precocious or rapid cleavage is a further explanation for an embryo with a non-canonical first mitotic division (1- > 2 cells) with the capacity for blastocyst formation. In contrast to an embryo undergoing multipolar mitosis, the cell lines produced following precocious division would not all be rendered sub-diploid. Partitioning of the genome would occur in only two of the resulting daughter cells and would still leave a diploid cell capable of continued normal development and blastocyst formation (Fig. 2. a.3; 2. b.3). For this reason, precocious divisions may be associated with blastocyst development more frequently than multipolar cleavage. It is therefore important to distinguish between the two types of direct cleavage as they will likely have different effects on the chromosome distribution in the blastomeres and developmental potential of the embryo.

Multipolar and precocious cleavages may also be uneven in nature. In such cases, the chromosomes may not be equally distributed among the blastomeres, resulting in a combination of daughter cells with extreme chromosome loss and others with a marginally sub-diploid or near-diploid chromosome complement (Fig. 2c). Single cells with as few as 8 chromosomes were observed by Ottolini et al. (2017) and may provide an explanation for the reduced potential of highly fragmented embryos, with larger fragments potentially containing segregated chromosomes. These near-diploid cells within an embryo may account for their continued development despite abnormal cleavage. Near-diploid cells (no nullisomy) would be expected to behave similarly to cells with meiotic aneuploidy and may result in blastocyst formation.

Other abnormal mitotic events, such as failed cytokinesis and reverse cleavage would result in aneuploidy and/or mosaicisim but are not associated with genome loss (Fig. 2. b.4; 2. b.5). Failed cytokinesis is characterised by multiple rounds of karyokinesis (≥ 2), without the associated cell cleavage and results in a polyploid cell. Depending on when and how many cells fail cytokinesis, it may still be possible for blastocyst development to occur if the failed cytokinesis event is followed by normal cleavage. However, we have recently witnessed a 1 cell embryo forming blastocoel-like vesicles and a structure similar to an expanded blastocyst following at least three rounds of mitosis with failed cytokinesis (Fig. 3.0). This demonstrates the propensity of cells without genome loss to develop to the blastocyst stage.

In the case of reverse cleavage, resorption of blastomeres is observed to occur after cytokinesis (Fig. 2. a.5; 2. b.5). If two cells, following normal mitotic division were to merge, a polyploid cell results which would be karyotypically indistinguishable from failed cytokinesis, and would persist throughout later embryo development cycles.

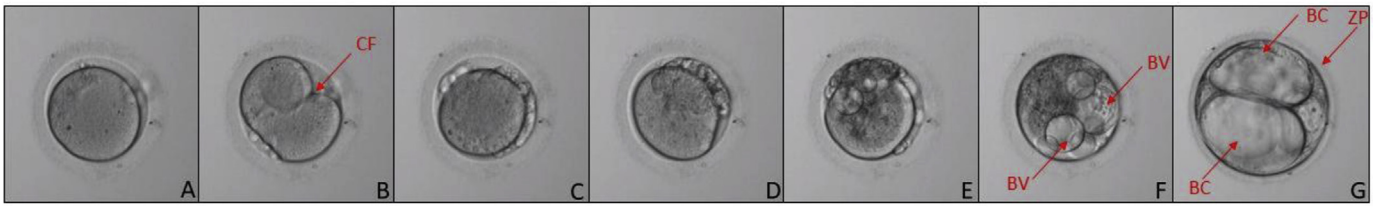

Fig. 3. Blastocyst-like structure forming from 1 cell embryo having undergone 3 rounds of mitosis with failed cytokinesis. A) 1 cell zygote prior to mitosis. B) Formation of cleavage furrow (CF) during first round of mitosis. C) 1 cell embryo after completion of first round of mitosis with failed cytokinesis. D) Embryo following second round of mitosis with failed cytokinesis. E) Embryo following third round of mitosis with failed cytokinesis. F) formation of blastocoel vesicles (BV) in single cell. G) Expanded blastocyst-like structure with large blastocoel-like cavity (BC) formed from the vesicles, and thinned zona pellucida (ZP) from a single cell.

However, if reverse cleavage occurs after precocious division for instance, the reformed blastomere could reconstitute the chromosome complement of the original cell; therefore, reinstating diploid karyotype and potential normal development. In this case, it can only be assumed that cytokinesis is complete and two stable cells are formed. It is therefore possible that these blastomeres never actually fully complete cytokinesis and thus the event may not represent true reverse cleavage resorption. Nevertheless, the resulting cells and their chromosomal complements would be the same in both situations.

These aberrant mitotic events represent commonly occurring phenomena observed by TLM of embryos in vitro. They can be used to account for the majority of embryo development outcomes and could potentially represent a novel methodology to assist with embryo selection, especially post PGT-A, helping to deselect embryos with lower implantation potential as result of mosaicism currently undetectable by current PGT-A methodologies (Rosenwaks et al., 2018).

5. Discussion

Research into early human embryo development and mosaicism has intensified in recent years, following the introduction of next generation sequencing (NGS) based tests for chromosome copy number for PGT-A, and the introduction of time lapse monitoring to embryo culture systems. While many factors may influence embryo development, aberrant cleavage divisions have now been shown to have a direct demonstrable influence on the human embryonic genome.

Effectively, abnormal cleavage leads to a form of mosaicism which results in extreme sub-diploid cells lines. If this involves genome loss, it is likely that the affected cells are unable to integrate into normal blastocyst development and would therefore arrest prior to the blastocyst stage. The hypothesised mechanisms of chromosome segregation throughout the developing embryo, supported by the use of retrospective time lapse analysis, can reasonably account for much of the high rates of embryo arrest observed during early embryogenesis, the high rates of aneuploidy in the arrested embryos, and the fact that chaotic or complex aneuploidies are more frequent at the cleavage stage of development (25%) than in blastocyst embryos (10%) (McCoy, 2017).

Recently, McCoy et al. (2018) have reported that maternal genetics may predispose some embryos to abnormal cleavage and early arrest. Analysis of over 41,000 embryo biopsy samples revealed that in embryos with complex aneuploidies there were distinct sub-diploid cells consistent with tripolar mitosis or sequential precocious mitosis events. The tripolar cleavage pattern was associated with maternal variants in haplotypes spanning the PLK4 centrosomal regulator which has been reported to be involved in aberrant mitosis in human embryos. It is highly likely that other genetic variants can predispose embryos to both meiotic and mitotic errors, and therefore not only contribute to the high levels of embryonic loss seen in human IVF but also in natural conceptions. What remains to be determined is what, if any, other genetic elements confer a predisposition to abnormal cleavage and genome activation patterns.

Although work on direct cleavage and genome loss has developed significantly over the last few years there is a limited understanding of how and why these events occur. An investigation of gene expression profiles in normally developing and arrested embryos might provide insight in establishing a link between genome loss and cellular arrest post zygotic genome activation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmg.2019.04.008.

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