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REVIEW

The origin and possible mechanism of embryonic cell-free DNA release in spent embryo culture media: a review

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Abstract

The presence of cell-free DNA in spent embryo culture media (SECM) has unveiled its possible utilization for embryonic ploidy determination, opening new frontiers for the development of a non-invasive pre-implantation genetic screening technique. While a growing number of studies have shown a high concordance between genetic screening using cell-free DNA (cfDNA) and trophectoderm (TE), the mechanism pertaining to the release of cfDNA in SECM is largely unknown. This review aims to evaluate research evidence on the origin and possible mechanisms for the liberations of embryonic DNA in SECM, including findings on the self-correction abilities of embryos which might contribute to the presence of cfDNA. Several databases including EMBASE, PUBMED, and SCOPUS were used to retrieve original articles, reviews, and opinion papers. The keywords used for the search were related to the origins and release mechanism of cfDNA. cfDNA in SECM originates from embryonic cells and, at some levels, non-embryonic cells such as maternal DNA and exogenous foreign DNA. The apoptotic pathway has been demonstrated to eliminate aneuploid cells in developing mosaic embryos which might culminate to the release of cfDNA in SECM. Nonetheless, there is a recognized need for exploring other pathways such as cross-talk molecules called extracellular vesicles (EVs) made of small, round bi-layer membranes. During in vitro development, embryos physiologically and actively expel EVs containing not only protein and microRNA but also embryonic DNA, hence, potentially releasing cfDNA of embryonic origin into SECM through EVs.

Keywords Apoptosis · Cell-free DNA · In vitro fertilization · Spent embryo culture media · niPGT-A

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Introduction

The genetic constitution of both chromosomes and genes highly influences the ability of embryos in vivo and in vitro to grow and successfully implant during the endometrial receptive phase. Notably, over 65% of early spontaneous miscarriages are due to chromosomal abnormalities in embryos [1], approximately 55% of cleavage-stage [2], and over 50% of blastocysts [3] generated from assisted reproduction programs carried abnormal chromosomes. Consequently, pre-implantation genetic diagnosis (PGD) and pre-implantation genetic screening (PGS) for chromosomal constitution were introduced in in vitro fertilization (IVF) to aid in selecting genetically normal embryos. At present, to improve the accuracy of the nomenclature, pre-implantation genetic testing is standardized into three categories as follows: pre-implantation genetic testing for monogenic disorders (PGT-M), pre-implantation genetic testing for chromosomal structural rearrangement (PGT-SR), and pre-implantation genetic testing for aneuploidy (PGT-A) [4].

The current PGT-A technique requires the biopsy of trophectoderm (TE) cells for testing and is therefore invasive, possibly inducing trauma to the embryos [5]. The technique is also quite time-consuming and demands highly skilled embryologists. In addition, the current PGT-A method also suffers from false positives and negatives due to embryo mosaicism. Embryonic TE cells might be less representative of the whole embryo chromosomal constitution due to downstream cell fate decisions. TE cells differentiate into cells forming the placenta, while the inner cell mass (ICM) differentiate into primitive endoderm and epiblast lineages which eventually form the fetus. Therefore, the possible utilization of non-invasive biomarkers to determine embryonic chromosomal status has long been considered to substitute the invasive TE cell biopsy methodology.

The presence of cell-free DNA in spent embryo culture media, among other biomarkers to distinguish euploid from aneuploid and mosaic embryos, has gathered considerable importance. The identification and possible use of cell-free DNA (cfDNA) in spent embryo culture media (SECM) and blastocoel fluid were first reported in 2013 by Stigliani et al. [6] and by Palini et al. [7], respectively. cfDNA in SECM has been presumed to arise from embryonic cells which include inner cell mass (ICM), and TE cells, indicating the authenticity of cfDNA in expressing the genetics of developing embryos. A growing number of recent studies have proven a high concordance rate between cfDNA and TE cells as samples for pre-implantation genetic testing [8, 9]. A 2019 study has reported high concordance rate of cfDNA with whole embryonic genome sequencing than that of TE cells (93.8% vs 82.0%, respectively) [8]. Likewise, a multicenter prospective study which recruited 1301 human blastocysts observed a good concordance between cfDNA and TE cells (78.2%, ranging from 72.5 to 86.3% across eight IVF centers) and also with ICM (84.4% using 81 donated aneuploid blastocysts) [9].

Despite the convincing concordance results in the aforementioned studies, the use of cfDNA for chromosomal analysis remains contradictory due to the lack of clear evidence on the origin of embryonic cfDNA and its release mechanism into SECM [10]. Both genomic and mitochondrial cfDNA have been sufficiently detected in SECM through either direct quantification or whole-genome amplification [6, 11]. Cellular fragmentation during embryo development has been presumed to cause the release of embryonic cfDNA into the SECM; hence, its quantity was theoretically thought to correlate positively with embryo fragmentation rate [6]. Unfortunately, studies have observed that DNA was detected at a very low level in the control culture droplet which had no contact with any embryos, suggesting DNA contaminations in the commercial culture media after manufacture [11, 12]. To bridge the knowledge gap, this review sought to evaluate current literature which elucidates the source of embryonic cfDNA and the possible mechanism for its release in SECM, hence strengthening its implication as a non-invasive biomarker for embryo ploidy status.

Methods

Search engines including EMBASE (https://www.embase.com/), PUBMED (https://pubmed.ncbi.nlm.nih.gov/), and SCOPUS (https://www.scopus.com/) were used to retrieve articles using the following keywords: (cell-free DNA or embryonic DNA or cfDNA or cfDNA origin or embryo mosaicism or embryo aneuploidy or blastocysts DNA or aneuploid cells embryonic DNA or embryo culture media or embryonic spent culture media or pre-implantation genetic screening for aneuploidy (PGT-A) or non-invasive pre-implantation genetic testing for aneuploidies (NiPGT-A)) AND (apoptosis or degeneration or cell death or active secretion or necrosis or embryo self-correction). Database searching was last accessed on December 17, 2022. This review focuses on evidence-based research of embryo self-correction mechanisms, the source, and the possible mechanism of cell-free DNA release in SECM. Only fully published English papers were reviewed without restriction in the year of publication.

Results

Origin of cell-free DNA in SECM during embryo culture

Establishing the origins of cfDNA in SECM is critical to validate its utilization for ploidy screening in routine clinical practice of IVF. A 2020 systematic review

suggested cfDNA to be detectable in SECM indicating that the growing embryos in vitro were likely to release cfDNA actively during the culture period [13]. This was supported by Hammond et al. [11] who observed increased cfDNA concentration in embryo-exposed SECM compared with non-exposed SECM suggesting for what they called embryo-associated structure to release cfDNA during the culture period. Vera-Rodriquez et al. [12] likewise confirmed that the control culture media (no contact with embryo) contained a lower amount of cfDNA than embryo-exposed SECM (median: 1.4 pg vs 6.7 pg) before DNA amplification. In emerging research pertaining to embryonic cfDNA in SECM, 3 DNA origins have been identified as follows: (1) blastomeres or embryonic cells, (2) maternal DNA from either the cumulus cells or polar body, and (3) at very low levels, exogenous DNA contamination in culture media probably derived from the added human serum albumin (HSA), a different working protocol for culture and sampling time (Table 1) [9, 11, 12, 14–16].

Rubio et al. [9] suggested that cfDNA was derived from both ICM and TE cells. The ICM components of 81 donated-aneuploid blastocysts were isolated and analyzed against the TE genetic analysis. Briefly, a high concordance rate was observed between cfDNA and both ICM and trophectoderm cells (84.4% and 87.5%, respectively) indicating that cfDNA was derived from both embryonic cell types. Chen et al. [15] also shed light on the cellular origin of cfDNA in SECM by utilizing the different DNA methylation profile patterns of TE and epiblast on the promoter region. Using only 61 SECM collected on day 6 which did not contain maternal DNA contamination, 29.5% SECM cfDNA (18 out of 61) correlated with TE, while 70.5% (43 out of 61) were shown to correlate with epiblast, thereby suggesting that both TE and ICM are the origins of embryonic cfDA in SECM. Intriguingly, another study demonstrated that the concordance rate of cfDNA-SECM with whole vitrifying-warming blastocysts for ploidy status was higher than that of TE biopsy (93.8% vs 82%) which implied that cfDNA represents the embryonic ploidy status more accurately [8]. However, comparing the whole embryonic genomes with TE might less precise.

Maternal DNA contamination was consistently reported in existing studies [9, 12, 14]. Maternal DNA contamination in SECM was evidently proven by Vera-Rodriguez et al. [12], using single-nucleotide polymorphism (SNP) analysis. In the experiment, embryonic allele-specific reference (haplotype) was derived from TE biopsy, while maternal-allele reference was obtained from follicular fluid samples. A total of 124 SNPs (90 on autosomal chromosomes while the remaining on Y chromosome) were checked to identify both embryonic and maternal haplotypes. It was found that maternal DNA contamination was higher in SECM (86–94%) with the median proportion of embryonic DNA haplotypes at approximately 8%. Polar body DNA contamination was also suggested, but given the nature of polar body existence, such contamination could be avoided by collecting SECM later in the embryo culture period, as clearly demonstrated in SECM collected on day 6 of culture [9]. Overall, the DNA methylation pattern analysis revealed that maternal DNA contamination originated largely from cumulus cells (Pearson correlation (R) = 0.93), and very low polar body contamination was detected in SECM collected on day 5 [15].

Clinical utilization of cfDNA for ploidy analysis was compared with polar body in advanced maternal age population [14]. Agreement in the genetic analysis between the two sample types was 72.2%, and a potential maternal DNA contamination was detected in the blastocysts SECM. Embryonic genotype profiling was attempted through the PGT-M cycle as a more advanced utilization of cfDNA in SECM. Capalbo et al. [17] noticed, however, that maternal DNA was detectable and could interfere with the genotyping analysis; hence, SECM has yet to be recommended for PGT-M analysis unless the maternal DNA contamination could be diminished.

In the context of exogenous DNA contamination, Hammond et al. [11] declared that there was a lack of cfDNA SECM studies with adequate controls to indicate whether the exogenous contamination originated from the culture media manufacturing, human serum albumin addition, or contamination obtained during the culture period. To address this, two types of controls from three different types of culture media (sequential, continuous, and human serum albumin/HSA solution) were designed: (1) fresh, external media controls which included the base culture media with and without 5 mg/mL HSA supplementation and 100% human serum albumin solution and (2) internal media controls exposed to the same conditions of embryo culture but had no contact with any embryos (supplemented with 5mg/mL HSA pre-manufacturing and post-manufacturing (added in the laboratory)). The external and internal media controls contained very low levels of DNA contamination, and increased levels were correlated with HSA supplementation in the culture media. This was expected given the high-affinity binding of the protein to DNA structures. Since most embryo culture media are supplemented with HSA, this potential contamination should be adjusted when considering the use of embryonic cfDNA in SECM for genetic analysis interpretation.

Embryonic cfDNA origin: does cfDNA reflect embryo self-correction ability?

The notion that human embryos are equipped with an innate self-correction capability has long been accepted [18, 19]. Yet, substantial proof to decipher the mechanisms of

Table 1 List of studies included in the review to address the origins of cfDNA in SECM

Authors	Sample size	Fertilization method	IVF patient characteristics	Day of sample collection	Genetic analysis for origin detection	Type of DNA detection	Origin of cell-free DNA
Hammond et al. [11]	227 blastocysts and 302 SECM	ICSI	Male infertility-induced ART	D3, D5, and D6	Short-tandem-repeat assay	Mitochondrial and nuclear DNA	Mixed of commercial media contaminations and embryonic DNA
Vera-Rodriguez et al. [12]	113 blastocysts matched with SECM	ICSI	Infertile couples following PGT-A	D5	Single-nucleotide polymorphism sequencing	Nuclear DNA	Mixed of embryonic DNA and maternal cumulus cells
Rubio et al. [9]	81 donated aneuploid blastocysts	ICSI	Advanced maternal age	D6/D7	Next-generation sequencing	Nuclear DNA	Originates from both inner cells mass and TE cells. Maternal DNA contamination could also be found in a few SECM samples.
Capalbo et al. [17]	72 SECM and 92 blastocyst fluid samples	ICSI	Infertile couples	D5	qPCR analysis designed specifically for targeted disorder	Nuclear DNA	Maternal and embryonic DNA
Feichtinger et al. [14]	22 SECM matched with oocyte polar body	ICSI	Advanced maternal age	D5	Comparative genomic hybridization	Nuclear DNA	Maternal and embryonic DNA
Chen et al. [15]	194 blastocysts SECM	ICSI	Infertile couples	Mostly in D5 and D6	DNA methylation sequencing	Methylation of whole-genome DNA	Mixed of embryonic, maternal cumulus cells and polar body DNA

ICSI intra-cytoplasmic sperm injection, *SECM* spent embryo culture media, *qPCR* quantitative polymerase chain reaction

self-correction is difficult to obtain [20]. A robust understanding of self-correction in embryos in vitro arises from the implementation of next-generation sequencing (NGS) in IVF which allows the discernment of embryo ploidy status into three categories (euploid, mosaicism, and aneuploid) [21]. TE biopsy typically aspirates more than one embryonic cell, each possibly comprising a different ploidy status. A euploid embryo has a complete, normal set of chromosomes (46 chromosomes, the common threshold is less than 20% of chromosomally abnormal cells), while a mosaic embryo represents a mixture of euploid and aneuploid cells (approximately 20–80% chromosomally abnormal cells). On the other hand, an aneuploid embryo contains largely of cells with additional or less chromosomes, with more than 80% of the cells being chromosomally abnormal. Restricted to data availability, the clinical practice of transferring mosaic embryos remains contradictory, with some research associating it with a high spontaneous abortion rate but could result in live birth babies even with a high-level mosaicism embryo [22]. One possible mechanism for this might be in the self-correction ability of human embryos. A well-described 2021 review has summarized several terms to describe the possible self-correction pathways in a mosaic embryo [23]. In this review, similar keywords were used to enhance the investigation of self-correction mechanisms in a developing embryo. Recently, several studies have just attempted to reveal such pathways in both human and animal model embryos with convincing evidence (Table 2) [24–28].

The most recent prominent findings on self-correction in embryos were discovered [24, 29]. NGS analysis was used to identify which structures of the blastocyst retained the abnormal cells in a total of 174 aneuploid blastocysts which were confirmed as aneuploid in the prior cleavage-stage biopsy [29]. It was found that peripheral cells and blastocoel cavity are the two structures with higher aneuploidy, suggesting a theory in which chromosomally abnormal cells are preferentially located distantly from the ICM and TE cell lineages. On the other hand, the investigation conducted by Yang et al. [24] revealed that aneuploid cells were abolished in the embryonic cell lineage through an apoptotic depletion mechanism, and preferential lineage allocation occurred in which aneuploid cells drifted to become extra-embryonic lineages. Utilizing the open access chromosome-wide analysis to capture gross abnormalities in gene expression of single cells from previous studies [30, 31], Yang et al. successfully demonstrated a higher cell aneuploidy rate of day 3 embryos (81.82%) which declined to 5.36% on day 7. In addition, the study confirmed that at day 6, none of the embryos contained 100% aneuploid cells. Consistent with the high rate of cell aneuploidy of embryos at the early stage of development, the incidence of embryo aneuploidy was 83.3% on day 3 and declined to 11.1% on day 4. The results reinforced the hypothesis that embryo mosaicism is common and that the aneuploid cells would be subsequently eliminated through apoptosis. Furthermore, the proposed apoptotic depletion pathway corroborated a previous study that observed a randomized activation of cell death in either the ICM or TE cells that could extensively occur in the ICM of human blastocysts [32].

Time-lapse incubators have also greatly helped in the understanding of an embryo's self-correction ability [33]. Partial compaction by electively extruding some cells was recorded at the morula stage using a time-lapse incubator system. Lagalla et al. [25] demonstrated that 5 out of the 7 expelled cells during compaction from a euploid blastocyst contained abnormal chromosomes. Supporting the previous evidence, Orvieto et al. [26] observed that 5 out of 9 euploid blastocysts had expelled aneuploid fragments or cell debris containing chromosomal abnormalities, suggesting that a self-correction was induced.

Extensive investigations to prove the self-correction capability in mouse embryo models have been conducted [27, 28]. Both studies confirmed that the elimination of aneuploid cells depended on the cell lineage fate. Aneuploid embryonic cell lineage was most likely to be abolished via apoptosis, while aneuploid trophectoderm cells were more resistant to apoptosis. Bolton et al. [27] highlighted that the progressive depletion of aneuploid cells occurred remarkably in the inner cell mass during blastocyst development. To understand the extent of apoptosis-induced elimination of aneuploidy cells, an experiment was carried out by Singla et al. [28]. The results indicated that there was a crosslinked cascade between p53-induced autophagy and apoptosis, prominently proving that another pathway was activated for aneuploid cell elimination in the inner cell mass. Furthermore, overexpression of p53, p21, and cyclin G1 and down-regulation of Bcl-2 expression were observed because of aberrant chromosome segregation in the cells; p53 activation leads to prohibition of cell proliferation.

Self-correction mechanisms in mouse embryos were proven through spindle assembly checkpoint (SAC) protein activation [34] and mitotic checkpoint kinase activation [35]. By inducing a slight increase of reactive oxygen species in the culture environment, both researchers had clarified the role of cell-cycle checkpoint apparatus in preventing aneuploid cells of in vitro developing mouse embryos. Zhang et al. demonstrated a cross-interaction between SAC and DNA damage response (DDR) proteins. SAC assembly involving MAD2 protein is a conversed pathway that prevents the early onset of anaphase in M-phase when mitotic aberrant occurs, while DDR protein activates checkpoint kinase 1 (CKH-1) to prevent cell cycle progression from G2 to M phase. SAC as a prominent pathway for self-correction in embryo was further demonstrated by Li et al. who proved that serine/threonine protein kinase Aurora B, a chromosome segregation key player, was involved in the activation of SAC; thus restricting the development of aneuploid cells in embryos.

Table 2 Substantial research evidence to prove self-correction pathway in embryos

Authors	Type of embryo utilized for the study	Suggested model for self-correction in embryo	Suggested model interpretations
Observation based on human embryos			
Yang et al. [24]	Human embryonic stem cells	Clonal apoptotic depletion of embryonic aneuploid cells and preferential lineage allocation	• Aneuploid cells were shown to down-regulated NANOG genes as a hallmark of pluripotency and allocated those aneuploid cells to trophectoderm lineage favorably • Depletion begins on day 3 of embryo growth and continues to occur toward embryonic development
Griffin et al. [29]	Human embryos from IVF cycle	Preferential allocation	• Aneuploid cells were preferentially located in the extra-embryonic peripheral cells and also into the blastocoel cavity
Lagalla et al. [25]	Human embryos obtained from IVF cycle	Embryonic aneuploid cell mortality	• Aneuploidy rescue through partial compaction by expelling aneuploid cells into perivitelline space
Orvieto et al. [26]	Human embryos obtained from IVF cycle	Embryonic aneuploid cell mortality	• Aneuploidy rescue through partial compaction by extruding embryo cells containing abnormal chromosomes as a fragmentation or cells debris as proof of embryonic self-correction
Observation based on animal model embryos			
Bolton et al. [27]	Mouse embryo	Clonal apoptotic depletion	• Apoptotic pathway was highly activated to eliminate aneuploid cells in embryonic cell lines in comparison to extra-embryonic cells lineage • Screen out aneuploid cells through apoptosis pathway
Singla et al. [28]	Mouse embryo	Clonal apoptotic depletion	• Eliminating aneuploid cells through p-53-dependent pathway for both autophagy and apoptosis • Aneuploid cells are favorably directed to non-embryonic cell lineage. In addition, a closed euploid-cell population is able to compensate for abnormal cell loss through proliferation to attain embryonic cell viability
Huang et al. [34]	Mouse embryo	Spindle assembly checkpoint (SAC) protein activation	• Mitotic arrest deficient 2 (MAD2) protein as a prominent component of SAC was activated to allow cells to delay metaphase-anaphase progression, thus ensuring proper alignment of chromosomes to mitotic spindles
Li et al. [35]	Mouse embryo	Mitotic checkpoint kinase protein activation	Aurora B protein involves in the activation of SAC function for aneuploidy prevention

The reliability of cfDNA in determining the ploidy of chromosomally normal embryos has been challenged [10, 36]. A group of researchers believed that cfDNA is more credible in reflecting the embryo's self-correction capability, especially since cfDNA possibly arose largely from the apoptosis of aneuploid embryonic cells. Tobler et al. [37] demonstrated a high discordance in embryonic DNA karyotyping between blastocyst fluid and either ICM or TE cells. Preferential positioning of aneuploid cells into the blastocoel cavity and peripheral cells also challenged the possible use of cfDNA [29]. The studies seem to support the concerns of Orvieto et al. [10]. However, a considerable agreement between the two types of samples for PGT-A is less likely to be observed without presuming that another pathway could exist to elucidate the phenomenon. In addition, a similar quantity of cfDNA in SECM

between euploid and aneuploid blastocysts before and after amplification seems to argue that the apoptotic pathway is the sole mechanism underlying cfDNA liberation [12, 16].

Possible mechanism of embryonic cell-free DNA release into spent embryo culture media

cfDNA abundance in SECM varies remarkably. Despite variability in timing and technical aspect of sample collection as well as DNA amplification protocols among the studies, the mechanism for the release of cfDNA into the SECM should be explored. According to the methylation patterns, cfDNA found in blood circulation of healthy individuals originates mainly from hematopoietic cells with minimal contribution from other tissues. This is because during apoptosis, dying cells send signals to attract phagocytic cells, and phagocytosis occurs to eradicate apoptotic bodies without releasing cfDNA in circulation [38]. In embryo culture media, on the other hand, where phagocytic cells are not present, the removal of apoptotic bodies is suggested to occur via secondary necrosis characterized by membrane ruptures (Fig. 1; Supplementary file 1) [39].

cfDNA is evidently released by cells with high metabolism or activity and is closely related to the cell death mechanism (Table 3) [40]. One study proposed that the amount of embryonic cfDNA correlated with the fragmentation rate, suggesting that cellular fragmentation of embryonic cells during culture contributed to cfDNA in SECM [6]. In 2013, Stigliani et al. [6] indicated that embryos with <5% fragmentation had lower levels of double-stranded DNA and mitochondrial DNA than embryos with >5% fragmentation. As fragmentation was associated with both apoptosis and apoptotic secondary necrosis [39, 41–43], it is reasonable to believe that cfDNA was shed as a consequence of apoptosis

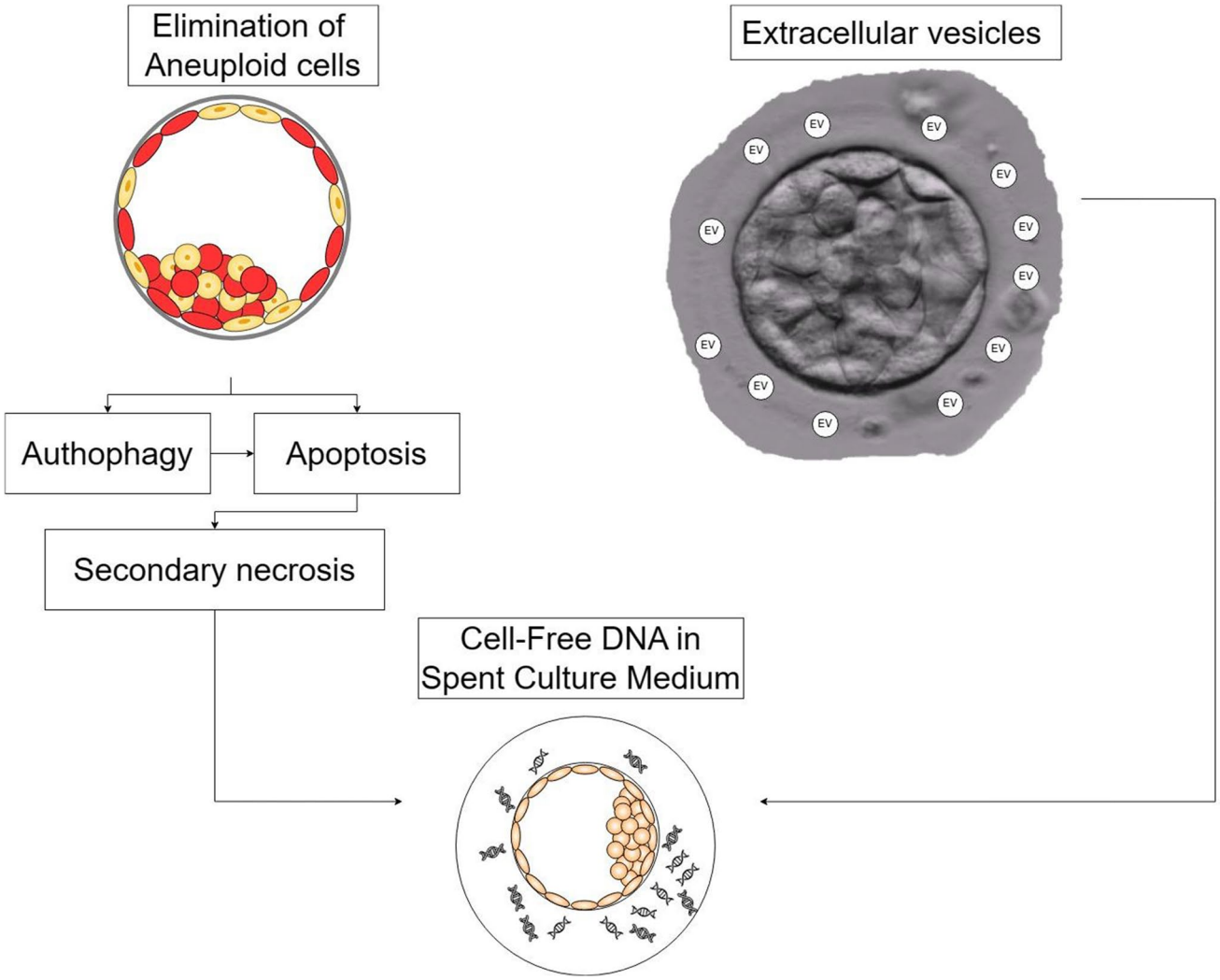

Fig. 1 Schematic representation of mechanisms for the release of cfDNA in SECM. EVs are reported to carry protein, mRNA, miRNA, mitochondrial DNA, as well as genomic DNA

Table 3 List of studies addressing the possible mechanism for the release of cfDNA in SECM

Suggested mechanisms	Researchers	Description
Cell death mechanism type I: apoptosis	Bolton et al. [27], Yang et al. [24], Singla et al. [28]	Inner cell mass aneuploid cells were confirmed to be removed via apoptosis pathway (Casp-3 activation)
Cell death mechanism type II: autophagy	Singla et al. [28]	p-53 dependent through apoptosis and autophagy
Secondary necrosis	Chi et al. [39], Jurisicova et al. [43], Fabian et al. [41, 42]	In the absence of phagocytic cells, embryonic apoptotic bodies undergo secondary necrosis by disrupting the membrane
Active release	Veraguas et al. [52], Vyas et al. [53], Giacomini et al. [46]	Extracellular vesicles (EVs) pass through zona pellucida. EVs could be presented as apoptotic bodies, microvesicles, and nanoparticles (exosomes) containing genomic DNA

as well as secondary necrosis events. In contrast, another study demonstrated that the number of mitochondrial DNA in SECM was unrelated to the embryo fragmentation rate [11].

The discovery of extracellular vesicles (EVs, 50–200 nm) in embryo culture medium could potentially elucidate the release of cfDNA in SECM [44, 45]. EVs are evidently released by pre-implantation embryos at the cleavage (day 3 embryos) and blastocyst stage [46]. Physiologically, EVs are signaling molecules for cell-to-cell communication which are shed by nearly all cell types and are normally found in biological fluids [47]. Notably, there are three types of EVs according to their size and unique properties including apoptotic bodies (500 nm–3 µm), microvesicles (100 nm–1 µm), and nanovesicles (mostly identical to exosomes, 30 nm–100 µm). While apoptotic body EVs represent the programmed cell death and thus contain genomic DNA, microvesicles and exosomes commonly carry specific proteins and micro-RNAs and less notably contain DNA [48]. Nonetheless, multiple evidence has shown that microvesicles and exosomes could also carry genomic DNA in order to boost the function of targeted cells [49–52].

Recently, Vyas et al. [53] investigated EVs in human embryos. The surface of both embryonic plasma membrane and zona pellucida expressed CD9-positive spherical vesicles consistent with the specific properties of EVs in the form of either singular or clustering. Moreover, the study proved that EVs were budded from the embryonic plasma membrane into the perivitelline space and were able to pass through zona pellucida to accumulate in the culture media. Embryo SECM collected on day 3 and day 5 were also demonstrated to contain spherical EVs with various sizes ranging from 50 to 500 nm. Interestingly, EVs only begin to shed from a zygote (post-fertilization) and not from metaphase II oocytes, suggesting the essential role of EVs as cross-talk signaling molecules to neighbor cells. In addition, the amount of EVs in SECM was more likely to be higher in the day-5 blastocyst culture compared to that of the day-3 cleavage stage culture media.

Corroborating the previous findings, the latest breakthrough provided essential data on EVs which carry detectable amounts of genomic DNA [52]. By using array-based comparative genomic hybridization, Veraguas et al. [52] evaluated whether chromosomal conditions of seven arrested day-3 cleavage-stage embryos correspond with EVs in the respective SECM. Three out of the seven embryos had normal ploidy status (euploid embryos) which coincide with the respective EVs carrying the normal 23 pairs of chromosomes. The remaining embryos were detected to carry chromosomal abnormalities of either a loss or gain of a chromosome which is similarly reflected in the EVs analysis. However, it was highlighted that EVs in SECM samples contained higher chromosomal abnormalities compared to the respective embryos. Thus, the possibility of EVs as a representative of embryo self-correction mechanism should also be explored [54]. Overall, that study raised a possible connection between EVs and cfDNA present in the culture medium (Fig. 1).

Direct correlations between EVs and circulating cfDNA were pointed out by Marcatti et al. [55] through various stages of lung adenocarcinoma. As cell-free floating DNA is easily degraded by active DNAase I in the serum, that study found that 90% of circulating cfDNA were protected from degradation, signifying the possibility of cfDNA being carried through EVs (either exosome, microvesicles, or apoptotic bodies). It was later found that indeed, the EVs contained 90% of circulating cfDNA. Since DNA isolation step is performed in niPGT-A procedures (likewise in TE-biopsy PGT-A), it is certainly possible to isolate cfDNA from free-floating DNA or EVs in SECM.

Discussion

The discovery of embryonic fragment DNA in SECM has broken new grounds for its application in the eagerly anticipated non-invasive pre-implantation genetic testing

for aneuploidy. The presence of fragmented genomic DNA of an embryo in SECM was confirmed with a mean size of 400 bp ranging from 100 to 1000 bp [6], while blood circulating cfDNA is approximately 167 bp (160–180 bp) [40]. Embryonic cfDNA in SECM is likely longer than the blood circulating cfDNA due to a negligible quantity of nucleases in embryo culture medium environment. Driven by the promising potential of an alternative approach for non-invasive chromosomal analysis, evaluation and validation on the diagnostic value of cfDNA to replace trophectoderm biopsy have been remarkably conducted while at the same time ruling out the essential question of why and how the cfDNA is released in SECM. It is now apparent that cfDNA in SECM constitutes a mixture of embryonic and non-embryonic DNA which complicates its utilization for genetic analysis without a proper prior sample preparation as concerned by several researchers [11, 12]. Advanced genetic analysis platforms are plausible to diminish such limitations. Currently, commercial kits for niPGT-A analysis using SECM are now available and have been observed to have a similar diagnostic ability (SECM cfDNA - Variseq (Illumina) vs SECM cfDNA - NICS (Yikon)) [56]. Interestingly, niPGT-A using cfDNA could be proposed as an alternative approach when the biopsy result is inconclusive to avoid a re-biopsy procedure [57].

Mosaic embryos which are defined by the presence of a mixture of euploid and aneuploid cells were more common in humans than in animals. The aforementioned studies have proved the complexity of the conserved mechanism against embryo mosaicism to uphold embryo viability. Cell death mechanisms, specifically apoptosis, are proven to eliminate aneuploid cells during embryo development, differentiation, and even during fetal development. It is important to note that the odds of chromosomal embryonic aneuploid cells in animal models are very low and are less representative for being used to elucidate the cellular pathway of human embryo aneuploid cell elimination; hence, different patterns or additional mechanisms might exist [24]. As regards the cfDNA, it is clear that the cfDNA in SECM may originate from apoptosis-eliminated aneuploid cells; thus, a concern was raised on the feasibility of embryonic cfDNA in representing embryo ploidy status. Nonetheless, it would be less presumable to attain a high concordance rate between cfDNA and biopsy samples (for either TE or ICM or whole embryo) if cfDNA solely arises from chromosomally abnormal cells.

As cfDNA is well-explored in cancer, there is conspicuous evidence to suggest that cfDNA shedding is not limited to dying cells as it is also released into circulation by actively proliferating clone tumors. Since a tumor mass could contain multiple cell lineages, competition among tumor cell types could occur through uncontrolled proliferation, thus inducing apoptosis [38]. Considering that mosaic embryo also comprise of different cell lineages, such cellular competition might occur, leading to stress-induced apoptosis in both the euploid and aneuploid embryonic cells. Several previous studies have presumably suggested that both euploid and aneuploid embryonic cells undergo apoptosis as a consequence of active proliferation during preimplantation development [8, 58]. Consequently, the genetic status between cfDNA and the embryo could be intercorrelated. Nevertheless, the present review has observed a lack of substantial scientific proof on whether apoptosis also occurs in euploid cells of an embryo in vitro contributing to the cfDNA constituent. Finally, this review is not able to confirm whether apoptosis also occurs physiologically in euploid cells of developing embryos in vivo due to the lack of current investigation.

Cell death mechanisms particularly apoptosis have been suggested to be a common pathway for embryonic cell-free DNA release. Even so, it is likely that additional mechanisms exist to underlie the presence of cfDNA in SECM. Apoptosis was the most intensively explored pathway in the current literature [24, 27, 28, 39], while other mechanisms have yet to be fully uncovered. The emergence of embryonic secretome studies revealed that embryos also release EVs which contain nuclear DNA fragments signifying a probable connection between embryonic EVs and the presence of cfDNA in SECM [46, 52, 53]. EVs are physiologically released from one cell to another targeted cell in response to environmental conditions. This review suggests that the mechanism of embryonic EVs in relation to embryonic cfDNA is worth exploring, as it might establish an explanation for the high concordance rate between the ploidy status of embryos and cfDNA.

Conclusion

The presence of aneuploid and euploid cells within a developing human embryo is common, and there exists a natural pathway to eliminate those cells during development which could contribute to the release of cfDNA in SECM. There is still a lack of well-designed studies to prove which embryonic cell types shed the cfDNA in SECM. Apoptosis is the most well-studied mechanism which could account for the release of embryonic cfDNA, while other pathways are less explored. EVs are another plausible mechanism to elucidate the release of cfDNA in SECM and the good concordance phenomena between embryos (both ICM and TE cells) and SECM cfDNA.

Abbreviations *cfDNA*: cell-free DNA; *Evs*: extracellular vesicle(s); *HSA*: human serum albumin; *ICM*: inner cell mass; *ICSI*: intra-cytoplasmic sperm injection; *IVF*: in vitro fertilization; *niPGT-A*: non-invasive pre-implantation genetic testing for aneuploidy; *NGS*: next-generation sequencing; *PGD*: pre-implantation genetic diagnosis; *PGS*: pre-implantation genetic screening; *PGT-A*: pre-implantation

genetic testing for aneuploidy; *PGT-M*: pre-implantation genetic testing for monogenic disorders; *PGT-SR*: pre-implantation genetic testing for chromosomal structural rearrangement; *qPCR*: quantitative polymerase chain reaction; *SAC*: spindle assembly checkpoint; *SECM*: spent embryo culture media; *TE*: trophectoderm cells

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Declarations

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