PREIMPLANTATION GENETIC TESTING

Single-cell technologies at the forefront of PGT and embryo research

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Abstract

While chromosomal mosaicism in the embryo was observed already in the 1990s using both karyotyping and FISH technologies, the full extent of this phenomenon and the overall awareness of the consequences of chromosomal instability on embryo development has only come with the advent of sophisticated single-cell technologies. High-throughput techniques, such as DNA microarrays and massive parallel sequencing, have shifted single-cell genome research from evaluating a few loci at a time to the ability to perform comprehensive screening of all 24 chromosomes. The development of genome-wide single-cell haplotyping methods have also enabled for simultaneous detection of single-gene disorders and aneuploidy using a single universal protocol. Today, three decades later haplotyping-based embryo testing is performed worldwide to reliably detect virtually any Mendelian hereditary disease with a known cause, including autosomal-recessive, autosomal-dominant and X-linked disorders. At the same time, these single-cell assays have also provided unique insight into the complexity of embryo genome dynamics, by elucidating mechanistic origin, nature and developmental fate of embryonic aneuploidy. Understanding the impact of postzygotically acquired genomic aberrations on embryo development is essential to determine the still controversial diagnostic value of aneuploidy screening. For that reason, considerable efforts have been put into linking the genetic constitution of the embryo not only to its morphology and implantation potential, but more importantly to its transcriptome using single-cell RNA sequencing. Collectively, these breakthrough technologies have revolutionized single-cell research and clinical practice in assisted reproduction and led to unique discoveries in early embryogenesis.

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Historical perspective on chromosomal mosaicism in preimplantation embryos

Reproductive medicine has recently celebrated 40 years since the introduction of in vitro fertilization (IVF) into clinical practice (Niederberger *et al.* 2018). Over the last few decades paralleling improvements in single blastomere analysis revealed that the genome of human IVF embryos is notoriously unstable. The first insights about the incidence of chromosomal aberrations in early human embryogenesis were obtained using fluorescent *in situ* hybridization (FISH) for preimplantation genetic testing for aneuploidy (PGT-A) or structural rearrangements (PGT-SR) of single interphase blastomeres of IVF cleavage-stage embryos (Delhanty *et al.* 1993, 1997, Munné *et al.* 1993, 1994, Schrurs *et al.* 1993, Harper *et al.* 1995, Gianaroli *et al.* 2001, Rubio *et al.* 2003, Baart *et al.* 2006). Using DNA probes to target chromosomes, these studies demonstrated that

at least 50% of embryos have chromosomal imbalances, with the majority being diploid-aneuploid mosaics. However, only a restricted set of differentially labeled probes can be applied in a single FISH protocol, so it mostly targets only chromosomes commonly found in spontaneous abortions. Considering that aneuploidy can affect any chromosome, a significant proportion of genomic abnormalities are inherently missed, thus the copy number state for the rest of the genome remained undetermined.

Several years later, owing to improvements in whole-genome amplification (WGA) protocols, it became possible to obtain enough input material for genome-wide platforms from just 6 to 7 pg of DNA of a single cell. Consequently, the development of new genomic technologies, such as comparative genomic hybridization (CGH) on metaphase chromosomes and DNA microarrays, began to overcome the limitations of targeted low-resolution techniques. These novel

methods provided the first view into the true extent of mosaicism in cleavage-stage embryos, as they allowed to assess all 24 chromosomes. First studies using comprehensive chromosome screening (CCS) revealed that 50% of embryos are fully aneuploid and 25% are diploid-aneuploid mosaic (Voullaire *et al.* 2000, Wells & Delhanty 2000), confirming the previously detected high chromosome aberration rate in FISH studies. Large segmental chromosome imbalances were also reported for the first time in approximately 7–32% of embryos (Voullaire *et al.* 2000, Wells & Delhanty 2000, Trussler *et al.* 2004, Wilton 2005, Daphnis *et al.* 2008, Rius *et al.* 2011). Subsequent implementation of array CGH (aCGH) further increased the resolution of single-cell analysis, showing a similar rate of segmental imbalances, ranging from 15 to 58% in cleavage-stage embryos (Vanneste *et al.* 2009, Voet *et al.* 2011, Rabinowitz *et al.* 2012, Fragouli *et al.* 2013, Mertzaniidou *et al.* 2013, Babariya *et al.* 2017), but declining to 7–19% by blastocyst stage (Fragouli *et al.* 2013, Fiorentino *et al.* 2014, Babariya *et al.* 2017). However, recent rapid uptake of next-generation sequencing (NGS) technology demonstrated that blastocysts seem to be more genetically heterogeneous than previously anticipated, harboring at least 30–40% of cells with a chromosome abnormality (Munné *et al.* 2017, Popovic *et al.* 2018, Fragouli *et al.* 2019). During this time PGT-A also gained a widespread momentum, triggering extensive research to evaluate the incidence, origin and impact of chromosomal mosaicism in early embryos, which has been systematically reviewed elsewhere (van Echten-Arends *et al.* 2011, Taylor *et al.* 2014, Fragouli *et al.* 2019, Popovic *et al.* 2020).

Single-cell genotyping reveals the nature of aneuploidy in embryos

Published PGT-A studies, using FISH, aCGH or NGS, have all been instrumental in understanding the incidence of chromosomal mosaicism in embryos, but they were mainly based on single biopsy analyses and provided little information on the nature(s) of aneuploidies. To compensate for these drawbacks, several groups successfully hybridized single-cell WGA products on high-density SNP arrays to disclose genotypes and copy number state of individual cells (Iwamoto *et al.* 2007, Vanneste *et al.* 2009, Johnson *et al.* 2010, Treff *et al.* 2010). The SNP-array technology allows to genotype hundreds of thousands up to millions of SNPs, which are polymorphic in the human population, across the entire genome. By using this information, it also became possible to identify copy-neutral losses of heterozygosity, such as uniparental disomy (UPD), and to determine the parental origin of aneuploidy. A landmark study explored the genomic constitution of all individual blastomeres from 23 good-quality cleavage embryos, using array-based approaches (Vanneste *et al.* 2009). The data revealed that 80% of

day three embryos bear signatures of chromosomal instability (CIN), which is manifested by an increased rate of whole chromosome aneuploidies and segmental imbalances that were reciprocal in sister blastomeres. A high aneuploidy rate (>70%) was also reported by Mertzaniidou *et al.* (2013), following the analysis of all blastomeres derived from human cleavage embryos. Compared to previous reports, the increased aneuploidy rate is the direct consequence of investigating all cells of the embryo, rather than a single biopsy. Moreover, mosaic UPD was detected for the first time (Vanneste *et al.* 2009), the frequency of which was later estimated to be around 3.6% in day three and 0.06% in TE biopsy, respectively (Rabinowitz *et al.* 2012, Gueye *et al.* 2014). In addition, triploidy (1–1.5%) and haploidy (<1%) were also reported, and genotyping disclosed that the majority of these abnormalities were of maternal origin (McCoy *et al.* 2015).

In general, meiotic whole chromosome aneuploidy in embryos predominantly involves maternal chromosomes and the frequency of meiotic errors in the oocyte is age-dependent (Franasiak *et al.* 2014). However, meiotic segmental aberrations mainly seem to affect paternal chromosomes (Kubicek *et al.* 2019), which likely implicates sperm DNA damage as chromosome breakages. In contrast, parent-of-origin analysis revealed that there is no allelic preference in mitotic aneuploidy, as both maternal and paternal chromosomes can be equally affected (Voet *et al.* 2011, McCoy *et al.* 2015). While mitotic non-disjunction and anaphase lagging are the two main mechanisms of postzygotic aneuploidy (reviewed in Mantikou *et al.* 2012), centric fission or peri-centric instability co-determine mitotic segmental imbalances. Chromosome breakage can also lead to complex deletion-duplication profiles by triggering the breakage-fusion-bridge cycle, which yields one daughter cell with a single terminal deletion and one daughter cell with terminal deletion and an inverted duplication (Voet *et al.* 2011). Finally, mitotic errors can be accompanied by abnormal embryo morphokinetics, such as direct unequal cleavage into three or more cells (Zhan *et al.* 2016) or embryo fragmentation and micronuclei formation (Chavez *et al.* 2012).

The era of genome-wide single-cell haplotyping

Single-cell haplotyping technology and its clinical implementation

The tremendous progress in genome analysis techniques further pushed the limits of single-cell research. From SNP genotype calls it is possible to reconstruct genome-wide haplotypes, which represent a stretch of genetic variants along a single chromosome, inherited either from the mother or the father. In the past decade, haplotyping methods have been developed to a single-cell level, enabling genome-wide linkage analysis of embryos from couples, who have single-gene disorders

running in the family (Vermeesch *et al.* 2016). There are currently two main single-cell haplotyping algorithms available that use SNP-array data: karyomapping (Handyside *et al.* 2010), and haplarithmisis (Zamani Esteki *et al.* 2015), which have been successfully validated and implemented into clinical practice for preimplantation genetic testing for monogenic disorders (PGT-M) (Natesan *et al.* 2014, Konstantinidis *et al.* 2015, Ben-Nagi *et al.* 2017, Dimitriadou *et al.* 2017). Nowadays, novel single-cell haplotyping techniques also aim to replace SNP-arrays with sequencing-based analysis. The first reports used low-coverage sequencing for aneuploidy screening combined with targeted haplotyping for mutation analysis (Yan *et al.* 2015, Ren *et al.* 2016). Recently, a novel generic NGS-based workflow was developed for comprehensive PGT, called Haploseek (Backenroth *et al.* 2018). Although embryos are subjected to low-pass sequencing, this methodology still relies on SNP microarray data from parental DNA to infer paternal and maternal haplotypes. Alternatively, genotyping-by-sequencing (GBS) can be used to reduce genome size and complexity for cost-effective SNP genotyping (Elshire *et al.* 2011). This principle was recently integrated into the haplarithmisis workflow for genome-wide haplotyping and copy number profiling (Zamani Esteki *et al.* 2019a), and was also developed by Agilent Technologies as OnePGT (Masset *et al.* 2019).

The development of haplotyping-based PGT-M represents a turning point in reproductive genetics. First, it is generic and can be applied to any single-gene disorder using one universal protocol. Thus, it excludes the need to develop customized patient- or locus-specific tests, greatly reducing the work-up time and cost of PGT-M cycles. Second, because the inheritance of pathogenic variants is deduced based on reconstructed haplotypes, rather than single point mutation analysis, it allows to overcome issues, associated with WGA, such as allele drop-in or drop-out. Third, meiotic homologous recombination patterns can be identified, which can break down the linkage of a disease-associated variant with its nearby SNPs. Finally, these assays can detect a wide range of chromosome aberrations across the genome, including whole or segmental aneuploidies, UPD or loss of heterozygosity regions, which can either lead to imprinting disorders or unmask a disease-causing recessive mutation, respectively. In turn, this paves the way to comprehensive PGT, which allows to combine PGT-M and PGT-SR/PGT-A to obtain information on monogenic disorders, chromosomal structural rearrangements and aneuploidy from a single biopsy using one single assay (Fig. 1) (Natesan *et al.* 2014, Konstantinidis *et al.* 2015, Ben-Nagi *et al.* 2017, Dimitriadou *et al.* 2017, Masset *et al.* 2019). The main difference between the two approaches is that karyomapping uses only discrete SNP genotypes, which for instance allows to identify meiotic trisomies following haplotyping, while haplarithmisis can also identify postzygotic mitotic trisomies by using haplotyped

SNP B-allele frequency values. Inclusion of aneuploidy information in PGT-M cycles can have additional benefits for embryo prioritization for transfer: embryos with a normal karyotype will have the highest priority (Fig. 2A). In case of meiotic errors, all cells of the embryo will be affected (Fig. 2B), and such embryo will be discarded, even if it does not carry the disease-allele. In case of mitotic aneuploidy, only a fraction of cells will be affected, but depending on the extent of the chromosomal mosaicism, such embryos may be less viable (Fig. 2C), hence they might have lower transfer priority. The clinical value of comprehensive PGT was also demonstrated by studies, showing increased pregnancy rates per embryo transfer or PGT cycle (Ben-Nagi *et al.* 2017, Dimitriadou *et al.* 2017, Minasi *et al.* 2017). Moreover, it can increase the number of available embryos for transfer per single IVF-PGT cycle, by including OPN and 1PN embryos, which can develop into normal biparental diploid embryos, but are often discarded from further clinical use (Destouni *et al.* 2018). The drawback of haplotyping-based PGT is that it requires family members, such as offspring or parents of the prospective parents, which are not always available. Although the possibility of alternative phasing via parental siblings was reported recently (Ding *et al.* 2020).

Single-cell haplotyping reveals the diverse origin of genomic anomalies in embryos

The implementation of haplotyping-based PGT shed novel biological insight into the origin of aneuploidy in preimplantation development. Based on recombination patterns, it became possible to distinguish meiosis I (MI) and meiosis II (MII) errors, and in case of haplarithmisis also meiotic errors from mitotic. By using karyomapping, a recent study reported an increased rate of maternal MI errors in TE biopsies, compared to MII errors (Kubicek *et al.* 2019), which was at odds with previously published data (Handyside *et al.* 2012). This discrepancy can be explained by different female age groups used in the two studies (mean age 32.7 vs 40.0). Namely, MI non-disjunction is more predominant in younger females, while premature separation of sister chromatid becomes more evident as women age (Gruhn *et al.* 2019). It was also suggested that a newly discovered non-canonical reverse segregation (Ottolini *et al.* 2015a), in which sister chromatids segregate at MI and homologous chromosomes at MII, acts as a 'corrective' mechanism in women with advanced age to account for cohesion loss (Zanders & Malik 2015). Hence, the age-dependent aneuploidy in oocytes and embryos arises by distinct mechanisms, which shape the U-curve of natural human fertility (Gruhn *et al.* 2019). Intriguingly, by using haplotyping a second polar body was detected at the blastocyst stage (Ottolini *et al.* 2015), which was an interesting finding, considering that polar bodies typically disintegrate following fertilization. In

Figure 1 Comprehensive PGT workflow. Comprehensive PGT is based on genetic analysis of single embryo biopsy. During the biopsy procedure, either one or two cells are removed at cleavage stage on day 3 or five to ten cells from the trophectoderm at blastocyst stage on days 5–6. Alternatively, polar bodies can be analyzed to screen for maternal genetic defects only. Subsequently, biopsied material is whole-genome amplified and processed via SNP-arrays or NGS protocols. Using genotype and haplotype information, comprehensive PGT enables to simultaneously perform PGT-M for Mendelian disorders, PGT-A for aneuploidy and PGT-SR for unbalanced translocations with deduced mechanistic and parental origin. Based on genetic analysis, embryos are then selected for transfer.

contrast, no difference between MI and MII error rates were observed for paternal trisomies (Kubicek *et al.* 2019), but this result can be biased by the low number of detected paternal aneuploidies, hindering the true estimation of MI and MII errors in spermatogenesis.

Apart from whole or partial chromosome losses and gains, haplotyping-based approaches also detected genome-wide ploidy violations, such as haploidy, triploidy and polyploidy, in approximately 1% of cleavage stage and TE biopsies (Dimitriadou *et al.* 2017, Kubicek *et al.* 2019). Based on parental haplotypes, haploid embryos can be classified as gynogenetic (carrying only maternal DNA) or androgenetic (carrying only paternal DNA), and triploid embryos as digynic or diandric, respectively. Most triploid embryos are digynic, originating as a result of meiotic failure in the oocyte and/or polar-body non-extrusion. Haploid

embryos were also mainly gynogenetic, suggesting that after intracytoplasmic sperm injection (ICSI), the sperm cell can likely activate the oocyte, but fails to decondensate and replicate its genome. These genome-wide findings are in agreement with previously published SNP-array data (McCoy *et al.* 2015). Currently only genotyping and haplotyping-based approaches can accurately identify ploidy abnormalities with parental origin, hence the true incidence of these events in preimplantation embryos is likely underestimated. However, it is worth noting that the haplotyping-based PGT data comes from embryo biopsies, which may not be representative of the whole embryo. Hence, application of this technology on multiple individual cells of both cleavage and blastocyst stage embryos might shed further insight into the true genomic landscape of embryo development.

Figure 2 The origin and consequences of genomic abnormalities in human embryos. (A) A haploid oocyte is fertilized by a haploid sperm that develops into a normal diploid zygote that undergoes normal cell divisions ultimately resulting in a normal euploid fetus. (B) Errors in meiosis will lead to zygotic inheritance of constitutional aneuploidy that affect all blastomeres within an embryo, resulting in developmental arrest, miscarriage or congenital birth defects. (C) Postzygotically acquired genomic aberrations will affect only a fraction of cells, leading to chromosomal mosaicism. Depending on the timing and extent of postzygotic aberrations, such embryos can have reduced developmental potential, causing developmental arrest, implantation failure and miscarriage, or leading to birth of a mosaic individual. (D) Alternatively, cells with postzygotic errors can be eliminated during later stages of development, resulting in a normal individual. (E) Non-canonical postzygotic division can lead to parental genome segregation into distinct maternal (pink) or paternal (blue) cell lineages with ploidy violations that can perpetuate in development, leading to developmental arrest, miscarriage and ultimately to molar pregnancy of androgenetic origin or mixoploid individuals.

Heterogoneic cell division

Single-cell haplotyping was also used to investigate the genomic stability of bovine in vitro embryos. Based on two initial studies, the frequency and nature of chromosomal anomalies in bovine and human is comparable, with 26% of cleavage-stage (Destouni *et al.* 2016) and 68% of blastocysts (Turner *et al.* 2019) showing biparental euploid profiles. Similar to human, abnormal bovine embryos also displayed a wide range of chromosome aberrations, including monosomies, trisomies, simple and complex segmental imbalances, UPD, triploidy and parthenogenetic activation of the oocyte. Intriguingly, it was discovered that androgenetic, gynogenetic and biparental blastomeres can co-exist within a single embryo (Destouni *et al.* 2016). This novel phenomenon was termed 'heterogoneic division', defining non-canonical postzygotic division that leads to segregation of parental genomes into distinct blastomere lineages, carrying either paternal or maternal chromosomes only. Heterogoneic division was especially evident in dispermic fertilized embryos, where the extra paternal genome was segregated into a separate androgenetic cell lineage, and the resulting embryo carried a combination of diploid, triploid and haploid cells with different paternal haplotypes. Aberrant parental genome segregation was not only triggered by abnormal fertilization, as also one monospermic fertilized embryo contained four androgenetic, four gynogenetic and three biparental blastomeres (Destouni *et al.* 2016). Later mixoploidy with gynogenetic and/or androgenetic blastomeres were detected in primate cleavage-stage IVF embryos (Daughtry *et al.* 2019). Mechanisms underlying such genomic heterogeneity remain elusive, and several hypotheses have been proposed, including gonameric spindle formation or asynchronous parental cell cycles (Destouni & Vermeesch 2017). Recently, an elegant microscopy study in mice demonstrated that male and female chromosomes are assembled on separate mitotic spindles during the first zygotic division, questioning the textbook concept of syngamy (Reichmann *et al.* 2018). Intriguingly, dual spindle formation also seems to exist in humans (Xu *et al.* 2019). Hence, failure to properly align two zygotic spindles may potentially provide a mechanistic basis for heterogoneic division and mixoploidy in embryos.

Consequence of chromosomal mosaicism on embryo development

The impact of embryonic chromosomal mosaicism in humans remains unclear, raising important questions regarding the burden and nature of genomic imbalances that can be tolerated by the embryo without compromising its survival. The clinical consequences of chromosomal mosaicism in embryos depend on a variety of factors, like the timing and severity of postzygotic

errors and whether they continue to propagate during embryo development (Fig. 2). Although chromosomal mosaicism is extremely evident in cleavage embryos, accumulating evidence suggests that it can be gradually depleted by the blastocyst stage (Fragouli *et al.* 2019, Popovic *et al.* 2019). A mouse model demonstrated that aneuploid cells can be eliminated during embryo development either via apoptosis in inner cell mass or a prolonged cell cycle in trophectoderm cells (Bolton *et al.* 2016). Apoptosis of aneuploid cells in mouse embryos is linked with autophagy-mediated pathways; however, whether the same 'rescue' mechanisms operate in human embryos remains to be determined (Singla *et al.* 2020).

The increased rate of mosaicism in IVF embryos also raised concerns that the in vitro environment can compromise their genetic integrity. A negative effect of in vitro procedures was observed in bovine cleavage stage embryos (Tšuiiko *et al.* 2017). However, there was no difference in aneuploidy levels between human IVF embryos and in vivo-derived embryos, obtained via uterine lavage (Munné *et al.* 2019). Similarly, no significant difference between IVF and natural conception was observed at live birth (Zamani Esteki *et al.* 2019b). Although the latter study identified sporadic *de novo* genomic aberrations in 10% of pregnancies, they were scattered across the genome, representing random events of postzygotic aneuploidy that had no functional consequences on placental biology or fetal health. Moreover, this result suggests that the high rate of aneuploidy observed in preimplantation embryos seems to diminish during pregnancy, likely due to negative selection of aneuploid cells (Fig. 2D). The fact that chromosomal mosaicism may not be preserved at later stages of prenatal and postnatal development is also well supported by birth of healthy children, following mosaic embryo transfer (Greco *et al.* 2015, Dimitriadou *et al.* 2017, Victor *et al.* 2019). At the same time, high-grade mosaicism has been associated with implantation failure or early pregnancy loss (Fragouli *et al.* 2017, Spinella *et al.* 2018, Victor *et al.* 2019, Munné *et al.* 2020). Close monitoring of mosaic embryo transfer may also be warranted due to potential risk of true fetal mosaicism (Kahraman *et al.* 2020). The likelihood that aneuploidy can affect fetal development depends on the chromosomes involved, nature of aneuploidy and the proportion of normal and aberrant cells within the embryo (Grati *et al.* 2018). Hence, understanding the impact of postzygotically acquired aneuploidy on embryo development is essential to determine the still controversial diagnostic value of mosaic genomic aberrations found with PGT-A.

Despite the low incidence of gross genomic abnormalities detected during prenatal testing (Engelbrechtsen *et al.* 2013, Zalel *et al.* 2016), these events might exist at a higher rate at the time of conception than anticipated. Due to selective pressure, embryos

with complex genomic abnormalities often arrest in development (Rubio *et al.* 2007, McCoy *et al.* 2015), resulting in their early demise and likely contributing to relatively low human fecundity (Macklon *et al.* 2002). Although androgenetic and gynogenetic embryos can reach blastocyst stage, they are incompatible with life likely due to gross imprinting abnormalities. Androgenesis is also implicated in the formation of molar pregnancy, characterized by abnormal trophoblast growth in the uterus (Hui *et al.* 2017), which can potentially arise as a result of heterogoneic division and proliferation of androgenetic cell lineages (Fig. 2E). Currently, it is unclear what drives these embryos to develop into these tissue masses, although some women can have a genetic predisposition toward for hydatidiform mole formation (Nguyen *et al.* 2018a,b). However, if present in a mosaic state, these genome-wide aberrations can bypass the natural developmental barrier, leading to chimaerism (Winberg *et al.* 2010, Yamazawa *et al.* 2010), mixoploidy (Järvelä *et al.* 1993, Edwards *et al.* 1994, van de Laar *et al.* 2002) and mosaic genome-wide uniparental disomy (Gogiel *et al.* 2013, Inbar-Feigenberg *et al.* 2013, Darcy *et al.* 2015, Bens *et al.* 2017, Spier *et al.* 2019), which have been observed in several individuals and newborns. The sequester of entire parental genomes to distinct daughter blastomeres can likely explain the existence of chimerism and mixoploidy in humans (Fig. 2E). In addition, it was recently proposed that heterogoneic division can also underlie rare cases of sequezygotic, or 'semi-identical' twins, when a medical evaluation revealed monochorionic twin pregnancy with discordant fetal sex (Gabbett *et al.* 2019). However, the frequency of such events in natural and IVF conception is currently not known.

Future perspectives

Understanding the genesis of embryonic aneuploidy as well as its fitness consequences at cellular level is fundamental to our knowledge of human development and fecundity. For that reason, considerable efforts have been put into linking the genetic constitution of the embryo not only to its morphology and implantation potential, but more importantly to its transcriptome. RNA is the first product of gene expression, which makes it a valuable and quantifiable indicator of phenotype at preimplantation stages.

Gene expression analysis to assess embryo competence

Multiple studies hunting for clinically relevant marker genes of an embryo's genomic composition and/or developmental competence are currently available. However, no consensus has been reached, likely due to substantial variation in terms of sample type and size, embryonic stages analyzed, genomic compositions of embryos, as well as the technologies used for gene

expression analysis. In a first attempt, qPCR of 84 genes related to DNA damage signaling pathways was performed on two pools of days 3–4 embryos, one containing poor morphological quality embryos with complex aneuploidies and the other containing good quality embryos with a single chromosome aneuploidy (Bazrgar *et al.* 2014). The first showed significant overexpression of five genes. Subsequent studies increased the resolution by analyzing the transcriptome of TE biopsies (Fuchs Weizman *et al.* 2019, Groff *et al.* 2019, Ntostis *et al.* 2019) and/or complete blastocysts (Licciardi *et al.* 2018, Groff *et al.* 2019) with known whole-genome composition from a previous biopsy. Licciardi *et al.* (2018) performed RNA-seq in 68 normally developing blastocysts having single monosomies or trisomies and three euploid blastocysts. They describe a gene set of six commonly up-regulated and 18 commonly downregulated genes in most of the aneuploid embryos compared to the ~3000 gene signature shared among normal blastocysts. Interestingly, viable trisomies, as well as sex chromosome aneuploidies, are transcriptionally most similar to euploid blastocysts (Licciardi *et al.* 2018). With the aim of relating gene expression to karyotype as well as other classical embryo phenotypic features (i.e. morphology and morphokinetics), Groff *et al.* (2019) performed RNA-seq and differential expression (DE) analysis in whole blastocysts ($n = 39$) for which digital karyotypes were inferred from the RNA-seq data. Interestingly, euploid vs aneuploid showed 53 differentially expressed genes, whereas high vs low morphological and morphokinetic quality showed only ten and nine differentially expressed genes, respectively (Groff *et al.* 2019), hinting toward a stronger link between gene expression and aneuploidy rather than with morphological/kinetic features. Additionally, a pilot study determined 47 differentially expressed genes between four implanted and four non-implanted euploid embryos using RNA-seq (Ntostis *et al.* 2019). Other authors propose that gene expression characterization of TE biopsies might serve as an additional embryo competence marker rather than a substitute for CCS (Marin *et al.* 2017, Fuchs Weizman *et al.* 2019). They provided proof-of-principle for methods for simultaneous gene expression determination and CCS of the same biopsy. The method of Marin *et al.* (2017) is qPCR-based and determines the expression of 30 genes plus CCS. Even though they described differentially expressed genes between monosomy and trisomy 21 blastocysts ($n=20$), no normal euploid blastocysts were included. In contrast, Fuchs Weizman *et al.* (2019) performed simultaneous gDNA and mRNA sequencing and DE analysis of TE biopsies (PGT-AT) on 17 blastocysts and revealed 1574 transcripts differentially expressed between euploid and aneuploid TE biopsies, but those were only aneuploid for chromosome 16. The first study tackling this question at a single-cell level across the whole preimplantation

development, used 53 embryos and processed half of each embryo cells for gene expression analysis of 90 genes by qPCR ($n=92$) and the other half for CCS using aCGH ($n=76$) (Vera-Rodriguez *et al.* 2015). They found a signature of 12 genes dysregulated in aneuploid embryos but only before day 3, which led to the hypothesis that maternal transcripts inherited from the oocyte might play a causal role in embryonic CIN (Vera-Rodriguez *et al.* 2015). Taken together, despite the very valuable samples and elegant experimental designs, none of the genes is confirmed to be dysregulated in the same direction under the same conditions in different studies. Therefore, the clinical application of those findings still requires further investigation.

Human embryo development uncovered by single-cell RNA-seq

Understanding the fate and effect of aneuploid cells in an environment of euploid cells during preimplantation development (Bolton *et al.* 2016, Singla *et al.* 2020) and beyond (Shahbazi *et al.* 2016, Popovic *et al.* 2019) will be of particular relevance to improve current PGT-A decision making when only mosaic blastocysts are available. Development and advances in single-cell omics technologies have revolutionized our understanding of cellular heterogeneity and gene expression regulation in health and disease. The application of these methods is paving the way toward deciphering the biological mechanisms and consequences of mosaicism in the human embryo.

Single-cell RNA-seq (Tang *et al.* 2009, Picelli *et al.* 2014) has been applied to human preimplantation embryos to disclose the most fundamental features of development, like gene expression networks and transcriptomic cell lineage differentiation. The first studies included only 3–30 cells per stage, ranging from oocytes to morula (Xue *et al.* 2013) or blastocysts (Yan *et al.* 2013). Nevertheless, sensitivity and coverage were substantially improved compared to previous microarray techniques (Vassena *et al.* 2011) detecting >10,000 transcripts per cell on average (Xue *et al.* 2013, Yan *et al.* 2013). Cluster analysis of the cells revealed major transcriptional differences first, between cells of different embryonic day of development and secondly, between cells before and after establishment of EGA at 8-cell stage. According to that, genes can be classified by their different expression patterns along with development, which relate with the course of maternal RNA degradation, EGA, and metabolic needs of the embryo (Xue *et al.* 2013, Yan *et al.* 2013). For example, genes related to oogenesis, cell cycle and mitosis decrease their expression from the zygote to the 4-cell stage, the point when genes related to EGA peak their expression (Yan *et al.* 2013). Subsequently, genes related to transcription, RNA processing, ribosome biogenesis and cell adhesion start their expression,

becoming maximum at early blastocysts and decreasing thereafter. Lastly, genes related to energy metabolism, oxidative phosphorylation and cellular respiration express specifically in late blastocysts (Xue *et al.* 2013). At the blastocyst stage, the first cell differentiation occurs to inner cell mass (ICM) or TE and ICM cells further differentiate in primitive endoderm (PE) or epiblast (EPI) (Shahbazi & Zernicka-Goetz 2018). With the aim to shed light on this cornerstone of human embryo development, Petropoulos *et al.* (2016) analyzed RNA-seq data of 1529 cells from 88 human embryos from the 8-cell to late blastocysts stage. They describe how differentiation of TE, EPI and PE occur simultaneously at the establishment of blastocyst stage and is preceded by co-expression of lineage-specific genes in cells during day five (Petropoulos *et al.* 2016). Moreover, single-cell transcriptome profiling has already been applied on in vitro cultured embryos through the peri-implantation stages (Shahbazi *et al.* 2016) revealing the regulatory networks acting on the further differentiation of trophoblast, primitive endoderm and epiblast up until the formation of the primitive streak (Xiang *et al.* 2020). Human embryo single-cell RNA-seq datasets (Xue *et al.* 2013, Yan *et al.* 2013, Petropoulos *et al.* 2016) are a very valuable resource and have been used by others not only to study gene signatures of cell differentiation (Blakeley *et al.* 2015, Stirparo *et al.* 2018), EGA (De Iaco *et al.* 2017), embryo metabolism (Zhao *et al.* 2019a) and expression dynamics of sex chromosomes (Zhou *et al.* 2019a), but also to provide insight on transcriptional regulation during human preimplantation development (Grow *et al.* 2015, Hasegawa *et al.* 2015, Bouckenheimer *et al.* 2016). In addition, the impact of aneuploidy on the transcriptome at single cell level has been investigated for the first time by computationally inferring the DNA copy number genome-wide from publicly available single-cell RNA-seq data (Petropoulos *et al.* 2016, Starostik *et al.* 2020). By doing so, similar signatures of aneuploidy were detected, which were previously described for other cell types, such as downregulation of genes involved in proliferation, metabolism and protein processing in addition to an upregulation of immune response genes (Santaguida & Amon 2015, Chunduri & Storchová 2019).

Single-cell multi-omics technologies

For the aforementioned transcriptional changes to occur and to restore its pluripotency, the zygote undergoes remarkable epigenetic changes, including erasure and re-establishment of DNA methylation, post-translational modifications of histones, chromatin remodeling and higher order genome reorganization (reviewed in Eckersley-Maslin *et al.* 2018). Since the development and application of single-cell RNA-seq, new technologies to measure each of these epigenetic signatures one cell at a time were created (reviewed

by Wen & Tang 2018). Their utilization helped to uncover key features in many cell types, including human embryos (Smith *et al.* 2014, Guo *et al.* 2017, Zhu *et al.* 2018). Thus, the interest on how these different molecular levels interact to regulate gene transcription, launched the field of single-cell multi-omics technologies (reviewed by Stuart & Satija 2019), which has been elected method of the year by Nature Methods ('Method of the Year 2019: Single-cell multimodal omics', 2020). Exciting multi-omics studies in human embryos are currently flourishing (Li *et al.* 2018, Zhou *et al.* 2019b) and will hopefully shed light on many remaining questions. In particular, multi-omics technologies that permit determining the genomic landscape of the cell together with other molecular changes such as RNA (Macaulay *et al.* 2015), epigenetics and/or chromatin structure (Li *et al.* 2018) – potentially together with CRISPR-Cas9 technologies (Fogarty *et al.* 2017) – will be of great interest to uncover the molecular aspects of mosaicism along human pre- and peri-implantation development.

Declaration of interest

T V and J R V are co-inventors on licensed patents WO/2011/157846 (Methods for haplotyping single cells); WO/2014/053664 (High-throughput genotyping by sequencing low amounts of genetic material); WO/2015/028576 (Haplotyping and copy number typing using polymorphic variant allelic frequencies). The other authors have nothing to disclose.

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Author contribution statement

O T and E F G drafted the original manuscript. J R V and T V provided feedback and performed critical manuscript revision. All authors approved the final version of the manuscript.

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