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Evolution of aneuploidy up to Day 4 of human preimplantation development

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STUDY QUESTION: What is the incidence of aneuploidy and mosaicism in all cells of top-quality Day-4 embryos analysed by array-based comparative genomic hybridization (array CGH)?

SUMMARY ANSWER: Our data show extensive abnormalities in Day-4 embryos.

WHAT IS KNOWN ALREADY: Numerous studies on human embryos at Day 3 and Day 5 of development show that they frequently contain aneuploid cells and are mosaic, although Day-5 embryos contain proportionally more normal cells than at Day 3. In contrast, only limited data exist on Day 4 of preimplantation development, despite the fact that it is the suggested stage for the initiation of the process of self-correction.

STUDY DESIGN, SIZE, DURATION: Thirteen embryos were analysed: four fresh good-quality preimplantation genetic diagnosis (PGD) embryos and nine good-quality surplus embryos cryopreserved on Day 3 and donated for research. On Day 4, following removal of the zona pellucida, all blastomeres were disaggregated and collected.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The genomic DNA of 283 single blastomeres from disaggregated embryos was amplified. Array CGH was carried out using 24SureTM Cytochip microarrays. After scanning of the microarray slides, the images were analysed using BlueFuse Software (BlueGnome). Combined with selective microsatellite analysis, hypothetical reconstructions of embryo chromosome complements were made following each of the first four cleavage divisions.

MAIN RESULTS AND THE ROLE OF CHANCE: No chromosome imbalance was detected for one PGD embryo, the other three were mosaic containing between 16 and 75% abnormal cells. All nine frozen–thawed embryos were abnormal. Six were mosaic with between 30 and 100% abnormal cells; three had abnormalities of meiotic origin, two of which displayed mitotic abnormalities. Evidence was also found of mitotic unbalanced structural chromosome rearrangements. The higher rate of abnormality of frozen–thawed embryos is based on a small number of embryos and cannot be tested statistically. The aneuploidy can mostly be explained by anaphase lag and non-disjunction. In some cases, we hypothesize endoreduplication followed by a cellular division with multipolar spindles to explain the results.

LIMITATIONS, REASONS FOR CAUTION: Array CGH technology determines relative quantification of chromosomal domains but does not allow for the visualization of chromosomal rearrangements, assessment of ploidy or detection of uniparental isodisomy. Conclusions drawn on segmental abnormalities should be treated with caution. The division trees presented are hypothetical models projecting back in time that try to explain observations in single blastomeres of Day 4 embryos. The limited number of embryos analysed does not allow drawing firm conclusions, but nevertheless provides valuable data on the origin of aneuploidy in human embryos.

WIDER IMPLICATIONS OF THE FINDINGS: Our data show extensive abnormalities in Day-4 embryos. We found no evidence of self-correction at this stage of development, suggesting that this process may start at a later stage of development.

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Key words: aneuploidy / chromosomal abnormalities / PGD

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Introduction

The high incidence of chromosome abnormalities in human gametes and early preimplantation embryos provides a partial explanation both for the relatively low fertility and for the low success of IVF treatment cycles. The development of preimplantation genetic diagnosis (PGD) and progress in cytogenetic techniques have offered valuable insight into the chromosomal status of human gametes and preimplantation embryos and demonstrated that errors leading to aneuploidy can arise not only during meiosis but also after fertilization, during preimplantation embryonic development (Delhanty *et al.*, 1997; Kuliev *et al.*, 2003; Vanneste *et al.*, 2009; Fragouli *et al.*, 2010).

The vast majority of the data on the chromosomal status of human preimplantation embryos come from studies on first and second polar bodies and oocytes, Day-3 cleavage-stage embryos and blastocysts on Day 5 and Day 6 of development (Voullaire *et al.*, 2000; Fragouli *et al.*, 2006, 2011). In contrast, the Day-4 embryo has received very little attention despite the fact that during this period, important morphological and developmental changes such as compaction and cavitation occur.

Oocytes show very variable aneuploidy rates, ranging from 22 to 72%, and embryos at Day 3 of development frequently contain aneuploid cells, up to 91% present in mosaic form, i.e. consisting of several different cell lines (Delhanty *et al.*, 1997; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Kuliev *et al.*, 2003; Gutierrez-Mateo *et al.*, 2004; Baart *et al.*, 2006; Fragouli *et al.*, 2006, 2010, 2011; Daphnis *et al.*, 2008; Vanneste *et al.*, 2009; Geraedts *et al.*, 2011; van Echten-Arends *et al.*, 2011; Mertzanidou *et al.*, 2013). At a later stage of development, blastocysts still show chromosomal abnormalities and mosaicism, although the data suggest a decrease in the proportion of aneuploid cells (Magli *et al.*, 2000; Coonen *et al.*, 2004; Fragouli *et al.*, 2008, 2011; Santos *et al.*, 2010; van Echten-Arends *et al.*, 2011).

To explain this apparent self-correction, several authors have proposed preferential growth of the euploid cells or preferential allocation of the normal cells to the inner cell mass (Wells and Delhanty, 2000; Fragouli *et al.*, 2008; Barbash-Hazan *et al.*, 2009). Furthermore, the embryonic genome is not yet fully active at the third cleavage stage (Tesarík *et al.*, 1986; Braude *et al.*, 1988; Vassena *et al.*, 2011), which may lead to depletion of the maternal transcripts responsible for the cell cycle control and thus to a high rate of chromosome abnormalities. Upon activation of the embryonic genome, the mechanisms of apoptosis and cell cycle checkpoints would become active leading to a decrease in abnormal cells at the blastocyst stage.

Mosaic embryos often arrest before reaching the blastocyst stage and it is suggested that this elimination occurs around the morula stage (Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002). In spite of these highly suggestive data pointing to the morula as a pivotal stage in preimplantation development, only limited data exist on Day-4 embryos (Baart *et al.*, 2007; Santos *et al.*, 2010). These studies analysed cryopreserved morula-stage embryos using fluorescent *in situ* hybridization for 15 chromosomes. Baart *et al.* (2007) analysed 12 morulas and found that all embryos were mosaic. Santos *et al.* (2010) analysed 18 morulas and reported a decreasing incidence of mosaicism from 83% on Day-4 embryos to 42% on Day-8 blastocysts.

The aim of this study was to investigate the incidence of aneuploidy and mosaicism in top-quality Day-4 preimplantation embryos by studying the full chromosomal content of each cell.

Materials and Methods

Source of cryopreserved and fresh embryos at Day 4

The experiments were approved by the local Ethical Committee and by the Federal Committee for Medical and Scientific Research on Human Embryos *in vitro*. Patients donating embryos for research signed an informed consent approved by the same committees. A total of 13 embryos were analysed. Four of these were fresh good-quality PGD embryos diagnosed as affected for the monogenic disease under consideration. The remaining nine embryos were good-quality surplus embryos, cryopreserved on Day 3 of development and donated for research after a legally determined period of 5 years. They were frozen–thawed using slow freezing–thawing dimethylsulphoxide standard methods (Van den Abbeel *et al.*, 1997) and cultured overnight. Care was taken to select embryos with excellent survival rate after thawing procedure and that cleaved further during their overnight culture. All embryos were cultured individually in 25 µl culture droplets in low oxygen conditions (5% O_2) under oil. Table I shows the information concerning the indications for IVF or PGD, maternal ages, the stimulation protocols, culture media, as well as embryo quality (Gardner and Schoolcraft, 1999; Balaban *et al.*, 2011) and cycle outcomes.

Array comparative genomic hybridization

Following morphological evaluation on the morning of Day 4 of development, the embryos were rinsed with and placed in 20 µl of Ca^{2+}/Mg^{2+} free buffered medium (Vitrolife G-PGD, Stockholm, Sweden) under oil at 37°C for up to 15 min (De Paepe *et al.*, 2012). After removing the zona pellucida using 5 µl of Tyrodes acid, all single blastomeres were washed and collected into sterile 0.2 ml polymerase chain reaction (PCR) tubes containing 2 µl of phosphate-buffered saline (Cell Signalling Technologies, Beverly, MA, USA) with 0.1% polyvinylpyrrolidone as previously described (Spits *et al.*, 2006). The genomic DNA of the blastomeres was amplified using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK), according to the manufacturer's instructions. Array comparative genomic hybridization (array CGH) was carried out using 24Sure™ Cytochip microarrays following the standard protocol (BlueGnome 24sure protocol, www.cytochip.com). After scanning of the microarray slides, the images were analysed using BlueFuse Software (BlueGnome). All genomic positions refer to the human genome build NCBI37. The minimum threshold to consider an aberration was set at 13 consecutive probes. The smallest segmental imbalance detected in this study was 6.57 Mbp. In the cases of unclear calls, or where due to the chaotic nature of the cells the BlueFuse Software did not produce any calls, a web-based implementation of the circular binary segmentation (CBS) algorithm was used to analyse the data (Olshen *et al.*, 2004; http://compbio.med.harvard.edu/CGHweb/). An aberration was called from the CBS method if it included at least 13 consecutive clones. In the case of sex-mismatch, the threshold to call an abnormality was set using the X chromosome separation, following the instructions of the manufacturer of the arrays. Otherwise, gain was called when the mean $\log_2$ ratio of the segment was >0.3 and loss was called for $\log_2$ ratios of less than -0.5. Two aberrations were called as one if the gap between them was <13 clones. Three representative examples of the analysis of the array CGH data can be found in the Supplementary data, Fig. S1. The GEO accession number for the array CGH data reported in this paper is GSE39518.

Table I Information of patients' characteristics, treatment protocols, cause of infertility and outcome of the IVF cycle.

	Frozen–thawed embryos									Fresh PGD embryos			
Patient information	**Embryo 1**	**Embryo 2**	**Embryo 3**	**Embryo 4**	**Embryo 5**	**Embryo 6**	**Embryo 8**	**Embryo 9**	**Embryo 7**	**Embryo 10**	**Embryo 11**	**Embryo 12**	**Embryo 13**
Female age	26	32	34	27	34(donor) 37(acceptor)		34		29(donor) 32(acceptor)	39	34	30	30
Stimulation	Agonist	Antagonist	Antagonist	Antagonist	Agonist		Antagonist		Agonist	Antagonist	Agonist	Antagonist	Antagonist
FSH IU/Oocyte	110	111.11	192.857	180	75		100		218.18	425	385.714	71.052	109.09
Insemination method	ICSI	ICSI	ICSI	ICSI	ICSI		ICSI		IVF	ICSI	ICSI	ICSI	ICSI
Medium until Day 3	Medicult	Sage	Vitrolife	Medicult	Vitrolife		Vitrolife		Vitrolife	Sage	Sage	Sage	Sage
Infertility cause	Male OAT	Female male PCO/ OAT	Male OAT	Male OAT	Female male Acceptor eggs		Male FNA		Female Acceptor eggs-POI	PGD OAT	PGD N	PGD N	PGD N
Embryo evaluation													
Day 1	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN	2PN
Day 1.5	2G1	2PN	1G1	2PN	2G1	2G1	2G1	1	2G1	NA	NA	NA	NA
Day 2	5G2	4G1	4G1	4G1	5G2	5G2	6G1	4G1	4G2	NA	8G1	NA	NA
Day 3	11G2	10G1	9G1	C2	9G1	11G2	12G1	7G1	Compacting G1	8G2	10G1	8G1	8G2
Day 4	16G1	10G1	13G1	11G1	Compacting G2	Compacting G2	B1	Compacted G1	Compacting G2	Compacted G1	Compacted G1	Compacted G1	B1
Survival after thawing	12/12 cells	12/12 cells	9/9 cells	8/8 cells	9/9 cells	8/8 cells	14/15 cells	11/12 cells	10/10 cells	–	–	–	–
Biopsy	–	–	–	–	–		–		–	2 cells	1 cell	2 cells	1 cell
Pregnant before	FET	FET	No	FET	Acceptor, no; donor unkown		No		Acceptor no, donor yes	Spontaneous	Spontaneous	Spontaneous	No
Cycle outcome	Live birth	No	Live birth	Live birth	Acceptor biochemical		Live birth		Acceptor no, donor live birth	Ongoing pregnancy	Ongoing pregnancy	No embryo transfer/OHSS	No

OAT, oligo-astheno-teratozoospermia; PCO, polycystic ovaries; POI, Premature Ovarian Insufficiency, donor oocytes in anovulatory patient; FNA, fine needle aspiration; NOA, non-obstructive azoospermia, N, normozoospermia; NA, not assessed; PGD, preimplantation genetic diagnosis; OHSS, ovarian hyper stimulation, no embryo transfer; FET, frozen embryo transfer. Embryos had been cultured in the following media until Day 3: G Series (Vitrolife, Stockholm, Sweden, fertility@vitrolife.com), BlastAssist (Medicult-Origio Benelux, Vreeland, The Netherlands) or Quinn's Protein Plus Cleavage Media (Sage *In Vitro* Fertilization, Inc. Trumbull, USA, www.coopersurgical.com), thawed embryos and PGD embryos were cultured in Quinn's Advantage Protein Plus Blastocyst Medium (Sage *In Vitro* Fertilization, Inc. Trumbull, USA, www.coopersurgical.com). Embryos were scored as follows: fertilized (two pronuclei or 2PN) on Day 1; cell stage and grade (G1 good-quality with <10% fragmentation, stage-specific cell size and no multinucleation; G2 fair quality with 10–25% fragmentation, stage-specific cell size for the majority of the cells and no multinucleation) on Day 1.5 (early cleavage); cell stage and grade (G1 good-quality with <10% fragmentation, stage-specific cell size and no multinucleation; G2 fair quality with 10–25% fragmentation, stage-specific cell size for the majority of the cells and no multinucleation) and the stage of compaction and grade (G1 good-quality, compacted, entered into the fourth round of cleavage and evidence of compaction that involves virtually all the embryo volume; G2 fair quality, compacting, entered into the fourth round of cleavage and compaction involves the majority of the volume of the embryo) or blastulation (B1 early blastocyst, the cavity represents <50% of the volume of the embryo) on Days 2–4.

Fluorescent microsatellite PCR

When indicated, the cells were also studied by fluorescent PCR for microsatellite markers. The Sureplex amplification material was purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Vilvoorde, Belgium) and used as a template for conventional PCR. Primer sequences and PCR conditions can be obtained upon request. The products were run on a capillary electrophoresis system ABI PRISM® 3130 XL Genetic Analyzer and the data interpreted using Genemapper 4.0 (Life Technologies, Ghent, Belgium).

Virtual reconstruction of cell divisions up to Day 4

Daily morphological evaluation of the embryos provided us information regarding their cell numbers, allowing us to determine the number of cleavages they underwent. This, along with the analysis of all cells, made it possible to reflect on the developmental history of each embryo and to create a virtual reconstruction of their day-by-day chromosomal status from zygote until Day 4, always choosing the simplest possible mechanisms (either non-disjunction, anaphase lagging, chromosome breakage or endoreduplication) that could explain the final outcome.

Results

Based on our results it would appear that top-quality fresh Day-4 embryos show lower levels of aneuploidy and mosaicism than frozen–thawed ones of the same morphological quality, although the small number of embryos does not allow for statistical analysis or firm conclusions. Figure 1 shows the breakdown of the types of abnormalities per blastomere, for frozen–thawed and fresh PGD embryos, categorized into single monosomies, single trisomies, complex aneuploidy (more than one chromosome involved) and segmental aberrations.

At the embryo level, one out of the four fresh PGD embryos had normal chromosomal complement in all the analysed cells. The other three embryos were mosaic containing 16, 50 and 75% of abnormal cells, respectively. None of the nine frozen–thawed embryos was completely normal. Only one embryo showed normal chromosomal complement in two-thirds of the analysed blastomeres. In four embryos, more than half and up to 76.5% of the analysed blastomeres contained chromosomal abnormalities. In one embryo, all the analysed cells were aneuploid. The remaining three embryos had abnormalities of meiotic origin, which in two of them co-existed with mitotic abnormalities in several cells.

Figures 2 and 3 propose a reconstruction of the evolution of chromosomal contents of embryos 1 and 6. These figures also show representative examples of the array CGH plots and PCR results. The hypothetical reconstructions for the other embryos in this study can be found in the Supplementary data, Figs S2–S6. Supplementary data, Table SI shows the detailed chromosomal complements per cell.

For embryo 1, we analysed cells 2, 3, 4, 5, 11, 12, 13 and 14 by PCR. We studied 11 markers for chromosome 18 and four markers for chromosomes 19 and X (Fig. 2). The combination of PCR results and $\log_2$ ratios supports the conclusion that all losses in these cells are in fact nullisomies. Moreover, chromosomes 19 and X show uniparental isodisomy for cells 11, 12, 13 and 14. Intriguingly, cells 11 and 12 contain one of the chromosomes and cells 13 and 14 the other. The most likely explanation for this striking pattern is that one cell underwent DNA replication without cell division (endoreduplication) and proceeded to divide with a tetrapolar spindle. During this division, numerous chromosomes failed to properly attach to the spindles. In some cases one of the cells would receive no chromosome at all, leading to nullisomy, and in other cases there was non-disjunction of sister chromatids leading to the observed isodisomy. An illustration of this process can be found in Fig. 2d.

To explain our findings in chromosome 18, we carried out PCR for seven microsatellite markers located on chromosome 18 on all cells of embryo 6 and 4 cells of embryos 8 and 9. The three embryos were donated by the same couple and were thus genetically related. We were able to identify four different chromosomes 18 in the three embryos, and proved that all cells of embryo 6 with two chromosomes 18 contained only one type of chromosome, indicating uniparental isodisomy (Fig. 3c). Furthermore, cells 2, 3 and 4 consistently failed to amplify for any of the markers on chromosome 18, whilst PCR for markers on chromosomes 19 and X did yield results. This, in combination with the fact that in these cells the $\log_2$ ratio of chromosome 18 is equal to the $\log_2$ ratio of the Y chromosome, suggests that they are nullisomic for chromosome 18. As can be seen from Fig. 3a, we propose that embryo 6 possibly developed from a zygote with monosomy 18. This may have been followed by a non-disjunction of chromosome 18, leaving three cells with a uniparental disomy for chromosome 18, and 3 sibling cells with a nullisomy 18.

Discussion

The reconstructions shown in Figs 2 and 3 and Supplementary data, Figs S2–S6 were generated following the principle of parsimony and assuming the involvement of well-known mechanisms of aneuploidy. In this study, chromosome non-disjunction and anaphase lagging were the mechanisms that most frequently could explain the chromosomal content of the embryos, and are also the most commonly described embryonic mitotic errors in the literature (Delhanty *et al.*, 1997; Daphnis *et al.*, 2005; Munné *et al.*, 2005; Mantikou *et al.*, 2012). Mitotic non-disjunction occurs when during mitosis the sister chromatids do not separate properly. As a result, one daughter cell has a gain and the other daughter cell has a reciprocal loss of a chromosome. Anaphase lagging takes place when one chromatid fails to attach to the spindle because of delayed movement during anaphase and is lost. This results in one daughter cell with a normal number of chromosomes and the other with a monosomy for the lagging chromosome. From our data, it is striking that whilst non-disjunction seemed to frequently involve large numbers of chromosomes in one cleavage (see for instance embryo 5), anaphase lagging typically affects only one or two chromosomes.

Endoreduplication occurs when one chromatid at the end of the S-phase is duplicated leading to the presence of three chromatids. Upon cell division, one cell ends up with the correct disomy while the other daughter cell is trisomic (Mantikou *et al.*, 2012). This phenomenon is quite frequent in cancer where it usually affects the whole chromosome set (Storchova and Pellman, 2004). Endoreduplication has been described in embryos and gametes in two singular studies in which metaphase spreads were obtained from human preimplantation material (Veiga *et al.*, 1987; Rosenbusch *et al.*, 1998). Interestingly, in one of these studies (Rosenbusch *et al.*, 1998), endoreduplication actually occurred only for two of the chromosomes.

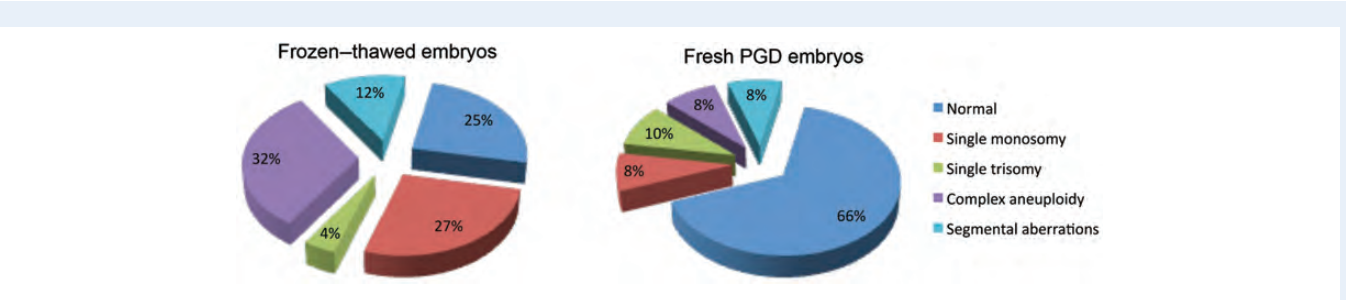

Figure 1 Breakdown of the types of abnormalities per blastomere for frozen–thawed and fresh PGD embryos, categorized into single monosomies, single trisomies, complex aneuploidy (more than one chromosome involved) and segmental aberrations. PGD, preimplantation genetic diagnosis.

Endoreduplication of single chromosomes could explain trisomies that appear in one cell without the reciprocal monosomies showing in the sibling cell. Endoreduplication of the full chromosome set followed by a cell division with a multipolar spindle may lead to cells with chaotic chromosomal content, as for instance seen in embryo 1 in this study.

Partial gains and losses that affect a whole or a small part of a chromosome arm can be explained, amongst others, by early mitotic unequal sister chromatid exchange and by chromosome breakage, sometimes followed by fusion (Voullaire *et al.*, 2000; Vanneste *et al.*, 2009; Mantikou *et al.*, 2012). In this study, 12 and 8% of the blastomeres of frozen–thawed and PGD embryos, respectively, contained segmental imbalances, which are lower than the reported frequencies in Day-3 embryos (Daphnis *et al.*, 2008; Vanneste *et al.*, 2009; Mertzanidou *et al.*, 2013).

A point to bear in mind is that array CGH only provides the copy number of the regions included in the array, and that it is not able to detect balanced chromosomal abnormalities and changes in ploidy state. Only visual inspection of the chromosomes would allow this, and could give information on the real structure of segmental imbalances (Scriven, 2013). It is possible that several of the abnormalities we found are, in fact, ring, dicentric or derivative chromosomes. For instance, cell 12 from embryo 6 may have a ring chromosome 1 [r(1)(p12q44)]; cell 2 of embryo 5 could carry a dicentric chromosome 7 [idic(7)(q21.1)]; and cell 9 of embryo 6 could have an unbalanced translocation with a derivative chromosome 5 [der(5)t(5;12)(q23.3;q12)].

To test our hypothesized virtual constructions, a number of chromosomes in several embryos of this study were haplotyped by PCR for microsatellite markers. The most interesting finding is several cases of uniparental isodisomy. In the case of embryo 6, the isodisomy most likely originated by a non-disjunction of chromosome 18 in an embryo with a meiotic monosomy 18. Embryo 1 showed several cells with chaotic chromosomal content, including several isodisomic chromosomes. The most striking feature of these isodisomies was that two cells contained one of the chromosomes, whilst the other pair of cells contained the other. This finding is identical to the uniparental isodisomy reported for embryo 20 in the work of Vanneste and collaborators (2009). Whilst these authors suggested that non-disjunction could be responsible for this, in a division reminiscent of meiosis I, in our case the most likely explanation for embryo 1 was that a cell underwent a tetrapolar division after a cycle of endoreduplication. Multipolar spindles have been commonly observed in different stages of human preimplantation development (Chatzimeletiou *et al.*, 2005, 2012). In our study, it could have been the mechanism behind the abnormalities seen in some of the cells of embryos 1, 2, 5 and 8.

When considering the results at the embryo level, at first glance, an adverse effect of the freezing–thawing procedure appears as a likely explanation for the higher aneuploidy rates in the frozen–thawed embryos when compared with the fresh PGD embryos (75.2 versus 35.1% per blastomere rate). However, the hypothetic reconstructions would suggest that in most embryos the abnormalities occurred prior to freezing and that in the consecutive cleavages aberrations were only added to the abnormal cell lines.

Alternatively, the higher rates of abnormalities found in the frozen–thawed embryos may be linked to patient and treatment characteristics such as maternal age, treatment indications, stimulation protocols or culture conditions. Gianaroli *et al.* (2010) reported an inverse correlation between the ratio of normal oocytes and female age, causes of female infertility, poor prognosis indications and the number of FSH units per oocyte. We did not find any apparent correlation to these factors, but our data set includes a small number of patients. On the other hand, the frozen–thawed embryos were donated from patients who underwent IVF treatment due to male and/or female infertility, while the fresh embryos were coming from PGD patients with proven fertility. Therefore, infertility possibly correlates with a higher incidence of chromosomal abnormalities during preimplantation development. The involved mechanisms are unknown, but are reminiscent of the higher incidence of children born after ART who have imprinting disorders such as Beckwith–Wiedemann syndrome. Here too, it has been suggested that the occurrence of these disorders is not related to ART in itself, but is rather related to the infertility of the couples (Horsthemke and Ludwig, 2005).

Based on studies of the chromosomal content of the same embryo on Day 3 and Day 5, it is generally accepted that preimplantation embryos become chromosomally more normal during their growth to blastocysts (Barbash-Hazan *et al.*, 2009). Our data on Day-4 embryos show that they are still very similar to Day-3 embryos, known to be mosaic in 73% and euploid in 22% (Van Echten-Arends *et al.*, 2011). Eleven of the embryos in this study had varying degrees of mosaicism (84%) and only one was uniformly euploid (7.5%). One more embryo had a majority of normal cells (32 out of 38 cells). At the blastomere level, the results on both frozen–thawed and fresh embryos fall in the same range as data coming from several studies analysing single blastomeres of Day-3 embryos by CGH and array CGH. In these, between 29.3 and 56% of the blastomeres were

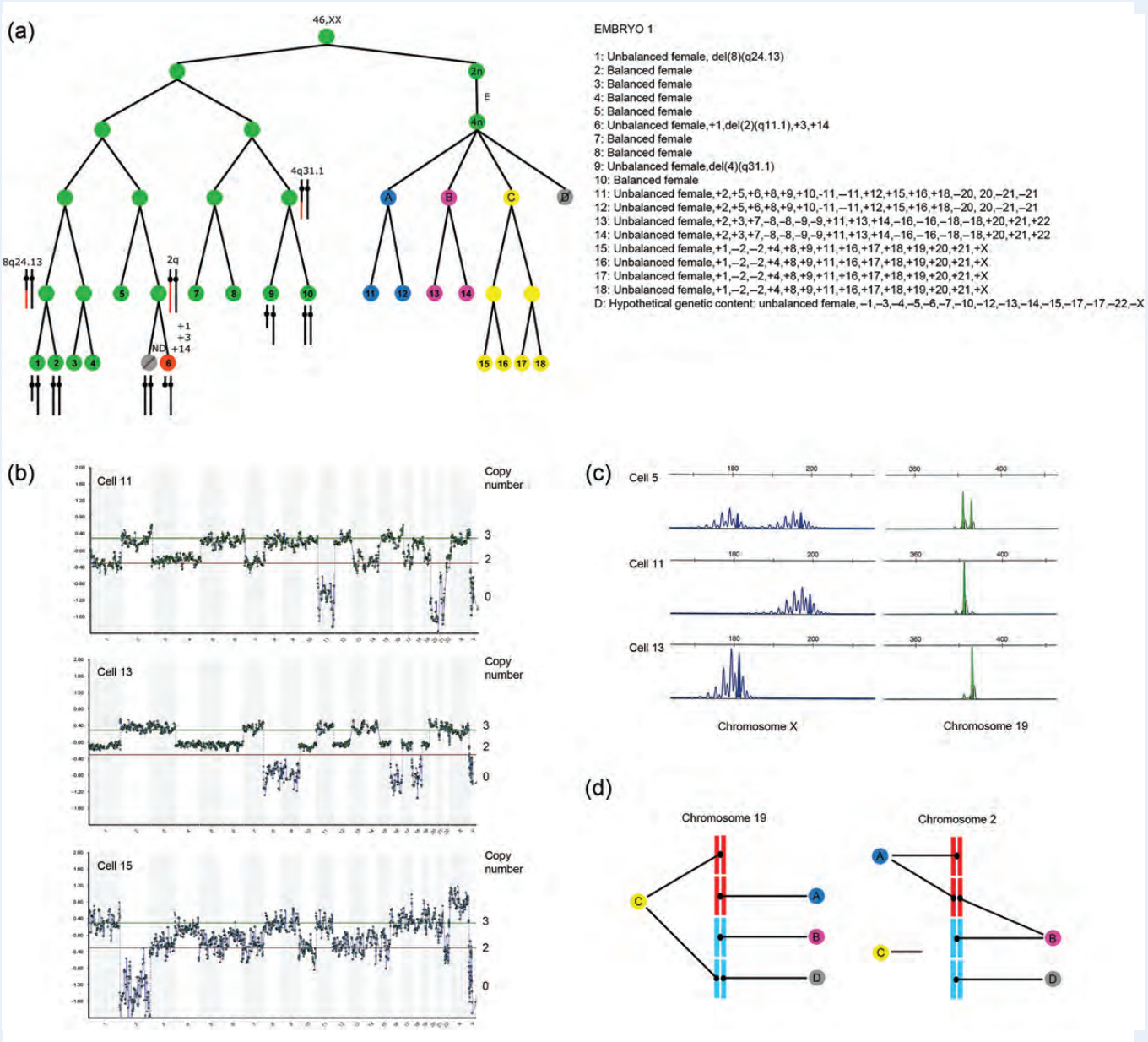

Figure 2 Reconstruction of the evolution of the chromosomal content of embryo 1. (**a**) The cell divisions tree. ND stands for non-disjunction and E for endoreduplication. Euploid cells (not taking segmental aberrations into account) are in green; aneuploid cells from the same lineage have distinct colours, different from green. Grey cells with a crossing line are hypothetical cells necessary to explain the final outcome. Segmental abnormalities are represented by schematic drawings of chromosome pairs. Regions of gain are coloured in green and that of loss in red. The numbered cells are the cells that have been analysed; the list on the right of the figure shows the chromosome complements for each cell. During the second cleavage, one of the cells of embryo 1 underwent DNA replication without cell division (endoreduplication). In the next cleavage, the cell underwent a tetrapolar division, resulting in cells A, B, C and D. D is a hypothetical cell containing 31 chromosomes and that we assume got lost as a fragment. (**b**) The array CGH plots for cells 11, 13 and 15. The plots for cells 12, 14, 16, 17 and 18 were identical to those of their sibling cells. (c) A representative example of the PCR analysis for chromosomes 19 and X of this embryo. Results for cells 1, 2, 3, 4, 6, 7, 8, 9 and 10 are identical to those of cell 5. Results of cell 12 are identical to those of cell 11 and results of cell 14 to those of cell 13. These results show that it is likely that cells 11, 12, 13 and 14 carry a uniparental isodisomy for at least chromosomes 19 and X. (**d**) We illustrate how the tetrapolar spindle could result in the haplotypes observed in the different cells. Segregation of chromosome 19: cells A and B would contain uniparental isodisomies, cell C a trisomy and cell D a monosomy. Segregation of chromosome 2 would result in cells A and B containing trisomies, cell C being nullisomic and cell D carrying a uniparental isodisomy. Non-disjunction (ND) occurred for all chromosome pairs for which the kinetochores were not occupied on both sides.

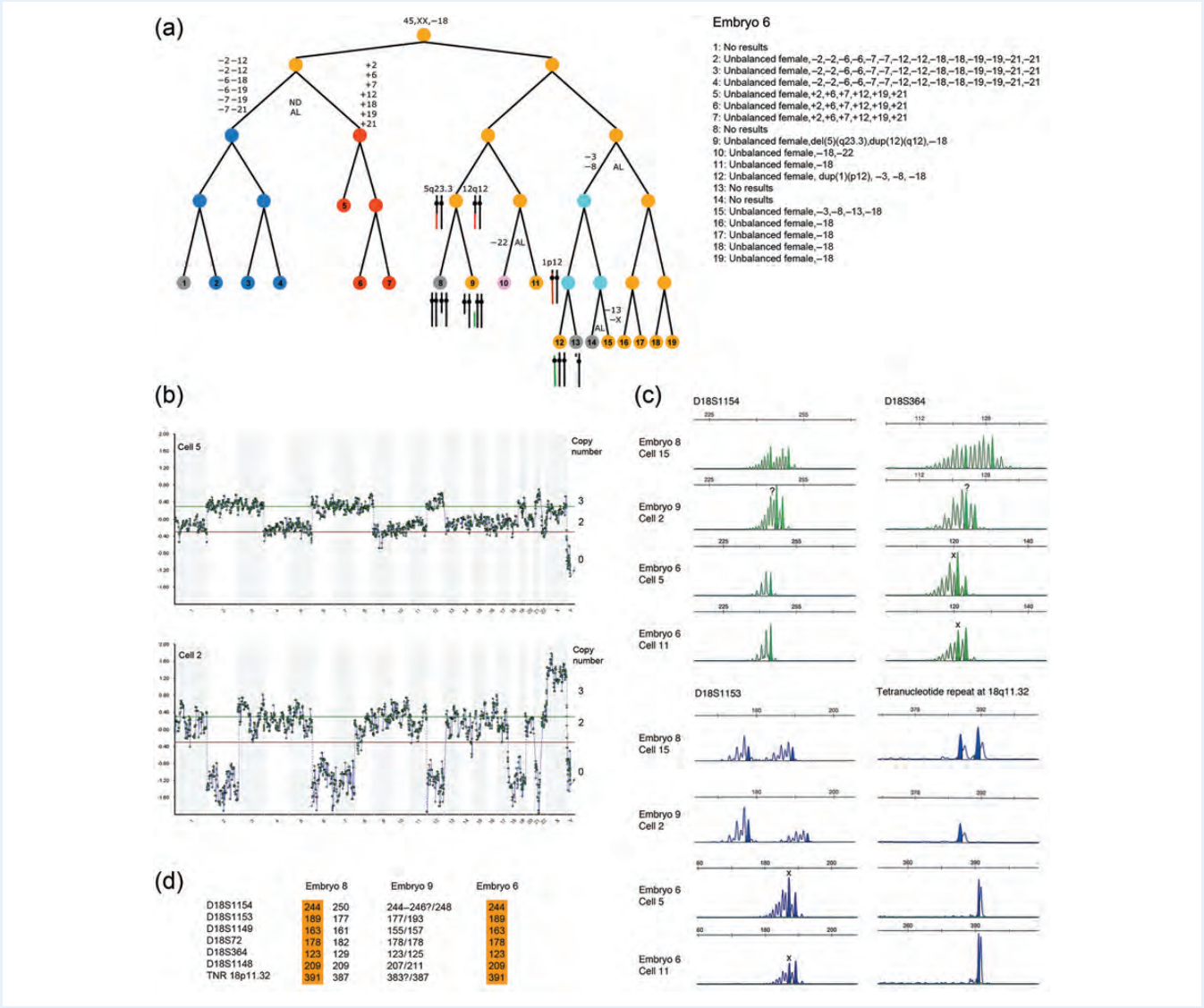

Figure 3 Reconstruction of the evolution of the chromosomal content of embryo 6. (**a**) The cell divisions tree. ND stands for non-disjunction and AL for anaphase lagging. Euploid cells (not taking segmental aberrations into account) are in green; aneuploid cells from the same lineage have distinct colours with different hues, other than green. Grey cells are cells for which we obtained no results, either because of unsuccessful preamplification or low-quality array CGH results. Segmental abnormalities are represented by schematic drawings of chromosome pairs. Regions of gain are coloured in green and that of loss in red. The numbered cells are the cells that have been analysed; the list on the right of the figure shows the chromosome complements for each cell. The array result for cell 12 indicates a gain of 1p12q44. The result could indicate a supernumerary derivative chromosome 1 or ring chromosome r(1)(p12q44), but only visual inspection of the chromosomes would be certain. (**b**) The array CGH plots for cells 2 and 5. The plots of the sibling cells were identical. (**c**) Part of the PCR results on four microsatellites for chromosome 18 of embryo 6 and its genetically related embryos 8 and 9. The most probable allele lengths were selected (filled peaks). After comparing results of all cells and all PCRs, haplotypes were obtained (**d**). The alleles with uninterruptable results are marked with a question mark; alleles that after haplotyping proved to be stutters rather than real alleles are marked with a cross. Cell 15 of embryo 8 and cell 2 of embryo 9 contains two different copies of chromosome 18, cells 5 and 11 of embryo 6 show only one haplotype for chromosome 18. Cells 2 (see also plot in b), 3 and 4 of embryo 6 consistently gave no amplification signal (data not shown), confirming the array CGH results that these cells are nullisomic for this chromosome. (d) The complete results of the haplotyping of chromosome 18 for these three embryos. In the haplotypes, the numbers are fragment lengths in basepairs for the microsatellite markers, those with a question mark are alleles with uncertain results. The haplotype of the chromosome 18 with uniparental isodisomy is highlighted in orange. Remarkably, all cells of embryo 6 show the same haplotype for chromosome 18, proving that there is one and the same chromosome 18 in all cells with monosomy 18, and two identical chromosomes 18 in the cells with diploid copy number for 18 (uniparental isodisomy).

euploid, 8–27% carried a single monosomy, 3–19.5% a single trisomy, 14–36.6% complex abnormalities and 5–6.3% segmental aberrations (Voullaire *et al*., 2000; Wells and Delhanty, 2000; Alfarawati *et al*., 2011). This suggests that the selective growth of normal cells and/or cell death of abnormal cells have not started at the Day 4 stage of development.

From the hypothetical reconstitution of the developmental history of the embryos shown in Figs 2 and 3 and Supplementary data, Figs S2–S6, it appears that except for the zygotes that come from gametes with meiotic errors, the first cleavage is predominantly normal. During the second and third cleavage, chromosomal abnormalities start to appear. From this time point on, abnormal cell lines tend to develop more complex abnormality patterns, while normal cell lines stably persist beyond the third cleavage.

Thus, the first three cleavages seem to be determining for normal embryo development as regards chromosomes. During the first cleavage divisions, the embryo depends highly on maternal gene transcripts and proteins already present in the oocyte (Braude *et al*., 1988). It has been hypothesized that when the maternal levels of necessary mitotic and cell cycle proteins are inadequate, the chromosome segregation is impaired and mitotic aneuploidies accumulate leading to mosaic embryos (Los *et al*., 2004; Delhanty, 2005; Baart *et al*., 2006). Gradually, the embryonic genome becomes active and cell cycle checkpoints are re-established. Consequently, the cells avoid new mitotic errors and chromosomally abnormal cells are eliminated.

Based on our findings, embryos with abnormal cells continue to develop up to Day 4, and Day-4 embryos do not seem to contain more normal cells than Day-3 embryos. Therefore, it is likely that the loss of abnormal cells starts at later stages of development (Ambartsumyan and Clark, 2008; Santos *et al*., 2010).

This unique data set of the chromosomal constitution of Day-4 embryos gives us an unprecedented insight into the natural evolution of chromosomes in early human development and reveals that self-correction does not occur at this time point of preimplantation development.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.M. and C.S. equally contributed to this work. A.M. participated in study design, execution, data analysis, manuscript drafting and critical discussion. C.S. participated in study design, data analysis, manuscript drafting and critical discussion. H.T.N. carried out all PCR analysis. H.V.d.V. participated in study design, material collection and manuscript critical discussion. K.S. participated in study design and manuscript critical discussion.

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Conflict of interest

None declared.

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