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| Complete List of Authors: | Voet, Thierry; University of Leuven, Center for Human Genetics  
Vanneste, Evelyne; University of Leuven, Center for Human Genetics  
Van der Aa, Niels; University of Leuven, Center for Human Genetics  
Melotte, Cindy; University of Leuven, Center for Human Genetics  
Jackmaert, Sigrun; University of Leuven, Center for Human Genetics  
Vandendael, Tamara; University of Leuven, Center for Human Genetics  
Declercq, Matthias; University of Leuven, Center for Human Genetics  
Debrock, Sophie; UZ Leuven, Leuven University Fertility Center  
Fryns, Jean-Pierre; University of Leuven, Center for Human Genetics  
Moreau, Yves; University of Leuven, ESAT  
D’Hooghe, Thomas; UZ Leuven, Leuven University Fertility Center  
Vermeesch, Joris; University of Leuven, Center for Human Genetics |
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Breakage-fusion-bridge cycles leading to inv dup del occur in human cleavage stage embryos

Thierry Voet,¹,a,b Evelyne Vanneste,¹,2a Niels Van der Aa,¹ Cindy Melotte,¹ Sigrun Jackmaert,¹ Tamara Vandendael,¹ Matthias Declercq,¹ Sophie Debrock,² Jean-Pierre Fryns,¹ Yves Moreau,³ Thomas D’Hooghe,² and Joris R Vermeesch¹,a,b

¹Center for Human Genetics, KULeuven-UZ Gasthuisberg, Leuven, Belgium; ²Leuven University Fertility Center, UZ Gasthuisberg, Leuven, Belgium; ³ESAT, KULeuven, Heverlee, Belgium

aboth authors contributed equally to this work

bsenior co-authors

*Correspondence to: Joris Vermeesch, Center for Human Genetics, Herestraat 49, 3000 Leuven, Belgium.

E-mail: Joris.Vermeesch@med.kuleuven.be
Abstract

Recently, a high incidence of chromosome instability (CIN) was reported in human cleavage stage embryos. Based on the copy number changes that were observed in the blastomeres it was hypothesised that chromosome breakages and fusions occur frequently in cleavage stage human embryos and instigate subsequent breakage-fusion-bridge cycles. In addition, it was hypothesized that the DNA breaks present in spermatozoa could trigger this CIN. To test these hypotheses, we genotyped both parents as well as 93 blastomeres from 24 IVF embryos and developed a novel SNP-array based algorithm to determine the parental origin of (aberrant) loci in single cells. Paternal as well as maternal alleles were commonly rearranged in the blastomeres indicating that sperm-specific DNA-breaks do not explain the majority of these structural variants. The parent-of-origin analyses together with microarray-guided FISH analyses demonstrate the presence of inv dup del chromosomes as well as more complex rearrangements. These data provide unequivocal evidence for breakage-fusion-bridge cycles in those embryos and suggest that the human cleavage stage embryo is a major source of chromosomal disorders.

Key Words

Breakage-fusion-bridge (BFB) cycle; inv dup del; cleavage stage embryogenesis; single-cell microarray analysis; chromosome instability (CIN); in vitro fertilization (IVF); preimplantation genetic diagnosis (PGD); preimplantation genetic diagnosis for aneuploidy screening (PGS)
Introduction

Recently, it was shown that chromosomal rearrangements occur frequently in the human cleavage stage embryo by microarray DNA-copy number profiling of all blastomeres separately from multiple embryos following in vitro fertilization (IVF) [Vanneste et al., 2009a]. Seventy percent of the top quality IVF embryos derived from young and fertile partners were mosaic for megabase-sized segmental aneuploidies that were characterized by complementary deletions and duplications or amplifications in different blastomeres of a single embryo. Frequently the embryo also carried blastomeres with a normal copy number of the chromosome that was rearranged in the sister blastomeres [Vanneste et al., 2009a]. Other studies that used microarray technology or metaphase comparative genomic hybridization (CGH) to type DNA copy number in single blastomeres revealed the occurrence of segmental aneuploidies in less than 38.5% of the embryos [Voullaire et al., 2000; Wells and Delhanty, 2000; Voullaire et al., 2002; Daphnis et al., 2008; Hellani et al., 2008; Johnson et al., 2010; Treff et al., 2010]. Based on the patterns of the DNA imbalances that were observed by single-cell array CGH, a number of predictions were made about the events leading to those imbalances. First, DNA double-stranded breaks (DSBs) would occur in the zygote or the first two cleavage cell cycles. The cause of these breaks remains unknown. Since approximately 36% of sperm nuclei derived from normospermic men contained DSBs which increased to ~57% in oligospermic men [Derijck et al., 2007] and parent-of-origin studies on postnatal cases of non-recurrent terminal deletion chromosomes demonstrated a preponderant loss of the paternal allele [Overhauser et al., 1990; Dallapiccola et al., 1993; Cody et al., 1997; Crow, 2000], we hypothesized that the postzygotic structural chromosome instability might be triggered by the DNA-breaks present in ejaculated human spermatozoa. If so, the...
majority of the rearrangements seen in the embryo should occur on the paternal allele. Second, it was hypothesized that breakage-fusion-bridge cycles occur. Some imbalances suggested that following the chromosome breakages, the replicated sister chromatids were fused resulting in terminal inverted duplications. When a centromere is present in such fragment this would lead to a dicentric chromosome that, when pulled to opposite spindle poles during the subsequent mitosis, would generate a terminal deleted chromosome in one blastomere and a chromosome with a terminal deletion and flanking inverted duplication in the other daughter cell. In addition, consecutive breakage-fusion-bridge cycles would lead to more complex rearrangements.

To test these hypotheses we developed a method that identifies in single cells the parent-of-origin of the copy number variations and analysed 93 blastomeres from 24 embryos. In addition, part of the chromosomal imbalances that were identified by single-cell array CGH in one or two blastomeres of a cleavage stage embryo were further characterized by FISH on the remaining cells of the same embryo. The rearrangements were observed on both the maternal and the paternal alleles, excluding sperm DSBs as the main driver of structural chromosome instability in cleavage stage embryogenesis. We prove the occurrence of breakage-fusion-bridge cycles resulting in inv dup del rearrangements in the human cleavage stage embryo, as well as more complex DNA rearrangements with a recurrent nature.

Materials and Methods

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Ethical Committee of the University Hospital Leuven.
on April 4, 2005. Patients provided written informed consent for the collection of samples and subsequent analysis.

**Collection of embryos**

For parent-of-origin analysis 24 embryos were used. Sixty-two blastomeres were biopsied from 11 PGD embryos following microdeletion screening or sex selection (all remaining blastomeres/embryo) [Vanneste et al., 2009a]. Embryos 6, 8, 9, 10, 14, 15, 17, 18, 19, 20 and 21 of this previous study were included because at least 2/3 of the blastomeres derived from one embryo were informative for copy number analysis and DNA from both parents was available.

Twenty-one blastomeres were biopsied from 11 embryos for array-based-PGD for a complex chromosomal rearrangement including an interchromosomal insertion and reciprocal translocation in the male partner (maximum 2 blastomeres per embryo). These embryos were numbered 24 till 34. Ten blastomeres were biopsied from 2 embryos following FISH-based-PGD for a reciprocal translocation in the male partner (all remaining blastomeres/embryo). These embryos were numbered 35 and 36. All embryos were derived from young couples showing no indication for preimplantation genetic diagnosis for aneuploidy screening (PGS). Semen quality was determined for two separate samples with at least a 1-week interval and interpreted according to the World Health Organization (WHO) criteria [World Health Organization, 2010].

For microarray-guided FISH, one or two blastomeres were biopsied per embryo and analyzed by microarray analysis. Thirteen embryos with a chromosomal anomaly by microarray analysis as well as FISH results in the majority of the remaining nuclei are described in this study.

Numbering of these embryos is according to Supp. Table S1. Of these embryos, (1) 3 were 4 or 5-day-old in vitro fertilized (IVF) embryos which were genetically unsuitable for uterine transfer following PGD and (2) 10 were spare embryos, defined as 3- or 4-day-old IVF embryos of
insufficient morphological quality for being transferred or frozen. Of these 13 embryos, 12 blastomeres of 11 embryos were analyzed on SNP array (1 blastomere per embryo, except for embryo 33 where two blastomeres were taken for array analysis), while the two other embryos were analyzed on BAC array (1 blastomere per embryo). The remaining cells were fixed for FISH analysis either simultaneously or after PGD.

Collection of single blastomeres from human embryos

Embryos were briefly incubated in acidic Tyrode’s solution (pH 2.4) (Vitrolife, Gothenburg, Sweden) to remove the zona pellucida. They were then transferred to droplets of Ca\(^{2+}\)- and Mg\(^{2+}\)-free medium supplemented with bovine serum albumine (Sigma-Aldrich) and gently pipetted to disaggregate the individual blastomeres. For array analysis, a single blastomere with a clearly visible nucleus was washed three times in the dissociation medium and then transferred using a mouth-piece to a 200 µl PCR tube containing 1.5 µl alkaline lysis buffer (50 mM dithiothreitol (Sigma), 200 mM KOH). For FISH analysis, the blastomeres were washed twice with culture medium to get rid of possible oil remnants and were fixed on a Superfrost plus microscope slide (LaboNord) using 0.01 mol/l HCl with 0.1% Tween-20 (Merck), as described [Melotte et al., 2004]. Briefly, following lysis of the blastomeres, the nuclei were allowed to dry and became fixed on the glass slide. Finally, slides were washed in 1 X phosphate-buffered saline (PBS) for 5 min and dehydrated by sequential washing in 70%, 90% and 100% ethanol. Fixed nuclei were pre-treated for 20 min with 0.5 mg pepsin/ml (Sigma) in 0.01 mol/l HCl at 37°C followed by a 3 min wash in 1 X PBS. Post-fixation was performed by incubating the slides for 10 min in a 1% formaldehyde solution with 0.05 mol/l MgCl\(_2\) and 1 X PBS at 4°C. Slides were subsequently washed in 1 X PBS and dehydrated by ethanol series.
Single cell array

A single blastomere was lysed and amplified following a multiple displacement amplification approach with Genomi Phi V2 (GE Healthcare) as described [Spits et al., 2006]. We did not use single cell amplifications yielding less than 2 µg DNA.

BAC arrays were performed in 36 hours as described [Vanneste et al., 2009a]. Briefly, 150 ng of single cell amplified DNA and non-amplified genomic reference DNA extracted from lymphoblasts of an individual with Klinefelter Syndrome were labeled for 2h by random primer labeling (BioPrime aCGH Genomic Labeling System; Invitrogen) using respectively Cy5- and Cy3-dCTPs (GE Healthcare). We mixed 1.75 µg of labeled single cell and reference DNA in hybridization solution and hybridized it overnight. Following scanning, we included only spots with signal intensities at least 2-fold above the autosomal median background intensity in the analysis which was performed using R (version 2.6.1, R development Core Team, 2007).

GeneChip Human Mapping 250K NspI arrays (Affymetrix) for single blastomeres were performed in four days according to manufacturer’s instructions using 250 ng of single cell amplified DNA. SNP-copy number analysis was performed to a publicly available reference pool of 41 HapMap females using either ‘Copy Number Analyzer for Genechip (CNAG) version 2.0’ [Nannya et al., 2005] or the ‘Copy Number Analysis Tool’ (CNAT4.0.1, Affymetrix). CNAT4.0.1 parameters were set as described [Vanneste et al., 2009a].

For SNP-typing, we analyzed probe intensities by the ‘GeneChip Genotyping Analysis Software (GTYPE) version 4.1 (Affymetrix)’ using the dynamic model [Di et al., 2005] with stringency P=0.12. LOH was performed using ‘Copy Number Analyzer for Genechip (CNAG) version 2.0’ [Nannya et al., 2005].
FISH

Fluorescence in situ hybridization (FISH) BAC DNA was amplified by DOP-PCR and the DNA was directly labelled by the Random Prime Labeling System (Invitrogen, Carlsbad, CA). Spectrum orange and Spectrum green (Vysis, Abbott laboratories, IL) were used as fluorochromes. Two to five clones of the same locus were mixed to obtain strong fluorescent signals. The final probe mixture was prepared as described [Melotte et al., 2004]. Briefly, the combined probemix, to which Cot-1 DNA was added, was dried and dissolved in hybridization buffer. Then, 1 µl of the probe was applied to the slide, covered with a coverslip (10 mm diameter) and sealed with rubber cement. The nuclei and probes were denatured simultaneously on a hot plate at 75°C for 5 min. Hybridization was allowed to take place overnight in a humid chamber at 37°C. After hybridization, excess or non-specific bound probe was removed by subsequent washes in 0.4 X standard sodium citrate buffer (SSC)/0.3% Igepal CA-630 (Sigma Aldrich) (73°C for 2 min), 2 X SSC/0.1% CA-630 (Sigma Aldrich) (room temperature for 1 min) and 2 X SSC (room temperature for 1 min) followed by dehydration through ethanol series. After drying, the slides were mounted in Vectashield anti-fading medium (Vector Laboratories, Peterborough, UK) containing 2.5 ng/ml 4’,6-diamidino-2-phenylindole (DAPI; Boehringer Ingelheim GmbH, Germany). Nuclei were examined using an Axioplan 2 microscope (Zeiss NV, Zaventem). For each additional FISH round, the coverslip was removed using PBS. The nuclei were denatured in 0.06 X SSC for 7 minutes in a warm water bath at 75°C, followed by a dehydration using sequential washes in 70% (ice cold), 90% and 100% ethanol, respectively. The probemix was denatured for 5 minutes in a warm water bath at 75°C. The quality of the probe mixture was first tested on nuclei derived from mitogen-stimulated blood lymphocytes. In 100 nuclei, the number of signals for each of the probes was counted. Detailed probe mix information
can be found in Supp. Table S1. FISH probes flanking a chromosomal breakpoint were
determined based on their position in NCBI35.

Parent-of-origin analysis

SNP probe intensities were analyzed by GTYPE 4.1 (Affymetrix) using the dynamic model
[Di et al., 2005] with stringency P=0.12. The parent-of-origin algorithm determines in single cell
genotypes the allelic origin of (aberrant) loci by identifying and visualizing in a parent-specific
manner SNPs which demonstrate a Mendelian error. The algorithm was developed in R (version
2.8.1 - http://www.r-project.org/) and is available upon request.

SNPs fulfilling the rules described in Table 1, were scored as either +1 or +0.5 (maternal) or -1 or -0.5 (paternal).

Subsequently, these scores are plotted genome-wide in pdf format per chromosome.

Rationale behind the algorithm: If for a particular SNP the maternal and paternal genotype are
respectively ‘AA’ and ‘BB’, one expects that the genotype of the SNP in a blastomere from an
embryo of those parents would be typed ‘AB’. However, if that SNP in the blastomere is called
‘AA’, this could be due to allele drop out (ADO) of the paternal B-allele, a preferential
amplification (PA) of the maternal A-allele, a true deletion of that locus on the paternal
chromosome or a true amplification of that locus on the maternal chromosome. Similarly, if for a
particular SNP the maternal and paternal genotype are respectively ‘AB’ and ‘BB’, while ‘AA’ is
obtained in the single cell, this could indicate a ADO of the paternal B allele, PA of the maternal
A allele, a true deletion of the paternal B allele or a true amplification of the maternal A allele. To
discriminate between single cell whole-genome DNA amplification artifacts and true DNA copy
number alterations only consecutive SNPs are scored. ADO and PA produce random parent-of-
origin artifacts (alternating positive and negative scores for consecutive SNPs), while true chromosomal aberrations stand out in consecutive SNPs and are assigned to either maternal or paternal origin. For nullisomic regions the algorithm will produce random noise (alternating positive and negative scores for consecutive SNPs), while the majority of SNPs within hemizygous deletions are collectively assigned to either bin of maternal or paternal origin. The parent-of-origin data was thus applied to interpret SNP-copy number profiles.

Accession numbers

Data discussed in this publication have been deposited in the US National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE11663.

Results

Parent-of-origin algorithm

To determine the parental origin of the rearrangements seen in the blastomeres, we genotyped both parents and the single blastomeres using Affymetrix 250K SNP arrays. Because single-cell DNA needs amplification for genome-wide SNP typing, errors are introduced in the single-cell genotyping due to allele drop out or due to biases in DNA amplification. Hence, to enable parent-of-origin analyses, we developed a novel algorithm that determines the allelic origin of (aberrant) loci by identifying and visualizing SNPs with a Mendelian error in a parent-specific manner at the genome wide level. To determine the accuracy of this approach, we typed a set of single blastomeres with known aneuploidies. For all hemizygous X-chromosomes in male embryos the maternal allele was correctly identified (26 blastomeres tested in 8 embryos). Parental origin
analysis of aneuploidies likely resulting from a meiotic non-disjunction further demonstrated that
the algorithm pinpointed the affected allele. In embryo 19 all six blastomeres with monosomy 20
were missing the maternal allele. In embryo 14 three blastomeres were lacking a maternal allele
and one remaining blastomere was typed to lack both alleles and hence was nullisomic for
chromosome 16. In embryo 18 all seven blastomeres carried an extra maternal allele explaining
the trisomy 15. The accuracy of this approach to characterize segmental aberrations was further
determined in ten blastomeres of two embryos derived from a couple carrying a paternal
translocation t(7;21)(p11.2;q11.2). In both embryos that inherited a derivative chromosome 21 as
well as a normal chromosome 7 by adjacent I segregation, the expected segmental 21q11.2-qter
deletion of the paternal allele was correctly identified. SNP copy number analysis revealed the
expected 7p11-pter duplication in seven blastomeres of which three could be confirmed by
paternal origin. One blastomere carried two copies for the 7p11-pter segment and one copy of the
7p11-qter segment. Parent-of-origin typing revealed the loss of the normal paternal allele of
chromosome 7 in this blastomere. In the two remaining blastomeres, the copy number profile for
chromosome 7 was uninformative. Similar results were obtained for 21 blastomeres derived from
embryos of another couple undergoing preimplantation genetic diagnosis (PGD) for a complex
chromosomal rearrangement in the male partner.

This demonstrates that the parent-of-origin algorithm accurately types the allele that is
affected by chromosome missegregation or rearrangement. It also distinguishes homozygous
from hemizygous deletions. Trisomic alleles could be determined with an accuracy of
approximately 50%. In total, 93 blastomeres derived from 24 embryos were analyzed.

Both paternal and maternal alleles were frequently rearranged
Parent-of-origin typing allowed the characterization of mitotic non-disjunction events by identifying the allele that was lost in one blastomere but gained in a sister blastomere (Fig. 1). The analyses of all whole chromosome imbalances (n = 164) which were mosaic in an embryo or present in one or both blastomeres biopsied from embryos 24 to 34, are listed in Table 2. No significant difference was detected between the prevalence of mitotic loss of the paternal or maternal allele in potential mitotic-nondisjunction or anaphase-lagging events. Two meiotic monosomies and one meiotic trisomy, all maternal in origin, were detected in three different embryos respectively.

Segmental aneuploidies were considered simple if unaccompanied by another DNA imbalance for the same chromosome in the same blastomere. Seventeen simple events were detected in 11 of the 24 embryos (46%; 11/24) and comprised 33 segmental imbalances of telomeric loci. The identical parental origin of recurrent or reciprocal segmental aneuploidies in the sister blastomeres of an embryo indicated that these copy number changes resulted from one chromosomal break or rearrangement in a precursor blastomere. Eight events were paternal and five were maternal in origin. For seven duplications in five simple chromosome rearrangement events the parental origin could not be determined.

Thirty-nine complex rearrangements, characterized by either multiple aberrations for a single chromosome in the same blastomere or segmental uniparental isodisomies were observed in 9 of the 24 embryos (37.5%; 9/24). They are listed in Fig. 2 and described further below. Chromatids that lost a p- or q-terminal segment, while the remainder of the chromatid was duplicated or amplified in the same blastomere, were detected in 25% of the embryos (n=11; in 6 embryos; 25%; 6/24). Additional loss of the homologous allele in the same blastomere, resulted in
segmental uniparental isodisomy-null or amplification-null profiles (Fig. 2A). Of the potential isodicentric chromosomes, six were paternal in origin and five occurred on the maternal allele.

**In general, no obvious correlation was apparent between post-zygotic chromosomal rearrangement and the semen quality of the couple (Supp. Table S2).**

**Terminal deletions flanking inverted duplications**

Bipolar segregation of the isodicentric chromosomes can instigate breakage-fusion-bridge (BFB) cycles that are characterized by pure terminal deletions and terminal deletions accompanied by inverted duplications (Fig. 3A).

Parent-of-origin typing suggests the presence of inv dup del chromosomes in single cells (Fig. 3B). Chromosome 1 in blastomere “g” of embryo 20 had a p-terminal deletion which was immediately flanked by a Mb-sized duplication and subsequently a normal copy number profile for the remainder of this chromosome. If the deleted and duplicated segments of this chromosome were related as expected for inv dup del chromosomes, the segments should have the same parental origin. Both the deletion and accompanying duplication were found to be of maternal origin (Fig. 3B). Consistent with a maternal event, a reciprocal pure deletion of the maternal allele was detected in blastomere “c” (Fig. 3B). This strongly suggests that isodicentric formation and related BFB-cycles do occur in early human embryogenesis following IVF.

Whereas the parent-of-origin typing strongly suggests the existence of inv dup del chromosomes, interphase FISH would provide ultimate proof. To identify interphase blastomeres with a potential inv dup del rearrangement we applied microarray-guided FISH. In this approach, one or two blastomeres were biopsied and arrayed to search for a chromosomal anomaly that can be the complement of an inv dup del rearrangement in one of the remaining cells that were fixed simultaneously.
for FISH either simultaneously or after PGD. Subsequently, the breakpoint(s) of the structural chromosomal anomaly detected in the arrayed blastomere was investigated by breakpoint-flanking FISH on the remaining blastomeres. In one blastomere of embryo 33 a q-terminal duplication on chromosome 1 was detected by microarray. Using differentially labeled FISH-probes separated by approximately 100kb and flanking proximal to the 1q-terminal breakpoint, which was determined by SNP-array analysis, as well as a centric FISH probe for chromosome 1, scars of a BFB-cycle were detected in a sister cell. One nucleus had two centric FISH signals, but three signals of the breakpoint flanking probes, of which four signals (two of each probe) were in close proximity (Fig. 3C). In a second FISH-cycle with two differentially labeled probes distal from the breakpoint, the q-terminal duplication detected by microarray analysis was confirmed by the detection of an extra third FISH signal for both probes in a nucleus that produced two signals for each probe in the first FISH-cycle (Fig. 3C).

**Signatures of ring chromosomes and other complex rearrangements**

In addition, more complex rearrangements were also observed. Parent-of-origin typing revealed chromosomes with multiple de novo DNA breaks during the first cleavages (n = 17; in 6 embryos; 25%; 6/24) (Fig. 2C, 2D). These rearrangements could involve both parental alleles or a single allele of a particular chromosome. No significant difference between the amount of rearranged paternal or maternal alleles was noticed (Fig. 2C, 2D).

Chromosome 1 in blastomere “c” of embryo 20 had lost both ends of the maternal allele and thus may have formed a ring chromosome. Chromosome 10 in blastomere “d” of embryo 10 lacked both parental alleles of a p-terminal segment and the q-arm, while the intervening amplified DNA segment was paternal in origin. Hence, this blastomere lacked the maternal chromatid, while the paternal allele was broken at a p- and pericentric q-locus. The amplification delete: blastomere

...
of the remaining paternal segment can be explained by ring formation and subsequent amplification by an odd number of sister chromatid exchanges. Chromosome 6 in blastomere “c” of embryo 14 carried an intact paternal allele as well as a paternal duplicated segment lacking large chunks of both the p- and q-arm, suggesting ring chromosome formation by breakage of one paternal allele of a uniparental isodisomy. Other complex rearrangements involving multiple de novo DNA breaks per chromosome, including frequent (peri-)alphoid DNA-breaks, were detected on both parental alleles and are shown in Fig. 2C-E.

Microarray-guided FISH further corroborated such complex rearrangements in human cleavage stage embryos. SNP-array analysis revealed a p- and q-terminal deletion as well as an amplification of the intervening piece for chromosomes 6 and 7 in one blastomere of an embryo (Fig. 4A,B). In the nucleus of sister blastomere “e” only one signal for the p- and q-terminal FISH probes and two signals for the centromere for both chromosomes 6 and 7 were detected. This, as well as 7q-breakpoint flanking FISH, confirmed both rearrangements. In the remaining FISHed nuclei, a normal copy number for chromosome 6 was detected, while chromosome 7 was normal in two blastomeres and monosomic in two other blastomeres. Furthermore, in the arrayed blastomere a homozygous 18pter-deletion was also detected, while the remainder of this chromosome was hemizygous. A 4th and 5th FISH cycle confirmed the rearrangement (Fig. 4C).

The probes for 18pter as well as the centromere of chromosome 18 produced aneuploid FISH signals consistent with a homozygous 18pter-deletion in blastomere “a”, an 18pter-truncated chromatid in blastomere “e” and an 18pter-amplification in blastomere “d”. Based on the location of the FISH signals in the nuclei a break on chromosome 18 was also apparent in blastomere “b” (Fig. 4C). The data for additional chromosomal rearrangements are presented in Supp. Figure S1.
Discussion

Breakage-fusion-bridge (BFB) cycles are a hallmark of tumors and drive amplifications of oncogenes [Bignell et al., 2007]. However, these rearrangements also underlie the origin of inverted duplications associated with a distal deletion, which is a frequent type of chromosomal rearrangement in patients with birth defects [Zuffardi et al., 2009]. With the advent of array-CGH technology for pre- and postnatal genetic diagnosis, this type of rearrangement has proven to be much more frequent than previously estimated [Ballif et al., 2003; Zuffardi et al., 2009; Vera-Carbonell et al., 2010]. Also, ring and translocation chromosomes that contain inverted duplications flanking the breakpoint have been identified [Rossi et al., 2007; Zuffardi et al., 2009]. The latter most likely result from inv dup del chromosomes that circularize or translocate for stabilization of the uncapped chromosomal end. The generally accepted origin for inv dup del chromosome creation involves a breakage-fusion-bridge (BFB) cycle through either (1) meiotic dicentric chromosome formation between sister chromatids or homologous chromosomes because of chromosomal breakage and illegitimate repair or because of homologue misalignment during pairing at meiosis I and subsequent non-allelic homologous recombination (NAHR) between inverted repeated sequences, or (2) pre-meiotic iso-dicentric chromosome formation between sister chromatids because of chromosomal breakage and non-homologous end joining (NHEJ) with the replicated but broken sister chromatid, or because of chromosomal breakage and intrastrand annealing [Ballif et al., 2003; Vera-Carbonell et al., 2010]. Following the BFB-cycle, the rearranged chromosome is stabilized by telomere healing or capture, or by circularization, resulting in gametes with a chromosomal inv dup del scar. Previously we postulated the formation of isodicentric chromosomes and suggested that breakage-fusion-bridges would occur in post-zygotic cells (Fig. 3A) which would be the origin of inv dup del chromosomes [Vanneste et al.].
et al., 2009a]. In this study we prove those predictions (Fig. 3B-D) and show that the non-recurrent inv dup del chromosomes are likely to result from postzygotic chromosomal breakage and isodicentric formation.

Parent-of-origin typing and microarray-guided FISH in blastomeres also revealed other complex chromosomal rearrangements. We show rearrangements involving multiple DNA-breaks on the same allele and provide ample evidence for (peri-)centric instability or centric fission resulting in i(p) or i(q) chromosome formation in human cleavage stage embryogenesis (n=16; 8 embryos; 33.3%). Centric fission has been characterized as an important driver of karyotype evolution [Perry et al., 2004] and i(p) and i(q) formation is an important mechanism in UPID etiology [Kotzot, 2001]. Hence, this study identifies chromosome instability in the cleavage stage human embryo as a major source of non-recurrent chromosomal disorders in addition to aberrant rearrangement and missegregation of chromosomes in pre-meiotic or meiotic germ cells. Furthermore, since this study includes cleavage stage embryos of good morphology, the data suggest that morphological scoring of a cleavage stage embryo following IVF is poorly suited to select cleavage stage embryos without genetic rearrangement.

These data are also in line with the growing indirect evidence of CIN in the in vivo conceived early human embryo [Pflueger S., 1999; Macklon et al., 2002; Bruder et al., 2008; Conlin et al., 2010; Mkrtchyan et al., 2010; Rodriguez-Santiago et al., 2010]. For instance, common genetic mosaicism for copy number as well as copy neutral anomalies that encompass large loci in the human genome are found in more than 1% of the individuals [Rodriguez-Santiago et al., 2010]. Yet, the high rates of de novo numerical and structural chromosomal aberrations that are formed during cleavage stage embryogenesis intuitively suggest that much more birth defects should occur. However, many of the cells with a rearrangement will be selected against because the altered gene content does not allow survival or efficient proliferation of the cell. In addition, the
majority of the cells with a numerical or structural anomaly contain not only additional chromosomal aberrations but also additional sister blastomeres with multiple chromosomal aberrations further decreasing the chance of cell or embryo survival. Only 3 out of 41 chromosomally abnormal blastomeres (derived from embryos for which at least 2/3 of the blastomeres were informative in this study; 62 blastomeres tested in total) contained a single chromosomal anomaly. Selection mechanisms against aneuploid cells have been uncovered. *De novo* aneuploidy in somatic cells can diminish their capacity to propagate in culture by a p53-dependent cell cycle delay which converges cell populations toward an overall diploid karyotype [Thompson and Compton, 2010]. Furthermore, human embryonic stem cells have been found to initiate caspase-dependent apoptosis of chromosomally abnormal cells upon differentiation, therefore suggesting that differentiation is a mechanism that selects for diploidy [Mantel et al., 2007]. Fifty percent of the cleavage stage embryos in our studies do carry at least one blastomere which is normal diploid [Vanneste et al., 2009a]. Dependent on which blastomere(s) populate the embryo proper, postzygotic *de novo* chromosome aberrations are likely to cause constitutional chromosomal aberrations in a fraction (mosaic) or all the cells of the newborn.

Understanding the factors that cause the chromosomal breakages preceding the rearrangements seen in early embryogenesis would provide insight into the etiology of the low human fecundity, genetic disease development and genome evolution and would contribute to a better understanding of the low success rate to improve baby-take-home rates by FISH-based preimplantation genetic diagnosis for aneuploidy screening (PGS) when performed on the cleavage stage embryo [Vanneste et al., 2009b]. Many reports indicate that DNA-breaks are common in ejaculated human spermatozoa [Derijck et al., 2007; Aitken and De Iuliis, 2010] and it is hypothesized that human sperm DNA damage is a major contributor to poor rates of conception, impaired embryonic development, increased incidence of miscarriage and genetic
disorders in the offspring [Aitken et al., 2009]. However, 35 paternal alleles as well as 23
maternal alleles were damaged by at least one chromosomal break during early embryogenesis in
this study and the majority of these structural variants only appear in a subset of sister
blastomeres suggesting that the DNA-breaks are instigated post-first cleavage division. Hence,
sperm DNA-breaks or other forms of sperm chromatin damage are unlikely to explain the vast
majority of de novo structural variants instigated during early embryogenesis.

Our data further question the clinical benefit of sperm DNA-testing which is performed in
many fertility clinics to predict the success rate of the assisted reproductive technology that is
offered to the couple [Sakkas et al., 2007; Aitken et al., 2009; Zini and Sigman, 2009]. Although
a number of reports claim an association between sperm DNA damage and an increased risk of
pregnancy loss in couples undergoing IVF or ICSI, there is no consensus and the impact of
damaged sperm DNA on reproductive outcome and human development in general remains
largely unknown [Sakkas et al., 2007; Aitken et al., 2009; Zini and Sigman, 2009]. Nevertheless,
the number of embryos analyzed in this study is relatively small and it remains possible that
sperm specific or male specific increases in paternal rearrangements would become apparent in
embryos from couples with fertility problems. Application of the parent-of-origin typing
developed in this study to each cell of cleavage stage embryos derived from fertile versus infertile
men with a high sperm DNA fragmentation index might shed light on whether such paternal
factors exist.

Many other mechanisms can underlie the chromosome breakages triggering the cleavage
stage CIN. Upon fertilization it is generally accepted that the mature metaphase II (MII) oocyte
delivers all the mRNA and proteins that are required for early cleavage cell divisions, as the
human zygotic genome is only activated at the 4- to 8-cell stage [Braude et al., 1988] and sperm
is largely void of transcripts [Ostermeier et al., 2004; Lalancette et al., 2008]. One possibility is
that the transmission of mRNA and proteins to the daughter cells during the first cleavage
divisions is not regulated which would result in stochastic differences amongst the cells. This
may result in insufficient damage or cell cycle control factors causing genomic chaos. Another
possibility could be that DNA damage response and repair pathways are not as active during
early embryogenesis as in later somatic cell types. Understanding the factors that cause the
chromosomal breakages preceding the rearrangements seen in early embryogenesis could be a
first step towards improving IVF success rates.

Acknowledgements

We thank all families who participated in the study, the LUFC for the technical assistance, and R.
Thoelen for her help with the FISHs. This work has been made possible by a IWT (SBO 60848),
FWO grant G.0320.07 and the SymBioSys Center of Excellence (Research Council, K.U.Leuven,
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References

Aitken RJ, De Iuliis GN. 2010. On the possible origins of DNA damage in human spermatozoa.
Mol Hum Reprod 16:3-13.

Aitken RJ, De Iuliis GN, McLachlan RI. 2009. Biological and clinical significance of DNA
damage in the male germ line. Int J Androl 32:46-56.

junctions suggest pre-meiotic breakage-fusion-bridge cycles are involved in generating


Figure legends

Figure 1. Parent-of-origin typing characterizes chromosome missegregations. Mitotic nondisjunction of the paternal allele of chromosome 1 in embryo 19. The paternal allele which is missing in blastomeres “b” and “e” is gained in blastomeres “a,c,d,f”. The left panel illustrates the interpreted copy number output (red indicates a monosomy, green a trisomy). The two middle panels show the parent-of-origin results in pink and blue. See the right panel as well as Table 1 for color interpretation. The copy number interpretation bars have been added to the parent-of-origin output (at y=0).

Figure 2. SNP-copy number and parent-of-origin profiles of complex rearrangements. Profiles characteristic of (A) isodicentric chromosomes, (B) inverse duplication-deletion chromosomes, (C) very complex rearrangements, (D) ring chromosomes, and (E) complex rearrangements encompassing chromosomal arms are shown. For each complex rearrangement, a chromosome ideograph, a bar representing the SNP-copy number interpretation (CN) and a bar representing the parent-of-origin interpretation (PO) is indicated from left to right. The color legend is depicted in (F). The embryo number (E), chromosome number (Chr) and affected blastomere are also indicated left of each chromosome ideograph. For each complex rearrangement, a table that describes the total number of detected copy number alterations and the parental origin of the affected allele is depicted. P: paternal; M: Maternal; N/D: Parent-of-origin not informative; S: Gain of both parental alleles; HoDel: Homozygous Deletion; UPID: Uniparental isodisomy.
**Figure 3.** Parent-of-origin and FISH-typing reveal breakage-fusion-bridge (BFB) cycles in human embryos

(A) A model for the genesis of breakage-fusion-bridge (BFB) cycles following a DNA double-stranded break (dashed arrow) is shown. Expected copy numbers are indicated. The model predicts the creation of dicentric inv dup isochromosomes by fusing replicated broken sister chromatids. According to our findings, such dicentric isochromosomes are frequently segregated to one spindle pole, resulting in a monosomy in the sister blastomere. However, if pulled towards opposite spindle poles, inv dup del chromosomes are generated. This figure is reproduced from Vanneste et al. [2009a].

(B) Evidence for inv dup del formation (chromosome 1 in embryo 20) due to bipolar segregation of an isodicentric chromosome. Parent-of-origin typing confirmed the inv dup del copy number profile of chromosome 1 in blastomere “g” and revealed that the maternal allele was affected. In blastomere “c” the reciprocal pure terminal deletion of the maternal allele was detected. The slight difference between the position of the proximal breakpoint of the duplication in blastomere “g” and the position of the breakpoint of the reciprocal deletion in blastomere “c” is most likely due to the inability of spot-on breakpoint mapping in the applied single-cell copy number analysis.

(C) FISH evidence for inv dup del formation. The microarray results on the left show a blastomere nucleus with a q-terminal duplication on chromosome 1. The FISH results for a selection of 5 nuclei from this embryo are shown. Centromeric (SA: Spectrum Aqua in light blue) as well as breakpoint flanking probes (SO: Spectrum Orange in red; SG: Spectrum Green in green) located proximal to the breakpoint were used in the first FISH round (upper panels), probes distal to the breakpoint in the second FISH round (lower panels). The interpreted amount of FISH signals as well as a chromosome ideograph based on this interpretation is indicated below each nucleus. The arrows beside the chromosome ideographs are added to illustrate the interpreted orientation.
shows three signals for each probe proximal to the breakpoint of which four signals (two of each probe) were in close proximity, but only two centromeric signals. The second nucleus shows two signals for each probe proximal to the breakpoint as well as two centromeric signals, but three signals for each probe distal to the breakpoint. (D) Confirmation of the model in (A) by parent-of-origin typing and evidence for isodicentric formation is presented (illustrated by chromosome 4 in embryo 20). Parent-of-origin indicates that the paternal allele is broken in blastomere “g”. A paternal q-terminal fragment is lost and two identical alleles of the remaining broken paternal chromatid are retained in this blastomere. This suggests that an isodicentric chromosome accompanied by a terminal deletion is present in blastomere “g”. Consistent with the breakage of the paternal allele, blastomere “d” contains an amplification of the reciprocal paternal q-terminal deletion in blastomere “g”, while blastomere “c” carries a maternal monosomy which is consistent with a monopolar segregation of the isodicentric chromosome to blastomere “g”.

These copy number and parent-of-typing profiles confirm the model proposed in (A). The slight difference between the position of the breakpoint of the 4q-terminal deletion in blastomere “g” and the position of the breakpoint of the 4q-terminal amplification in blastomere “d” is most likely due to the inability of spot-on breakpoint mapping in the applied single-cell copy number analysis. (E) Color legend to panels (B) and (D).

Figure 4. FISH profiles of complex genetic rearrangements in human cleavage stage embryos.

The copy number bar interpreted from the microarray analysis of one biopsied blastomere, followed by a chromosome ideograph and the physical position as well as the color-label (SO: Spectrum Orange in red; SG: Spectrum Green in green; SA: Spectrum Aqua in light blue) of the FISH probes are shown on the left. Five FISH rounds were performed on this embryo which are depicted in consecutive order from top to bottom. The complex chromosomal rearrangement
detected by microarray analysis of one blastomere is confirmed by FISH analysis of the
remaining sister blastomeres. (A) pter and qter deletions on chromosome 7, (B) pter and qter
deletions on chromosome 6, (C) pter homozygous deletions on chromosome 18 are confirmed by
FISH. The amount of interpreted FISH signals is depicted below each nucleus as well as a

chromosome ideograph according to the detected FISH signals. (*)(**)(***) For nucleus “e” in
panel A (*), panel B (***) and panel C (***) the overlapping signals indicated by the arrow were
interpreted as a FISH artefact. (****) One SA signal for nucleus “e” in panel C was considered
unreliable (arrow) because of the artifact depicted in (*, ** and ***). (') For nucleus “e” in panel
B one additional SG- and SA-FISH signal is masked by the large stain (arrow). (’’) FISH-
artefacts are indicated with an arrow. (’’’) For nucleus “e” in panel C one SA-signal (arrow) is
considered unreliable because of the stain in the previous FISH cycles (’’ and ’’’). (°) For nucleus
“d” in panel C the SG-FISH signals were too weak to be visualized.
References


Table 1. Rules for parent-of-origin scoring

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<th>Plot color</th>
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Table 2. Affected alleles in whole chromosome imbalances uncovered by parent-of-origin analysis

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<td>9; 100%</td>
<td>-</td>
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<td>18; 20%</td>
<td>22; 24.4%</td>
<td>50; 55.6%</td>
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<td>Tetrasomy or more</td>
<td>11; 100%</td>
<td>2; 18.2%</td>
<td>1; 9.1%</td>
<td>8; 72.3%*</td>
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</table>

*Represent most likely amplifications of both maternal and paternal alleles
Figure 1
218x78mm (600 x 600 DPI)
Figure 1
218x78mm (600 x 600 DPI)
Figure 2
214x287mm (600 x 600 DPI)
Figure 2
214x287mm (600 x 600 DPI)
Figure 3
962x1113mm (600 x 600 DPI)
Figure 3
962x1113mm (600 x 600 DPI)
Figure 4
177x248mm (600 x 600 DPI)
Figure 4
177x248mm (600 x 600 DPI)
Supp Figure S1:

FISH analysis of chromosomal rearrangements detected by microarray analysis of a single blastomere

(A,B) The copy number bar generated from the microarray analysis of one biopsied blastomere, followed by a chromosome ideograph and the physical position as well as the color-label (SO: Spectrum Orange in red; SG: Spectrum Green in green; SA: Spectrum Aqua in light blue) of the FISH probes are shown on the left.

(A) One blastomere of this embryo demonstrated for chromosome 5 a p-terminal duplication by microarray analysis. FISH-analysis of 15 nuclei from sister blastomeres using 5p- and 5q-terminal probes confirmed this rearrangement. The reciprocal deletion, which is characterized by one 5p FISH signal and two signals for 5q, was found in 3 intact and 1 damaged nucleus of this embryo. One nucleus also demonstrated 2 FISH signals for 5p and

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only one for 5q suggesting it contains a monosomy 5 and the 5p-fragment which is duplicated in the arrayed blastomere. An amplification of the q-terminal part of this broken chromatid was uncovered in 3 nuclei revealing two 5p and four 5q signals. A monosomy for chromosome 5 was detected by FISH in one nucleus, while 4 nuclei demonstrated the amount of FISH-signals expected for a normal copy number status. The remaining nuclei showed signs of degradation. These findings are consistent with a post-fertilization DNA-break on one of both homologous chromosomes 5 in a particular blastomere. In subsequent cell cycles, the segmental aneuploidies accumulated.

(B) SNP-array analysis of one blastomere of embryo 40 revealed a complex chromosomal rearrangement for chromosome 2 consisting of a duplication of the entire short arm, an additional q-terminal duplication and a normal copy number state for the intervening segment of this chromosome. Consistent with this rearrangement detected by SNP-array, we discovered two nuclei each revealing only one FISH signal for the p- and q-terminal probes, but two FISH signals for the centromere of chromosome 2. Hence these nuclei lack the reciprocal p-and q-terminal segments which are detected in excess in the arrayed blastomere, but retain the centric fragment. In contrast, another nucleus reveals no FISH signals for the centromere of chromosome 2, but two signals of the p-terminal segment and one signal of the q-terminal segment. One nucleus reveals 2 FISH signals for p-terminal and centric probes, but multiple signals of the q-terminal probe. Collectively, these FISH signals corroborate the complex chromosomal rearrangement detected by SNP-array analysis of the sister blastomere. In addition, one nucleus produces two signals for all FISH probes indicating it contains the normal amount of chromosome 2, while in one nucleus no FISH signals for none of the probes could be detected suggesting it is nullisomic for chromosome 2.
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For Peer Review

Vysis

self labeled mix

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RP11-304L17

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RP11-77F05

RP11-486H09

RP11-555O15

RP11-18K10

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### Supp. Table 2: Semen characteristics

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<sup>a</sup> a “+” indicates that at least one embryo related to the semen characteristics has a whole chromosome aberration

<sup>b</sup> a “+” indicates that at least one embryo related to the semen characteristics has a segmental chromosome aberration

<sup>c</sup> numbering of the embryos is described in Materials and Methods
ND: Not determined, morphology of the spermatozoa was assessed in the first routine semen sample.
self labeled mix

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RP11-495E11

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