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Chromosomal analysis of blastocysts from balanced chromosomal rearrangement carriers

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Abstract

Balanced chromosomal rearrangements (CRs) are among the most common genetic abnormalities in humans. In the present study, we have investigated the degree of consistency between the chromosomal composition of the blastocyst inner cell mass (ICM) and trophectoderm (TE) in carriers with balanced CR, which has not been previously addressed. As a secondary aim, we have also evaluated the validity of cleavage-stage preimplantation genetic diagnosis (PGD) based on fluorescence *in situ* hybridization (FISH) of blastocysts from CR carriers. Blastocyst ICM and TE were screened for chromosomal aneuploidy and imbalance of CR-associated chromosomes based on whole-genome copy number variation analysis by low-coverage next-generation sequencing (NGS) following single-cell whole-genome amplification by multiple annealing and looping-based amplification cycling. The NGS results were analyzed without knowledge of cleavage-stage FISH results. NGS results for blastocyst ICM and TE from CR carriers were 86.49% (32/37) consistent. Of the 1702 (37×46) chromosomes examined, 99.47% (1693/1702) showed consistency. However, only 40.0% (18/45) of all embryos had consistent results for chromosomes involved in CR, as determined by blastocyst NGS and cleavage-stage FISH. Of the 85 CR-affected chromosomes analyzed by FISH, 37.65% (32/85) were incongruous with NGS results, with 87.5% (28/32) showing imbalanced composition by FISH but balanced composition by NGS. These results indicate that chromosomal composition of blastocyst ICM and TE in balanced CR carriers is highly consistent, and that PGD based on cleavage-stage FISH is inaccurate; therefore, using blastocyst TE biopsies for NGS-based PGD is recommended for identifying chromosomal imbalance in embryos from balanced CR carriers.

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Introduction

Balanced chromosomal rearrangements (CRs), which include Robertsonian translocation (Rob), reciprocal translocation (Rcp) and inversion (Inv), are among the most common genetic abnormalities in humans, with an incidence of around 0.19% of newborns (Hamerton *et al*. 1972, 1975, Jacobs *et al*. 1974), a rate that is higher among infertile couples (De Braekeleer & Dao 1991) and patients with implantation failure and recurrent miscarriage (Campana *et al*. 1986, Stern *et al*. 1999). Preimplantation genetic diagnosis (PGD) based on fluorescence *in situ* hybridization (FISH) (Conn *et al*. 1998, Van Assche *et al*. 1999, Coonen *et al*. 2000, Alves *et al*. 2002, Otani *et al*. 2006, Bernicot *et al*. 2010, Bernicot *et al*. 2012, Ko *et al*. 2013) and microarray-based profiling (Alfarawati *et al*. 2011, Fiorentino *et al*. 2011, Treff *et al*. 2011, Colls *et al*. 2012, van Uum *et al*. 2012, Huang *et al*. 2013, Tan *et al*. 2013) are widely used to select normal/balanced embryos for CR carriers to transfer.

Chromosomal mosaicism is frequently observed in cleavage-stage embryos when screening for aneuploidy (Bielanska *et al*. 2002, van Echten-Arends *et al*. 2011). Data from single-cell comparative genomic hybridization (CGH) have also revealed that cleavage-stage embryos from translocation carriers exhibit mosaic patterning of the translocated chromosomes, with their distribution varying considerably in different blastomeres even from the same embryo (Malmgren *et al*. 2002). This can compromise the accuracy of genetic diagnoses that are based on cleavage-stage embryos. An alternative approach is to use blastocyst trophectoderm (TE) biopsies when performing PGD for CR carriers (Huang *et al*. 2013). However, very little is known about the consistency between the inner cell mass (ICM) and TE of blastocysts from CR carriers in terms of chromosomal composition. Previous studies in cleavage-stage embryos have found a higher frequency of mosaicism and abnormal segregation for translocation-associated chromosomes as compared to other chromosomes that

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were not associated with translocation from the same embryo (Iwarsson *et al*. 2000) or embryos from couples with a normal karyotype (Emiliani *et al*. 2003). Some explanations have been proposed to explain the phenomenon, for example, a higher propensity of CR-associated chromosomes to missegregate as a result of rearrangements themselves during mitosis/meiosis (Iwarsson *et al*. 2000) or the greater likelihood of acrocentric chromosomes (such as chromosomes 13, 14, 15, 21 and 22) that are frequently involved in translocation to missegregate and show numerical abnormalities (Boue *et al*. 1985), resulting in more diverse cell lines (Iwarsson *et al*. 2000, Emiliani *et al*. 2003). Thus, mosaicism may be common in blastocysts from CR carriers, especially for CR-associated chromosomes. Previous investigations indicated the chromosomal composition of ICM and TE from patients without known chromosomal abnormalities, for example, balanced CR shows a high degree of consistency, and TE testing is therefore unlikely to be confounded by mosaicism (Fragouli *et al*. 2008, Northrop *et al*. 2010, Capalbo *et al*. 2013*b*). However, microarray analyses of the TE and ICM have detected mosaicism in a high proportion of aneuploid blastocysts, suggesting that TE biopsies do not accurately predict the chromosomal content of the ICM in aneuploid embryos (Liu *et al*. 2012). Thus, before widespread clinical application of TE biopsies in PGD for CR carriers, it is worth noting whether TE represents the state of ICM in blastocysts of CR carriers.

Despite the clinical application of cleavage-stage FISH in PGD for CR carriers, there is little evidence to support its validity in predicting the chromosomal composition in later development stages. Previous studies have shown that aneuploidy screening of cleavage-stage embryos by FISH yields results that are poorly consistent with those obtained by comprehensive chromosomal screening (CCS) of corresponding blastocysts (Northrop *et al*. 2010). These discrepancies may be due to mosaicism, FISH artifacts or self-correction of chromosomal segregation errors (Munne *et al*. 2005*b*, Barbash-Hazan *et al*. 2009). Therefore, PGD based on cleavage-stage FISH for CR carriers may not accurately detect the imbalance of CR-associated chromosomes and effectively reflect the chromosomal composition in the corresponding blastocysts. Moreover, aneuploidy is common in embryos from CR carriers (Pujol *et al*. 2003), even those that are normal/balanced for CR-associated chromosomes (Pujol *et al*. 2006, Treff *et al*. 2011), and CR-associated chromosomes can exert inter-chromosomal effects during meiosis and mitosis (Lejeune 1963, Pellestor *et al*. 1989, Conn *et al*. 1998, Munne *et al*. 2005*a*), leading to an increased risk of chromosomal abnormality. As such, aneuploidy screening is recommended in parallel with the detection of CR-associated imbalances.

Emerging single-cell next-generation sequencing (NGS) technology provides a comprehensive approach to chromosomal analysis (Zong *et al*. 2012) and has shown clinical applicability in concurrent screening for comprehensive chromosomal aneuploidy (Fiorentino *et al*. 2014*a*,*b*, Huang *et al*. 2014, Wells *et al*. 2014) and chromosomal/segmental imbalances in CR carriers (Wang *et al*. 2014). The high throughput, automation and cost-effectiveness of NGS make it a promising alternative to currently available methods that can potentially improve the efficiency of CCS.

The primary aim of this study was to investigate the consistency of chromosomal composition between the ICM and TE of blastocysts from CR carriers using NGS-based concurrent screening of chromosomal aneuploidy and CR-associated chromosomal imbalances, and to evaluate the validity of cleavage-stage FISH-based PGD for CR carriers by examining the corresponding blastocysts. As a secondary aim, this study assessed whether NGS-based PGD using TE biopsies accurately reflects ICM chromosomal composition.

Materials and methods

Patients and embryos

Single blastomeres were biopsied from embryos obtained from CR carriers and subjected to FISH-based PGD. Blastocysts previously diagnosed as unbalanced by FISH or exhibiting developmental arrest were donated for research. A total of 51 blastocysts from 13 couples were evaluated between December 2013 and October 2014 at the Reproductive Medical Center of Xiangya Hospital, China. Basic genetic information is provided in Table 1.

Ethical approval

This study was approved by the Institutional Review Board of Xiangya Hospital, Central South University. All subjects provided written informed consent for study participation.

Embryo culture, blastomere biopsy and FISH analysis

Embryos were cultured in G1 medium (Vitrolife, Kungsbacka, Sweden) at 37 °C in a humidified atmosphere of 6% CO_2. As a routine PGD procedure, good-quality embryos from the two-pronuclear zygotes with $\geq$6 cells and $\leq$15% fragments on day 3 (Fig. 1A) were biopsied for FISH analysis of CR-affected chromosomes. The biopsy was performed by opening a hole in the zona pellucida with a series of single, millisecond pulses delivered from a 1–3-μm diode laser (Hamilton-Thorne Research, Beverly, MA, USA) in Ca^{2+}-/Mg^{2+}-free hydroxyethyl piperazineethane sulfonic acid (HEPES)-buffered human tubal fluid medium (Irvine Scientific, Santa Ana, CA, USA). Single blastomeres were aspirated into the biopsy pipette (Humagen, Charlottesville, VA, USA) and detached from the embryos. Embryos were transferred to G2 medium (Vitrolife) and cultured until days 5–6 using the same conditions after biopsy. Biopsied blastomeres were subjected to interphase nuclear FISH, as described previously (Ko *et al*. 2013). All probes (Table 1) were prepared from our laboratory's repository of

Table 1 Carrier karyotypes and FISH probes used for PGD.

Karyotype	No. of patients	No. of embryos	Translocated segment[a] of Chr A[b] (Mb)	Translocated segment of Chr B[c] (Mb)	Probes
45, XX, der(13;14)(q10;q10)	3	10	97.3	89.7	LSI 13q SO, LSI 14q SG
45, XY, der(13;14)(q10;q10)	4	15	97.3	89.7	LSI 13q SO, LSI 14q SG
45, XX, der(14;22)(q10;q10)	1	9	89.7	36.6	LSI 14q SG, LSI 22q SO
46, XY, *t*(1;19)(p36;q13)	1	3	28.0	26.7	Tel 1p SG, Tel 1q SR, Tel 19q SO
46, XX, *t*(8;22)(q24.1;q11.2)	1	3	28.7	33.4	LSI 8p SO, Tel 8q SR, Tel 22q SG
46, XY, *t*(1;10)(p34;q26)	1	6	46.8	16.4	Tel 1p SG, Tel 1q SR, Tel 10q SO
46, XX, inv(11)(p15q11)	1	1	21.7	81.3	LSI 11p SO, Tel 11q SG
46, XY, inv(20)(p13q13.1)	1	4	5.1	21.3	LSI 20p SG, Tel 20q SO

[a]Expected minimum size of the imbalance in mega bases (Mb) according to the University of California Santa Cruz (UCSC) database (http://genome.ucsc.edu/index.html). [b]First chromosome of the karyotype formula (or short arm of chromosome with inversion). [c]Second chromosome of the karyotype formula (or long arm of chromosome with inversion).

bacterial artificial chromosome libraries and labeled with fluorescein using a Nick-Translation kit (Abbott Molecular), according to the manufacturer's instructions.

Blastocyst biopsy and whole-genome amplification

Blastocysts were biopsied as described previously (Northrop *et al.* 2010), with slight modifications. TE and ICM samples (each consisting of 5–8 cells) from expanded blastocysts (Fig. 1C and E) were biopsied and evaluated for chromosomal mosaicism. The ICM was judged as being free of TE cells by morphological assessment carried out by an embryologist. For early-stage blastocysts that were not expanded and did not show distinguishable ICM and TE, a single sample was biopsied as described above. Samples were washed three times in Ca^{2+}-/Mg^{2+}-free 1×PBS and placed in nuclease-free 0.2 ml PCR tubes for whole-genome amplification (WGA). Samples were amplified by the multiple annealing and looping-based amplification cycling single-cell WGA method, which has been validated for PGD/preimplantation genetic screening (Huang *et al.* 2014), using a kit (Yikon Genomics, Taizhou, China) according to the manufacturer's protocol.

NGS and chromosomal copy number variation analysis

The amplified genome of each blastocyst TE and ICM sample was sequenced using an Illumina HiSeq 2500 platform for a total of two million reads. Chromosomal copy number variation analysis was carried out, as described previously (Zong *et al.* 2012). Reads were aligned to the human genome hg19 reference sequence and filtered to remove reads that were unmapped, duplicated or had low mapping scores. Filtered reads were counted and displayed with a bin size of 1 Mb across the whole-genome. Data in each bin were normalized by guanine-cytosine (GC) content to *in silico* reference data in order to remove bias. Copy number gain from two to three copies of a genome segment resulted in a 50% increase in read counts as compared to those for normal chromosomes, and copy number loss from two copies to one copy resulted in a 50% decrease in read counts. Copy number analysis was

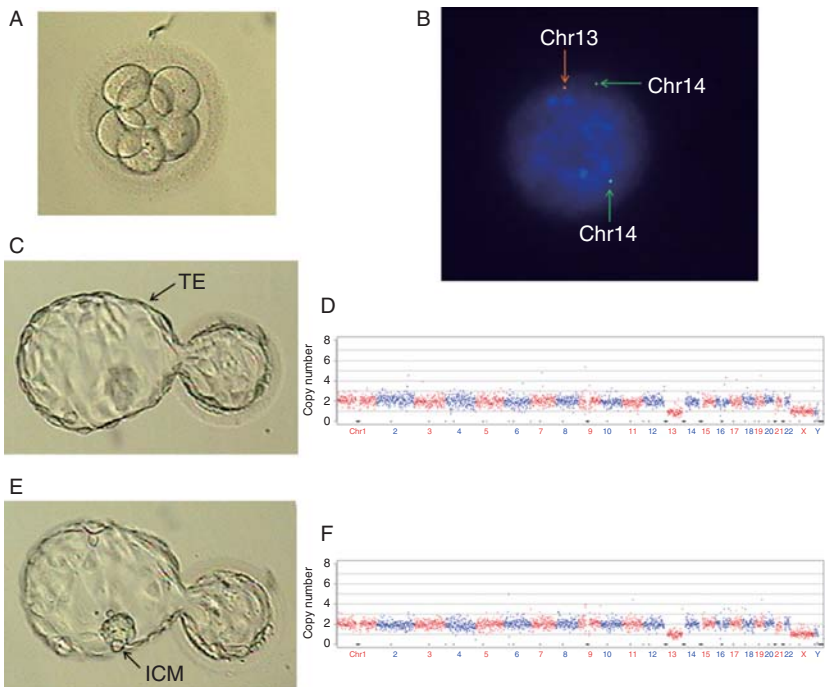

Figure 1 Fluorescence *in situ* hybridization (FISH) and next-generation sequencing (NGS) analyses of an embryo from a Robertsonian translocation carrier. (A) Micrograph of an 8-cell embryo on day 3 from a der (13; 14) (q10; q10) carrier. (B) Interphase nuclear FISH of the same 8-cell embryo showing monosomy of chromosome (Chr) 13. (C) Micrograph of the same embryo at the blastocyst stage with trophectoderm (TE) being in focus. The TE was biopsied for whole-genome amplification (WGA) and NGS. (D) Chromosomal screening for the TE by NGS; only one copy of chromosome 13 was detected, consistent with results of the FISH analysis. (E) Micrograph of the same embryo at the blastocyst stage with inner cell mass (ICM) being in focus. The ICM was biopsied for WGA and NGS. (F) Chromosomal screening for the ICM by NGS, consistent with results of the TE.

Table 2 FISH and NGS results of embryos from carriers with balanced rearrangement.

Embryo No.	FISH result	NGS results		Agreement between FISH and NGS[a]	Agreement between ICM and TE[b]	Aneuploidy[c]
		ICM	TE			
Rob						
der(13;14)(q10;q10)						
1	+14	NB	NB	NO	YES	NO
2	−14	NB	NB	NO	YES	NO
3	Multi-nuclei	+2	+2	–	YES	YES
4	−13	+14, +2p (mos, 40%)[d]	+14	NO	Mosaicism	Mosaicism
5	+13	−15	−15	NO	YES	YES
6	−13	−13	−13	YES	YES	NO
7	+13,−14	−5	−5	NO	YES	YES
8	No signal	NB	NB	–	YES	NO
9	No signal	−13	−13	–	YES	NO
10	+13	+13	+13	YES	YES	NO
11	−13	+2,+14	+2,+14	NO	YES	YES
12	−14	−14	−14	YES	YES	NO
13	−13	−13	−13, +14q(q23.3-q32.33) (mos, 70%)[d]	YES	Mosaicism	NO
14[e]	NB	NB		YES	–	NO
15	−13	NB	NB	NO	YES	NO
16	No signal	+6p (mos, 50%)[d], −13 (mos, 30%)[d], +17 (mos, 60%)[d]	NB	–	Mosaicism	Mosaicism
17[e]	Multi-nuclei	+2,+6,+9,+15, −22		–	–	YES
18	−14	+13	+13	NO	YES	NO
19[e]	−13	NB		NO	–	NO
20	−14	NB	NB	NO	YES	NO
21	−14	−14	−14	YES	YES	NO
22	+13	NB	NB	NO	YES	NO
23	−13	+6	+6	NO	YES	YES
24[e]	−14	−8		NO	–	YES
25[e]	−13	NB		NO	–	NO
der(14;22)(q10;q10)						
26	−14	−8 (mos, 50%)[d],+17 (mos, 50%)[d]	−8q(q21.2-q24.3) (mos, 60%)[d], +17 (mos, 50%)[d]	NO	Mosaicism	Mosaicism
27[e]	−14,−22	−14,−22		YES	–	NO
28	−22	−22	−22	YES	YES	NO
29[e]	−14	NB		NO	–	NO
30[e]	−14	−14,+16,−18		YES	–	YES
31	−22	−21	−21	NO	YES	YES
32	−14	−2p(p22.3-p25.3)	−2p(p22.3-p25.3)	NO	YES	segment loss
33	−22	−22	−22	YES	YES	NO
34	−14	−14 (mos, 30%)[d], +16p (mos, 40%)[d]	+16p (mos, 30%)[d]	NO	Mosaicism	Mosaicism
Rcp[f]						
t(1;19)(p36;q13)						
35	−1p, +19q	−1p(p33-p36.33),+ 19q(q13.11-q13.43)	−1p(p33-p36.33), +19q(q13.11-q13.43)	YES	YES	NO
36	−1p,−1p, −19q	NB	NB	NO	YES	NO
37	Multi-nuclei	NB	NB	–	YES	NO
t(8;22)(q24.1;q11.2)						
38[e]	+22q	+8p(p11-p23.3),+8q(q11-q24.11), −22q(q11-q11.21)		NO	–	NO
39[e]	−8q	−8q(q24.11-q24.3), +22q(q11.21-q13.33)		NO	–	NO
40[e]	+8q,−22q	+8q(q24.11-q24.3), −22q(q11.21-q13.33), +2,+11,+17		YES	–	YES
t(1;10)(p34;q26)						
41	−1p;+10q	−1p(p31.1-p 36.33), +10q(q25.3-q26.3)	−1p(p31.1-p 36.33), +10q(q25.3-q26.3)	YES	YES	NO
42	+1p,−10q	+1p(p31.1-p 36.33), −10q(q25.3-q26.3), −21	+1p(p31.1-p 36.33), −10q(q25.3-q26.3), −21	YES	YES	YES
43	−10q	−21	−21	NO	YES	YES
44	+1p,−10q	+1p(p31.1-p 36.33), −10q(q25.3-q26.3), +22	+1p(p31.1-p 36.33), −10q(q25.3-q26.3), +22	YES	YES	YES
45[e]	+1p,−10q	+1p(p31.1-p 36.33), −10q(q25.3-q26.3)		YES	–	NO
46	−10q	NB	NB	NO	YES	NO
Inv[f]						
inv(11)(p15;q11)						
47	−11p,−11q	-X,-X / -X,-Y	-X,-X / -X,-Y	NO	YES	YES

Table 2 *Continued.*

Embryo No.	FISH result	NGS results ICM	NGS results TE	Agreement between FISH and NGS[a]	Agreement between ICM and TE[b]	Aneuploidy[c]
inv(20)(p13;q13.1)						
48	+20p,−20q	NB	NB	NO	YES	NO
49	−20p	NB	NB	NO	YES	NO
50[e]	NB	NB		YES	–	NO
51[e]	NB	NB		YES	–	NO

[a]Agreement indicates that the FISH result is consistent with NGS results from ICM or TE or both. [b]Mosaicism refers to the incongruity of ICM and TE NGS results due to mosaic copy number of chromosomes/chromosomal segments in the ICM or TE. [c]Aneuploidy of chromosomes that are not involved in balanced rearrangement. [d]The percentage of mosaicism (aneuploidy) is estimated on the assumption that the biopsied tissue contains two distinct cell lines (diploid and triploid, or diploid and haploid) with regard to the chromosome analyzed. [e]Only one NGS result is shown for embryos at the early blastocyst stage (without distinguishable ICM and TE). [f]FISH results for embryos from carriers with reciprocal translocation and inversion are presented as gains or losses of chromosome arms based on fluorescence signals. For these embryos, FISH and NGS results were considered to be consistent when the copy number of translocation/inversion-related regions detected by NGS was consistent with that of chromosome arms inferred from fluorescence signals.

performed in a blinded manner with respect to the results of the cleavage-stage FISH analysis.

Results

A total of 37 expanded and 14 early-stage blastocysts were biopsied for NGS, with all samples yielding interpretable results in the copy number analysis. FISH results of 45 embryos were readable while the remaining six showed multiple nuclei or no signal (Table 2).

Consistency between blastocyst ICM and TE

The consistency between blastocyst ICM and TE was 86.49% (32/37) by NGS (Figs 1D, F and 2B, C, E, F); five embryos exhibited ICM or TE mosaicism (Fig. 3B, C, E and F) and were from Rob carriers, with concordance rates of 80.77% (21/26), 100% (8/8) and 100% (3/3) for blastocysts from Rob, Rcp and Inv carriers respectively (Table 2). Of the 1702 (37×46) chromosomes that were detected, 99.47% (1693/1702) showed consistency (Table 3). Of the nine mosaic chromosomes in 26 expanded blastocysts from Rob carriers, three of a total of 26×2 (5.77%) were Rob-related and six of a total of 26×44 (0.52%) were Rob-free, representing a statistically significant difference ($\chi^2=18.320$; Fisher's exact test; $P=0.005$).

Validity of cleavage-stage FISH-based PGD

In the analysis of CR-associated chromosomes by blastocyst NGS and cleavage-stage FISH, 40.0% (18/45) of all embryos showed concordant results, with concordance rates of 34.48% (10/29), 54.55% (6/11) and 40.0% (2/5) for embryos from Rob, Rcp and Inv carriers respectively (Table 2 and Fig. 1B, D and F).

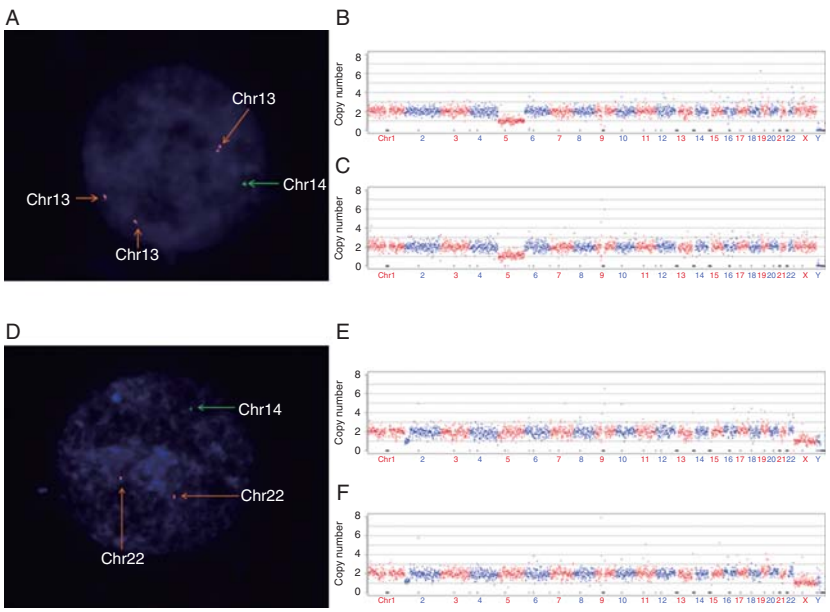

Figure 2 Disparity between results obtained by fluorescence *in situ* hybridization (FISH) and by next-generation sequencing (NGS). (A) FISH analysis of a cleavage-stage embryo from a der (13; 14) (q10; q10) carrier showing trisomy of chromosome (Chr) 13 and monosomy of chromosome 14. (B) and (C) Chromosomal screening by NGS of the inner cell mass (ICM) (B) and trophectoderm (TE) (C) from the same embryo at the blastocyst stage as (A); chromosomes 13 and 14 had normal copy numbers, but monosomy was observed for chromosome 5. (D) FISH analysis of a cleavage-stage embryo from a der (14; 22) (q10; q10) carrier showing monosomy of chromosome 14. (E) and (F) Chromosomal screening by NGS of the ICM (E) and TE (F) from the same embryo at the blastocyst stage as (D); chromosome 14 had a normal copy number, but a segmental loss was detected for chromosome 2, −2p (p22.3–p25.3).

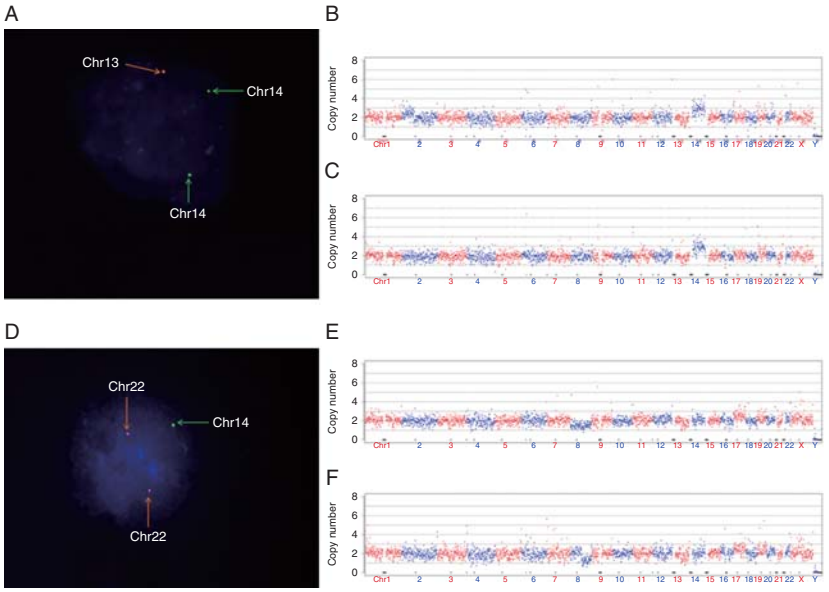

Figure 3 Disparity between results of inner cell mass (ICM) and trophectoderm (TE) copy number analyses due to mosaicism. (A) Fluorescence *in situ* hybridization (FISH) analysis of a cleavage-stage embryo from a der (13; 14) (q10; q10) carrier showing monosomy of chromosome (Chr) 13. (B) and (C) Chromosomal screening by NGS of the ICM (B) and TE (C) from the same embryo at the blastocyst stage as (A); chromosome 13 had a normal copy number, but trisomy was observed for chromosome 14. A segmental gain of chromosome 2 (+2p) was detected in the ICM but not the TE; however, the copy number in this region was <3 owing to mosaicism. (D) FISH analysis of a cleavage-stage embryo from a der (14; 22) (q10; q10) carrier showing monosomy of chromosome 14. (E) and (F) Chromosomal screening by NGS of the ICM (E) and TE (F) from the same embryo at the blastocyst stage as (D); chromosome 14 had a normal copy number, but mosaicism was observed for chromosomes 8 and 17 in the ICM and TE.

Of the 85 CR-associated chromosomes (80 chromosomes from 40 embryos of Rob/Rcp carriers and five chromosomes from five embryos of Inv carriers) analyzed by cleavage-stage FISH, 37.65% (32/85) were inconsistent with the blastocyst NGS results; of these, 87.5% (28/32) showed imbalanced chromosomal composition by FISH but balanced composition by NGS (Table 3 and Fig. 2A, B, C, D, E and F). Of the 25 chromosomes in embryos obtained from Rob/Rcp carriers (after excluding three chromosomes in embryos from Inv carriers) showing imbalanced composition by FISH but balanced composition by NGS, 80.0% (20/25) exhibited copy number loss by FISH (Fig. 2D, E and F), in contrast to 20.0% (5/25) that exhibited copy number gain (Fig. 2A, B and C). A total of 31.91% (15/47) of embryos were abnormal, showing whole or segmental aneuploidy (Fig. 2B, C, E and F) of chromosomes that were not involved in CR.

Discussion

This study investigated the chromosomal composition of the blastocyst ICM and TE in CR carriers by NGS and found a high degree of consistency between the two tissues (99.47% for individual chromosomes and 86.49% for individual embryos), which is in line with previous studies of embryos from karyotypically normal couples (Fragouli *et al.* 2008, Northrop *et al.* 2010, Capalbo *et al.* 2013*b*). These findings indicate that TE biopsies reliably reflect ICM chromosomal status and can be used for PGD of CR carriers. All inconsistencies (five embryos) were attributable to chromosomal/segmental copy number mosaicism in the ICM or TE. Embryonic mosaicism is derived from a variety of mechanisms including chromosome anaphase lagging (Coonen *et al.* 2004, Capalbo *et al.* 2013*a*), non-disjunction (Bean *et al.* 2001) or endoreplication. Anaphase lagging could account for the vast majority of chromosome mosaicism (Coonen *et al.* 2004). Several checkpoints control the proper alignment of chromosomes during mitosis and meiosis and block entry into anaphase when the chromosomes are not appropriately attached to the spindle (Encalada *et al.* 2005, Wei *et al.* 2011). However, these mechanisms may be deficient to avoid all the errors, supported by findings of lagging chromosomes in human embryos (Coonen *et al.* 2004). These lagging chromosomes may be encapsulated into embryonic micronuclei sequestering from the blastomeres into cytoplasmic fragments (Chavez *et al.* 2012). Such fragments may remain or be absorbed by a neighboring blastomere (Wong *et al.* 2010, Chavez *et al.* 2012), creating euploid–aneuploid mosaicism, aneuploid mosaicism or even chaotic chromosome distribution pattern. Previous study also indicated that the lack of functional cell cycle checkpoint mechanisms may lead to chromosomal segregation errors during the mitotic divisions of human preimplantation embryos and thus to mosaicism (Harrison *et al.* 2000). We estimated the percentage of mosaicism (aneuploidy) on the assumption that the biopsied tissue contains two distinct cell lines (diploid and triploid or diploid and haploid) with regard to the chromosome analyzed. If the error occurs during the first mitotic divisions, the embryo would be more likely to exhibit ~50% of abnormal cells (such as the ICM of embryo 26). If the abnormal cells account for ~70% or only ~30% of the total cells, it could be inferred that the error occurs during the later stage of embryo development (such as the ICM and TE of embryo 34) or that the embryo is subjected to aneuploidy rescue (such as the TE of embryo 13). Furthermore, we found that Rob-affected chromosomes exhibited a higher rate of mosaicism in the ICM and TE (5.77%) than Rob-free chromosomes (0.52%), suggesting that consistency

Table 3 Number of individual chromosomes with consistent/inconsistent results by FISH and NGS or between ICM and TE of embryos from balanced rearrangement carriers.

	Agreement between FISH and NGS			Agreement between ICM and TE			
Embryo No.	Number of consistent chromosomes	Number of inconsistent chromosomes (CN: from 3/1/0 to 2)	Number of inconsistent chromosomes (CN: from 2 to 3/1)	Number of consistent chromosomes	Number of inconsistent chromosomes	Number of mosaic chromosomes	**Number of aneuploidy/ segment imbalance**
1	1	1	0	46	0	0	0
2	1	1	0	46	0	0	0
3	–	–	–	46	0	0	1
4	0	1	1	45	0	1	–
5	1	1	0	46	0	0	1
6	2	0	0	46	0	0	0
7	0	2	0	46	0	0	1
8	–	–	–	46	0	0	0
9	–	–	–	46	0	0	0
10	2	0	0	46	0	0	0
11	0	1	1	46	0	0	1
12	2	0	0	46	0	0	0
13	2	0	0	45	0	1	0
14[a]	2	0	0	–	–	–	0
15	1	1	0	46	0	0	0
16	–	–	–	43	0	3	–
17[a]	–	–	–	–	–	–	5
18	0	1	1	46	0	0	0
19[a]	1	1	0	–	–	–	0
20	1	1	0	46	0	0	0
21	2	0	0	46	0	0	0
22	1	1	0	46	0	0	0
23	1	1	0	46	0	0	1
24[a]	1	1	0	–	–	–	1
25[a]	1	1	0	–	–	–	0
26	1	1	0	44	0	2	–
27[a]	2	0	0	–	–	–	0
28	2	0	0	46	0	0	0
29[a]	1	1	0	–	–	–	0
30[a]	2	0	0	–	–	–	2
31	1	1	0	46	0	0	1
32	1	1	0	46	0	0	1
33	2	0	0	46	0	0	0
34	1	1	0	44	0	2	–
Rob Subtotal	35	20	3	1187	0	9	15
35	2	0	0	46	0	0	0
36	0	2	0	46	0	0	0
37	–	–	–	46	0	0	0
38[a]	1	1	0	–	–	–	0
39[a]	1	0	1	–	–	–	0
40[a]	2	0	0	–	–	–	3
41	2	0	0	46	0	0	0
42	2	0	0	46	0	0	1
43	1	1	0	46	0	0	1
44	2	0	0	46	0	0	1
45[a]	2	0	0	–	–	–	0
46	1	1	0	46	0	0	0
Rcp Subtotal	16	5	1	368	0	0	6
47	0	1	0	46	0	0	2
48	0	1	0	46	0	0	0
49	0	1	0	46	0	0	0
50[a]	1	0	0	–	–	–	0
51[a]	1	0	0	–	–	–	0
Inv Subtotal	2	3	0	138	0	0	2
Total	53	28	4	1693	0	9	23

[a]Only one NGS result is shown for embryos at the early blastocyst stage (without distinguishable ICM and TE).

is lower for chromosomes affected by Rob. This may be due to the higher propensity for acrocentric chromosomes involved in Rob (such as chromosomes 13, 14, 15, 21, and 22) to missegregate during mitosis/meiosis (Boue *et al*. 1985), resulting in more diverse cell lines and a higher frequency of mosaicism (Iwarsson *et al*. 2000, Emiliani *et al*. 2003).

We evaluated the validity of cleavage-stage FISH-based PGD for embryos from CR carriers by examining their blastocysts and comparing the corresponding

results, and found a validity of only 40% in all embryos, with only about 62% of CR-associated chromosomes showing consistency between cleavage-stage FISH and blastocyst NGS results. The copy number of ~87% of CR-associated chromosomes or chromosomal segments was imbalanced on day 3 but balanced on day 5/6, which was likely due to genetic mosaicism or FISH artifacts. A high rate of cleavage-stage mosaicism for CR-associated chromosomes has also been observed in other studies (Iwarsson *et al*. 2000, Emiliani *et al*. 2003); thus, analysis of one or two blastomeres may not provide an accurate indication of whole embryo chromosomal composition. Nonetheless, FISH may overestimate true cleavage-stage mosaicism (Treff *et al*. 2010); we found that 80% of chromosomes with copy number imbalance on day 3 and a balanced copy number on day 5/6 showed loss of copy number on day 3. Technical limitations associated with FISH, including inefficient probe hybridization or signal overlap, may account for this discrepancy. Although the efficiency of the in-house locus-specific probes was pre-validated using mitotic metaphase chromosomes of cultured peripheral blood lymphocytes, they may be suboptimal with interphase nuclei of single blastomeres. Alternatively, chromosomal repair processes such as mitotic checkpoints (Encalada *et al*. 2005, Wei *et al*. 2011) and reabsorption of chromosome-containing cytoplasmic debris (Chavez *et al*. 2012), which are active during embryonic development to the blastocyst stage, may be involved. The percentage of chromosomally balanced blastocysts observed in the present study (~60%) exceeds the predicted incidence of Rob carriers (1/6 normal, 1/6 carrier) (Bint *et al*. 2011). However, many previous studies have reported high incidence of alternate segregation in Rob carriers (Munne *et al*. 2000, Roux *et al*. 2005, Ogur *et al*. 2006, Chen *et al*. 2007, Anton *et al*. 2010, Pilip *et al*. 2014), which produces balanced gametes. In addition, the majority of embryos from Rob carriers are normal/balanced (Jin *et al*. 2010), which is consistent with the current findings.

Our analyses indicate that the widely used cleavage-stage FISH has limited effectiveness in PGD for embryos from balanced CR carriers. Results from cleavage-stage FISH and blastocyst NGS from the same embryos were poorly correlated, in agreement with other studies comparing cleavage-stage FISH and blastocyst-stage microarray results (Northrop *et al*. 2010), as well as comparative FISH analyses of cleavage- and blastocyst-stage embryos (Baart *et al*. 2004). However, in one study, blastocyst-stage embryos previously analyzed by array CGH were reanalyzed by FISH with remarkably consistent results (Fragouli *et al*. 2011), indicating that technical constraints of FISH are not solely responsible for the observed incongruence between cleavage-stage and blastocyst chromosomal composition. In addition, FISH-based PGD benefits CR carriers with improved pregnancy outcomes, which provide clinical evidence for the efficacy of FISH (Scriven *et al*. 2013). Nonetheless, cleavage-stage FISH is not the ideal PGD protocol for CR carriers, given that a significant proportion of embryos exhibiting balanced composition of chromosomes involved in CR had abnormalities in other chromosomes. The NGS-based PGD method was effective in detecting chromosomal imbalances as small as ~7 Mb (i.e. the loss of 22q11–22q11.21 in embryo 38), and the TE biopsy accurately reflected the chromosomal composition in the ICM. Therefore, blastocyst TE biopsies for NGS-based PGD can be used for the reliable detection of all chromosomes and are effective in PGD for balanced CR carriers. However, mosaic copy numbers of CR-associated chromosomes in the TE must be interpreted with caution; in addition, clinical validation of NGS-based assays is required, while the segment size of the chromosomal imbalance that can be reliably detected by NGS remains to be determined.

In conclusion, our findings demonstrate that chromosomal composition of blastocyst ICM and TE from balanced CR carriers was highly consistent. In addition, PGD results of cleavage-stage FISH analysis were poorly correlated with those obtained by NGS, with a significant proportion of blastocysts showing an imbalance by cleavage-stage FISH but judged as balanced according to NGS analysis. Thus, using blastocyst TE biopsies for NGS-based PGD is recommended for identifying chromosomal imbalance in embryos from balanced CR carriers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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