Natural selection between day 3 and day 5/6 PGD embryos in couples with reciprocal or Robertsonian translocations

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Abstract

Purpose For translocation carriers, preimplantation genetic diagnosis (PGD) provides the opportunity to distinguish between normal/balanced and unbalanced embryos prior to implantation and, as such, increases the likelihood of a successful ongoing pregnancy. The data presented here compares autosomal reciprocal and Robertsonian translocation segregation patterns in day 3 versus day 5/6 IVF-PGD embryos to determine if there is a difference in the chromosome segregation patterns observed at these developmental time points.

Methods A retrospective analysis on PGD translocation carriers at Monash IVF was performed. Segregation patterns were compared between day 3 and day 5/6 embryos to ascertain whether selection against malsegregants exists.

Results For reciprocal translocations, 1649 day 3 embryos (139 translocations) from 144 couples and 128 day 5/6 embryos (59 translocations) from 60 couples were analysed. Day 3 segregation analysis showed that 22.3% of embryos were normal/balanced (consistent with 2:2 alternate segregation) and 77.7% were unbalanced (malsegregation). Day 5/6 segregation analysis showed that 53.1% of embryos were normal/balanced and 46.9% were unbalanced. For Robertsonian translocations, 847 day 3 embryos (8 translocations) from 54 couples and 193 day 5/6 embryos (6 translocations) from 31 couples were analysed. Day 3 segregation analysis showed that 38.7% of embryos were normal/balanced (consistent with

2:1 alternate segregation) and 61.3% were unbalanced. Day 5/6 segregation analysis showed that 74.1% of embryos were normal/balanced and 25.9% were unbalanced.

Conclusions This data demonstrates an increase in the proportion of genetically normal/balanced embryos at day 5/6 of development. This suggests a strong natural selection process between day 3 and day 5/6 in favour of normal/balanced embryos. These findings support performing PGD testing on day 5/6 of embryo development.

Keywords Preimplantation genetic diagnosis · Reciprocal translocation · Robertsonian translocation · Segregation · Unbalanced embryos

Introduction

Reciprocal and Robertsonian translocations occur in approximately 0.2 and 0.1% of the human population, respectively [1]. Translocation carriers are considered ‘balanced’ because all their genetic information is present; however, they are at risk of producing unbalanced gametes during meiosis due to malsegregation of the chromosomes involved in the translocation. Preimplantation genetic diagnosis (PGD) is an alternative to invasive prenatal diagnosis for couples who carry a translocation and provides the opportunity to distinguish between normal/balanced embryos (which have the potential to result in a successful ongoing pregnancy) and unbalanced embryos (which result in implantation failure, miscarriage or the birth of a chromosomally unbalanced child).

To understand the mechanism by which unbalanced embryos are formed, it is first necessary to understand how the translocation chromosomes behave during meiosis. For reciprocal translocations, the translocation chromosomes come together to form a quadrivalent at the pachytene stage of

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meiosis. Five defined modes of segregation can then occur, resulting in the production of 16 possible gametes which, once fertilised, can lead to the formation of 32 possible embryo outcomes (refer to Fig. 1) [2]. Alternate segregation (2:2) is the only mode of segregation that can result in a normal/balanced embryo and genetically healthy ongoing pregnancy. The other modes of segregation, namely 2:2 adjacent-1, 2:2 adjacent-2, 3:1 and 4:0 segregation, are all forms of malsegregation that lead to the generation of chromosomally unbalanced embryos. For Robertsonian translocations, the translocation chromosomes come together to form a trivalent at the pachytene stage of meiosis. The chromosomes can then segregate by four modes: 2:1 alternate, 2:1 adjacent-1, 2:1 adjacent-2 and 3:0 segregation, resulting in eight different embryo outcomes (refer to Fig. 2). Again, alternate segregation is the only mode that can result in a genetically healthy ongoing pregnancy. The remaining modes of segregation are all forms of malsegregation that lead to the generation of chromosomally unbalanced embryos.

In recent years, there has been a significant change in the approach to PGD testing with the majority of clinics moving from cleavage stage blastomere biopsy on day 3 of development to trophectoderm biopsy on day 5/6 of development. The PGD technology used has also changed, with clinics moving from targeted fluorescent in situ hybridisation (FISH)-based testing to more comprehensive screening techniques such as single-nucleotide polymorphism (SNP) arrays, array comparative genomic hybridisation (array-CGH), qPCR or next-generation sequencing (NGS)/targeted-NGS. These changes in testing have contributed to a significant improvement in implantation rates per transfer [3–8] and a decrease in miscarriage rate after preimplantation genetic screening [9]. Monash IVF has offered PGD for translocation carriers as a clinical procedure since January 2000. From 2000 to 2011, Monash IVF performed blastomere biopsy on day 3 of embryo development followed by PGD testing using FISH. Since 2011, Monash IVF has performed blastocyst biopsy on day 5/6 of embryo development followed by PGD using SNP array, array-CGH or NGS. The aim of this study was to compare autosomal reciprocal and Robertsonian translocation segregation patterns in day 3 versus day 5/6 IVF-PGD embryos to determine whether selection against malsegregants exists between day 3 and day 5/6 of embryo development.

Materials and method

Study design

A retrospective analysis of reciprocal and Robertsonian translocation patients having testing at Monash IVF between 2000 and mid 2016 was performed in order to compare the translocation segregation modes in IVF-PGD embryos at day 3 and

day 5/6 of embryo development. Patients who had an unbalanced karyotype or mosaic karyotype or couples wherein the partner also had a known chromosome abnormality were excluded from the data set. PGD testing was performed either on day 3 using FISH or day 5/6 using SNP array, array-CGH or NGS. This study was approved by the Monash Surgical Private Hospital Human Research Ethics Committee (approval number: 07078).

Preimplantation genetic diagnosis test development/feasibility

Before commencing an IVF-PGD cycle, all couples underwent genetic counselling and PGD feasibility testing in order to determine if PGD was possible for their particular translocation. PGD feasibility testing using FISH was performed as per the protocol previously described by Brodie et al. [10]. PGD feasibility testing using SNP array or array-CGH involved an assessment of the translocation breakpoints to determine if the microarray platform had adequate resolution to distinguish the normal/balanced embryos from all unbalanced forms of malsegregation. NGS was considered a suitable testing platform for Robertsonian translocation carriers as unbalanced embryos would display a chromosome imbalance involving a whole chromosome arm (refer to Fig. 2), which was significantly larger than the 20-Mb detection resolution reported by Illumina. The suitability of NGS for reciprocal translocation carriers was assessed on a case-by-case basis. This involved using NGS to reassess embryonic DNA from embryos diagnosed as unbalanced by array-CGH in a previous IVF-PGD cycle. If the NGS platform had adequate resolution to detect the chromosome imbalance and distinguish the normal/balanced embryos from all unbalanced forms of malsegregation, this testing platform was offered in subsequent IVF-PGD cycles. Couples were able to commence an IVF-PGD cycle once feasibility testing was complete.

Blastomere biopsy and fluorescent in situ hybridisation

For patients accessing FISH-based testing, embryo biopsy was performed on day 3 post-oocyte collection on embryos that were ≥ 5 cells. Embryos with 5 or 6 cells underwent single blastomere biopsy, while embryos with ≥ 7 cells had two blastomeres biopsied. Embryos < 5 cells were considered unsuitable for biopsy. Single blastomeres were fixed to glass slides using Carnoy's fixative, pre-treated using pepsin (if required) and hybridised using the optimised conditions determined during the feasibility testing process. Individual nuclei were analysed independently by two scientists using an Olympus BX51 fluorescent microscope (Olympus Optical CO. LTD, Tokyo, Japan). FISH analysis guidelines followed those published by Munne et al. [11]. The embryos were considered normal/balanced and genetically suitable for transfer when

two signals were present for each of the FISH probes used. The embryos were considered unbalanced and not suitable for transfer when missing or additional probe signals were

observed. Embryos without a FISH result (7.6% of biopsied embryos) were excluded from this study. Outcomes were measured as the number of embryos with a FISH signal pattern

Fig. 1 Segregation of a reciprocal translocation. **a** Alternate segregation (2:2) is the only segregation mode that leads to gametes with a complete genetic complement. Following alternate segregation, one zygote will have a normal chromosome complement while the other will carry a balanced form of the reciprocal translocation. **b** Adjacent-1 segregation (2:2) results in the formation of embryos with a trisomy for one translocated segment and a monosomy for the other. **c** Adjacent-2 segregation (2:2) results in the formation of embryos which are trisomic for one of the chromosomes involved in the translocation and monosomic for the other. Segregation (3:1): **d, f** Tertiary trisomy results in the formation of a zygote with 47 chromosomes. Tertiary monosomy results in the formation of a 45 chromosome zygote. **e, g** Interchange trisomy results in the formation of a zygote with 47 chromosomes. This form of segregation is only viable if a trisomically viable chromosome (i.e. 13, 18 or 21) is involved in the translocation. Interchange monosomy results in the formation of a zygote with only 45 chromosomes. In 4:0 segregation (**h**), all four chromosomes migrate together to one daughter cell. This mode of segregation results in the production of zygotes which are either trisomic for both chromosomes involved in the translocation or monosomic for both chromosomes involved in the translocation

consistent with a meiotic mode of translocation segregation. Embryos with a FISH signal pattern that could not be attributed to a meiotic mode of translocation segregation were classified as ‘unknown segregation’. It was assumed that the unknown segregation profile was due to embryonic aneuploidy, mosaicism or technical limitations of FISH-based PGD testing.

Blastocyst biopsy and single-nucleotide polymorphism array/array-comparative genomic hybridisation/next-generation sequencing

For patients accessing SNP array, array-CGH or NGS testing, embryo biopsy was performed on day 5/6 post-oocyte collection. On day 3 post-oocyte collection, the embryo's zona pellucida was breached using a low-intensity laser beam (Zilos laser, Hamilton Thorne, UK, or Saturn Active laser4, Research Instruments, UK). On day 5 and/or day 6, the embryos were assessed for suitability for biopsy. Embryos were considered suitable for biopsy if they had developed to the blastocyst stage and contained a clearly defined inner cell mass (i.e. the inner cell mass was identified as a tightly compacted ball of cells clearly that separate from the trophectoderm cells) and an appropriate number of trophectoderm cells to enable the removal of approximately three to seven cells for PGD testing while retaining an adequate number of trophectoderm cells to support ongoing embryo development. The biopsied blastocysts were vitrified post-biopsy.

Single-nucleotide polymorphism array Each biopsy sample was washed through a series of 20 µl wash buffer drops

Fig. 2 Segregation patterns for a Robertsonian translocation. A carrier of a balanced Robertsonian translocation can produce unbalanced gametes, resulting in the formation of zygotes which are either trisomic or monosomic for one or both of the chromosomes involved in the translocation

(Natera Inc., USA) before being transferred to a sterile 0.2- μ l PCR tube containing 5 μ l of wash buffer. The tubes containing the biopsy samples were frozen and shipped to Natera Inc. for testing. SNP array testing was performed by Natera Inc. using the method previously described [12]. Embryos were considered suitable for transfer if they were normal/balanced for the chromosome rearrangement and euploid for the remaining chromosomes.

Array-comparative genomic hybridisation Each biopsy sample was washed through a series of 20 μ l 1 $\times$ PBS drops (Cell Signaling Technologies, USA) before being transferred to a sterile 0.2- μ l PCR tube containing 2.5 μ l of 1 $\times$ PBS. Whole-genome amplification (WGA) was performed using the SurePlex DNA Amplification System (Illumina, UK). For reciprocal translocations, WGA products (as well as male SureRef reference DNA (Illumina, UK)) were fluorescently labelled with Cy3 or Cy5 fluorophores using the Fluorescent Labelling System (Illumina, UK) and competitively hybridised to 24sure+ arrays (Illumina, UK). For Robertsonian translocations, WGA products (as well as male and female SureRef reference DNA (Illumina, UK)) were labelled and hybridised to 24sure V3 arrays (Illumina, UK). Arrays were washed and scanned with an Agilent C DNA microarray scanner (using two colour scan settings at 10- μ m resolution). WGA, labelling, hybridisation, array washing and array scanning were all performed using the standard protocol available at www.illumina.com. Samples were analysed independently by two scientists using BlueFuse Multi Software (Illumina, UK). Analysis was performed using the manufacturer's recommendations published in 'A technical guide to aneuploidy calling with 24sure V3' and the technical note, '24sure +translocation detection' (also available at www.illumina.com) [13, 14]. Embryos were considered suitable for transfer if they were normal/balanced for the chromosome rearrangement and euploid for the remaining chromosomes. Embryos with a mosaic result were not recommended for transfer.

Next-generation sequencing Each biopsy sample was washed through a series of 20 μ l 1 $\times$ PBS drops (Cell Signaling Technologies, USA) before being transferred to a sterile 0.2- μ l PCR tube containing 2.5 μ l of 1 $\times$ PBS. Whole-genome amplification (WGA) was performed using the SurePlex DNA Amplification System (Illumina, UK). Following WGA, NGS was performed using VeriSeq (Illumina, UK). Tagmentation, sample barcoding, parallel sequencing and alignment were all performed using the standard protocol available at www.illumina.com [15]. Samples were analysed independently by two scientists using the BlueFuse Multi Software (Illumina, UK). Analysis was performed using the manufacturer's recommendations published in 'A technical guide to aneuploidy calling with VeriSeq PGS'

[16]. Embryos were considered suitable for transfer if they were normal/balanced for the chromosome rearrangement and euploid for the remaining chromosomes. Embryos with a mosaic result were not recommended for transfer.

Embryos without a PGD result (0.9% biopsied embryos) were excluded from this study. Outcomes were measured as the number of embryos with a SNP array, array-CGH or NGS profile consistent with a meiotic mode of translocation segregation. Embryos with a profile that could not be attributed to a meiotic mode of translocation segregation were classified as unknown segregation. It was assumed that the unknown segregation profile was due to embryonic aneuploidy or technical limitations of PGD testing.

In order to have comparable data between the FISH and microarray/NGS groups, this study only took into account the PGD results obtained for the chromosomes involved in the particular translocation (as FISH was not capable of providing simultaneous comprehensive chromosome screening). Therefore, aneuploidy and mosaicism involving other chromosomes were not assessed in this study.

Limitations

A limitation of this study is the varying capacities of each of the PGD testing platforms to detect triploidy and the capability of each of these platforms to distinguish triploidy from the 3:0 segregation for Robertsonian translocation carriers. As FISH solely analysed the chromosomes involved in the translocation, this testing did not enable the distinction between 3:0 segregation and triploidy for Robertsonian translocation carriers. Array-CGH and NGS, on the other hand, were capable of detecting triploidy if the embryo was male, while SNP array testing was capable of distinguishing between diploidy and triploidy irrespective of embryo gender. Another limitation of this study is the difference in detection capabilities between array-CGH and NGS technologies. NGS has been reported to be superior to array-CGH for the detection of chromosomal mosaicism [17, 18]. Given that mosaic embryos are reported to be associated with an increased risk of implantation failure and miscarriage compared with true euploid embryos [19–21], the improved detection of mosaic embryos from true euploid embryos has the potential to provide patients with an additional embryo selection tool. At the time of this study, embryos diagnosed as normal/balanced for the chromosome rearrangement but mosaic for other chromosome/s were diagnosed as 'mosaic' and not recommended for transfer. The fate of these embryos (i.e. whether or not they were considered eligible for transfer) was at the discretion of the treating IVF clinic. While some clinics will not transfer mosaic embryos, others may consider these embryos eligible for transfer as a second preference in the absence of an embryo with a normal/balanced and euploid result.

Statistical analysis

Statistical analysis was performed using the Fisher exact test. A *P* value of <0.05 was considered statistically significant.

Results

Table 1 shows a breakdown of the specific patient groups. Overall, 2817 embryos were analysed from 289 couples (212 different translocations). The reciprocal translocation group consisted of 1649 day 3 embryos (139 translocations) and 128 day 5/6 embryos (59 translocations). The Robertsonian translocation group consisted of 847 day 3 embryos (8 translocations) and 193 day 5/6 embryos (6 translocations). The details of each specific translocation are listed in the Supplementary data Table S1.

Reciprocal translocation results

Table 2 shows the breakdown of day 3 versus day 5/6 embryos, and Table 3 shows the segregation modes observed in embryos from reciprocal translocation carriers. On day 3, only 22.3% (368/1649) of embryos were diagnosed as either normal or balanced (alternate segregation) for the translocation chromosomes. The remaining 77.7% (1281/1649) of embryos were diagnosed as unbalanced for the translocation chromosomes. On day 5/6, 53.1% (68/128) of embryos were diagnosed as either normal or balanced (alternate segregation) while 46.9% (60/128) of embryos were diagnosed as unbalanced for the translocation chromosomes. There was a statistically significant difference in alternate segregation between day 3 and day 5/6 embryos. This was also observed for the 3:1 ($P = 0.0010$) segregation mode, as well as for the unknown segregation mode ($P < 0.0001$). This represents a statically significant reduction in the proportion of malsegregants on day 5/6 compared with day 3 of development ($P < 0.0001$).

Robertsonian translocation results

Table 4 shows the breakdown of day 3 versus day 5/6 embryos, and Table 5 shows the segregation modes observed in embryos from Robertsonian translocation carriers. On day 3, 38.7% (328/847) of embryos were diagnosed as either normal or balanced (alternate segregation) for the translocation chromosomes. The remaining 61.3% (519/847) of embryos were diagnosed as unbalanced for the translocation chromosomes. On day 5/6, the majority of embryos 74.1% (143/193) were diagnosed as normal or balanced (alternate segregation) and 25.9% (50/193) of embryos were diagnosed as unbalanced for the translocation chromosomes. There was a statistically significant difference in alternate segregation between day 3 and day 5/6 embryos. This was also observed for the 3:0

Table 1 Reciprocal and Robertsonian translocation study groups

	Reciprocal		Robertsonian		Total
	Day 3	Day 5/6	Day 3	Day 5/6	
Mean maternal age	34.0	33.5	35.4	32.9	
Mean PGD cycle number	1.9	1.7	3.2	2.1	
Translocations	139	59	8	6	212
Couples	144	60	54	31	289
Embryos	1649	128	847	193	2817

segregation mode ($P = 0.0097$) as well as for the unknown segregation mode ($P < 0.0001$). This represents a statically significant selection away from malsegregants in the day 5/6 embryos ($P < 0.0001$).

Discussion

This PGD study compares the segregation patterns of embryos from autosomal reciprocal and Robertsonian translocation carriers at day 3 and day 5/6 of embryo development. The majority of day 3 embryos were diagnosed as unbalanced for the translocation chromosomes (reciprocal 77.7%, Robertsonian 61.3%), whereas by day 5/6, the majority of embryos were diagnosed as normal or balanced (reciprocal 53.1%, Robertsonian 74.1%) ($P < 0.0001$). The proportion of unbalanced embryos with more complex malsegregation patterns also decreased as embryo development progressed. For example, for reciprocal translocation carriers, adjacent-2, 3:1 and 4:0 segregation was observed in 29.3% of day 3 embryos but only 18.0% of day 5/6 blastocysts ($P = 0.0059$). Similarly, for Robertsonian translocation carriers, the more unbalanced 3:0 segregation was observed in 5.1% of day 3 embryos but was only observed in 1.0% of blastocysts tested ($P = 0.0097$). The exclusion of complex malsegregants on day 5/6 and increase in the least unbalanced form of malsegregation (i.e. reciprocal 18.7% adjacent-1 at day 3 versus 27.3% at day 5/6, Robertsonian 28.7% adjacent at day 3 versus 24.9% at day 5/6) suggest a selection for malsegregants towards the least unbalanced form. The observed increase in the proportion of normal/balanced embryos and concomitant decrease in complex malsegregants on day 5/6 of development relative to day 3

Table 2 Day 3 versus day 5/6 data for reciprocal translocation carriers

	Day 3	Day 5/6	Total
Normal/balanced	368 (22.3%)	68 (53.1%)	436
Unbalanced	1281(77.7%)	60(46.9%)	1341
Total	1649	128	$P < 0.0001$

Table 3 Segregation modes for day 3 vs. day 5/6 for reciprocal translocation carriers

Segregation mode	Day 3	Day 5/6	<i>P</i> value
Alternate	368 (22.3%)	68 (53.1%)	$P < 0.0001$
Adjacent-1	309 (18.7%)	35 (27.3%)	$P = 0.0204$
Adjacent-2	156 (9.5%)	16 (12.5%)	$P = 0.2758$
3:1	295 (17.9%)	7(5.5%)	$P = 0.0001$
4:0	31 (1.9%)	0 (0.0%)	$P = 0.1635$
Unknown	490 (29.7%)	2 (1.6%)	$P < 0.0001$
Total	1649	128	

of development suggest that for translocation carriers, there is a strong natural selection process between these two developmental time points. Based on this, the shift in PGD testing from blastomere biopsy on day 3 to blastocyst biopsy on day 5/6 is beneficial for translocation carriers. Blastocyst biopsy not only enhances the likelihood of identifying a normal/balanced embryo for transfer, but also avoids many of the pitfalls and limitations of day 3 blastomere biopsy and FISH analysis, which are well documented [22, 23]. Indeed, a significant limitation of this and similar studies is the potential for technical inconsistency when analysing a single cell using FISH. This limitation is no doubt partly responsible for the increased rate of ‘unknown’ segregation modes detected following day 3 biopsy. While the limitations of single-cell-based FISH analysis further back the transition to day 5/6 biopsy for translocation patients, it is important to acknowledge that the successful clinical implementation of a blastocyst biopsy PGD program is inherently dependent upon the use of a high-quality culture system that supports the growth of embryos to the blastocyst stage of development [24]. If a sub-optimal culture system is in use, the benefits associated with blastocyst biopsy may be lost due to the significant reduction in embryo numbers and/or quality due to inefficient and harmful culture conditions.

This study supports the results of a previous study by Tan et al. [25], who performed a retrospective study on 575 translocation carriers to determine if PGD was more effective using day 5/6 biopsy with SNP array-based analysis and a frozen embryo transfer compared to traditional day 3 biopsy with FISH-based analysis and a fresh embryo transfer. They

Table 4 Day 3 versus day 5/6 data for Robertsonian translocation carriers

	Day 3	Day 5/6	Total
Normal/balanced	328 (38.7%)	143 (74.1%)	471
Unbalanced	519 (61.3%)	50 (25.9%)	569
Total	847	193	$P < 0.0001$

Table 5 Segregation modes for day 3 vs. day 5/6 for Robertsonian translocation carriers

Segregation mode	Day 3	Day 5/6	<i>P</i> value
Alternate	328 (38.7%)	143 (74.1%)	$P < 0.0001$
Adjacent	243 (28.7%)	48 (24.9%)	$P = 0.3284$
3:0	43 (5.1%)	2 (1.0%)	$P = 0.0097$
Unknown	233 (27.5%)	0 (0.0%)	$P < 0.0001$
Total	847	193	

compared PGD results as well as clinical outcomes and found that day 3 biopsy with FISH analysis had significantly ($P < 0.001$) more embryos with an unbalanced chromosome complement (reciprocal 80%, Robertsonian 64%) compared to day 5/6 biopsy with SNP array analysis (reciprocal 64%, Robertsonian 58%). Tan et al. concluded that SNP array-based PGD with day 5/6 blastocyst biopsy and a frozen embryo transfer has the potential to increase clinical outcomes when compared to FISH on blastomeres and a fresh embryo transfer.

Even though the shift to day 5/6 blastocyst biopsy has been shown to improve patient outcomes [26–28], there are differences in the technologies used for PGD testing. Extensive validation studies have been performed to compare NGS to other technologies. Our clinic has used NGS to reassess embryonic DNA from 52 embryos initially diagnosed as unbalanced for the chromosome rearrangement using array-CGH. NGS confirmed the unbalanced result for the embryo in 52/52 (100%) cases. Other groups have performed similar validation studies. Fiorentine et al. [29] performed a retrospective study of NGS against array-CGH and reported a high level of concordance (207/208; 99.5%) between the two technologies. Based on the success of this initial study, the same group [30] performed a prospective trial to assess the clinical application of NGS and array-CGH in parallel on day 5/6 blastocyst biopsy samples. Again, their results for blastocysts were concordant (191/195; 99.5%) between the technologies. Yang et al. [31] took this further and performed a randomised clinical study comparing clinical success rates following NGS compared with array-CGH. Both the NGS group and the array-CGH group consisted of 86 blastocysts. Their study showed that NGS was capable of screening all 24 chromosomes accurately and that ongoing pregnancy rates (74.7 vs. 69.2%; $P > 0.05$) and implantation rates (70.5 vs. 66.2%; $P > 0.05$) were similar between technologies. In a further study by Yin et al. [32], the diagnostic accuracy of SNP array and NGS was assessed on 38 blastocysts. SNP array and NGS results were concordant for 32 samples (26 euploid and 6 with simple aneuploidies). NGS was also capable of identifying 6 embryos with unbalanced chromosome abnormalities due to translocations (1 which was not identified by SNP array). They concluded that NGS and SNP array could identify aneuploidy with 100% consistency; however, for unbalanced

chromosome rearrangements, NGS may provide higher accuracy for some chromosome regions.

While the high levels of concordancy between NGS and array-based technologies are encouraging, it is important to note that there are some diagnostic differences between the different testing platforms. NGS has been shown to be more sensitive than array-CGH for the detection of chromosome abnormalities such as mosaicism [17, 18]. This improved sensitivity has been reported to lead to a decrease in the post-PGS miscarriage rate (~10% for array-CGH compared to ~4% following NGS) [17]. Both array-CGH and NGS are capable of detecting haploidy and triploidy but only if the embryo is male, while SNP array is capable of distinguishing between haploidy, diploidy and triploidy irrespective of embryo gender. The resolution of detection also varies between technologies. Array-CGH has been reported to detect segmental aneuploidies 5–6 Mb in size [33, 34], SNP array has been reported to detect segmental aneuploidies 2.4–5 Mb in size [35, 36], and NGS has been reported to detect segmental aneuploidies down to ~3 Mb in size [30].

The pattern of selection against chromosomally abnormal embryos in our study is supported by findings on aneuploidy rates at different embryo developmental stages [5, 26–28]. Two large studies by Fragouli et al. [26, 37] demonstrate a significant increase in euploid embryos at day 5/6 compared with day 3 of embryo development. The first study showed day 5/6 euploidy rates of 41.9% compared to 17.2% for day 3 embryos. The second study supported this with euploidy rates of 43.9% for day 5/6 embryos and only 16.3% for day 3 embryos. This group also reported a lower proportion of complex abnormalities among the aneuploid embryos on day 5/6 compared to day 3: 17.3 versus 49.7% [26] and 16.3 versus 50.8% [37]. This is consistent with our findings in the translocation data that there is selection against chromosomally abnormal embryos. Overall, taking both the translocation data and aneuploidy data together, this suggests that there is a fundamental selection process occurring between day 3 and day 5/6 of embryo development, which significantly increases the proportion of normal/balanced embryos and concomitantly decreases the proportion of aneuploid or unbalanced embryos.

While there are limited studies reporting on translocation segregation in day 3 versus day 5/6 embryos, the process of natural selection against chromosomally abnormal conceptuses is well documented. Gardner, Sutherland and Shaffer demonstrate this effective selection process through an analysis of aneuploidy rates at different stages of gestation [1]. Their figures show that 50% of day 3 embryos are chromosomally abnormal compared with 30% of conceptuses, 10% of first trimester fetuses and 0.4% of newborns. Several studies have also reported a correlation between pregnancy loss and chromosomal abnormalities at different gestational ages [38, 39]. Azmanvo et al. reported that 62% of blighted ovum, 36.2% of first trimester missed abortions and 34% of second trimester

abortions showed chromosomal abnormalities [40]. These observed patterns of pregnancy loss also demonstrate that the type of chromosome abnormality influences the gestation at which the pregnancy is lost. For example, double aneuploidy is observed at significantly higher rates in the first trimester compared with the second trimester [27, 40]. These findings are consistent with ours that as development progresses, there is an increase in normal/balanced chromosome compliments (alternate segregation) and a concomitant decrease in complex malsegregants. Studies such as these offer insight into a process of natural selection that begins in gametogenesis and continues well into the second trimester. This natural selection results in cessation of pregnancy due to non-viable genetic compliments with a clear correlation between gestational age and selection against chromosomal aberrations. The current study furthers this discussion by demonstrating a significant shift towards normal/balanced embryos at day 5/6 of development when compared with day 3, suggesting that there is a process of natural selection against chromosome abnormalities in early embryogenesis (i.e. specifically between blastomere day 3 and blastocyst stage day 5/6 embryos). It also demonstrates the selection for malsegregation between day 3 and day 5/6 to the least unbalanced form of the translocation.

Previous studies investigating the relationship between gestational age and pregnancy loss have highlighted the value in understanding the mechanisms that cause an unbalanced chromosome constitution to lead to foetal loss. This is of particular interest in the PGD setting where selection is occurring in the very early stages of embryogenesis. The natural selection process occurring between day 3 and day 5/6 is presumably due to impaired viability, leading to some abnormal embryos undergoing developmental arrest before reaching the blastocyst stage. McCoy et al.'s group found a high proportion of complex aneuploidies in day 3 embryo biopsies. They concluded that some of these aneuploidies are purged by selection prior to blastocyst formation [41]. Fragouli et al. have proposed that cell cycle regulatory mechanisms, which act to monitor and maintain accurate chromosome segregation, become active following the switch from the maternal genome to the embryonic genome at the blastomere stage, removing genetically unbalanced cells [26]. While this presents as a potentially feasible explanation, future research is required to investigate the genetic and biochemical pathways by which this natural selection occurs between day 3 and day 5/6 of embryological development.

Overall, this study shows that there is a selection process occurring between day 3 and day 5/6 of embryo development which increases the proportion of normal/balanced embryos, which supports the shift in PGD from day 3 blastomere embryo biopsy to day 5/6 blastocyst biopsy. Testing at this more advanced stage of development represents a greater predictor of ongoing viability. That is, if there are embryos available for biopsy on day 5/6 of development, they are more likely to be

normal/balanced. If there are no embryos available for biopsy on day 5/6 of development, the couples have not spent money on PGD testing embryos that are unbalanced and/or do not have the capacity to develop to the blastocyst stage in culture. This provides an improvement in testing for translocation carriers.

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Author's contribution Claire E. Beyer participated in study concept and design, data acquisition, study execution, analysis, manuscript drafting, critical discussion and interpretation of data.

Elissa Willats participated in study design, manuscript drafting, critical discussion and interpretation of data.

Compliance with ethical standards This study was approved by the Monash Surgical Private Hospital Human Research Ethics Committee (approval number: 07078).

Conflict of interest The authors declare that they have no conflict of interest.

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