Mosaicism: "survival of the fittest" versus "no embryo left behind"

Current techniques of preimplantation genetic diagnosis (PGD) for aneuploidy (PGD-A) combined with blastocyst biopsy have been shown to significantly improve implantation rates and ongoing pregnancy rates in at least three randomized clinical trials as well as several case-controlle d prospective studies and two metanalyses. Additionally, there is also strong evidence that the incidence of miscarriage is reduced in in vitro fertilization (IVF) cycles using PGD-A. A paper from the U.S. Centers for Disease Control and Prevention has provided further insight into the value of PGD-A for patients of advanced maternal age. The investigators analyzed IVF data from the United States between 2011 and 2012 and compared results for treatment cycles with or without PGD-A. They concluded that a statistically significant decrease in miscarriage rates and increased odds of live birth were apparent in cycles of women over 37 years old when PGD-A had been used (1). In addition, it has been demonstrated that the transfer of a chromosomally normal blastocyst results in an implantation rate that remains constant irrespective of maternal age (Fig. 1). This represents conclusive evidence in support of the hypothesis that PGD-A assists in the identification of viable embryos, and demonstrates that the major reason (perhaps the sole reason) for the decrease in embryo implantation rates per transfer with advancing maternal age is aneuploidy (2).

Despite these studies, results from a larger multicenter randomized clinical trial may be the only way to convince the most skeptical members in the reproductive medicine community that PGD-A has clinical value. Such a trial is currently under way and is close to completion (clinicaltrials.gov as NCT02268786). Despite the large and rapidly growing weight of evidence in support of PGD-A, some continue to have reservations about the clinical utility of preimplantation aneuploidy testing. When considering the effectiveness of PGD-A, it is important to understand the purpose or the process. It is intended as a selection tool to assist in the identification of the embryos with the greatest likelihood of viability, allowing them to be prioritized for transfer (ideally in a single-embryo transfer).

At the time PGD-A was conceived, embryo cryopreservation was associated with reduced implantation rates relative to fresh transfers; consequently, the concept of replacing one embryo at a time, a notion that relies on nondetrimental cryopreservation, was not considered to be a viable strategy. Today, with modern vitrification methods, the idea of cryopreserving all embryos followed by multiple rounds of single-embryo transfer seems more feasible. This has led some to argue that all methods of assessing embryo viability (including aneuploidy testing) should now be considered unnecessary. Eventually, the cumulative ongoing pregnancy rate after multiple embryo transfers would be expected to equal that obtained after transfer of viable embryos identified using techniques such as PGD-A. Even if only one embryo from a cohort is viable, eventually it will be transferred. Furthermore, if the results obtained from chromosome analysis were sometimes erroneous, resulting in euploid embryos being incorrectly discarded, a one-at-a-time embryo transfer strategy could actually be superior to PGD-A. However, this viewpoint fails to acknowledge that 15% to 40% of the nonselected embryos miscarry (a rate two to six times higher than observed after PGD-A), resulting in a significantly delayed time to conception. Additionally, many patients cannot face the emotional stress of multiple embryo transfers, leading to an appreciable rate of dropout from treatment, thus resulting in worse cumulative rates than PGD-A.

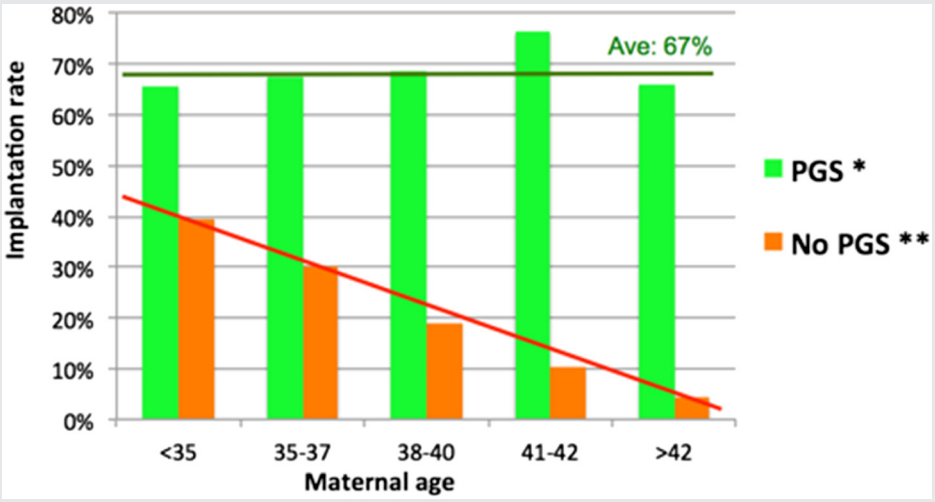
Above and beyond its use for embryo viability assessment, the capacity of PGD-A to reduce the incidence of miscarriages and all but eliminate the risk of children born with severe congenital abnormalities associated with trisomy represents the principal reasons why patients find this approach attractive. Pregnancy loss or, worse still, termination of a much-wanted pregnancy due to a serious genetic abnormality is perhaps the worst possible outcome of assisted reproductive treatment.

Although the arguments against PGD-A based upon "cumulative pregnancy rate" are not without a logical basis, it must be acknowledged that the transfer of a chromosomally normal embryo eliminates the impact of advancing female age on implantation rate (see Fig. 1), and the reduced time to pregnancy and lower risk of miscarriage represent significant advantages.

The second remaining source of criticism directed at PGD-A is that the error rate is still not 0, so some euploid embryos, which might have produced viable pregnancies, are incorrectly discarded while other aneuploid ones are inadvertently replaced. Most proponents of PGD-A have a background in clinical genetics, a field where the traditional emphasis is on sensitive detection of abnormalities. In most areas of clinical genetics, false positives, while undesirable, are nonetheless tolerated if in so doing the sensitivity of testing is increased. Perhaps unsurprisingly, this has led to a focus on minimizing false negatives during PGD-A analyses, reducing the risk of replacing abnormal embryos. The error rates of PGD-A have fallen from about 7% of reanalyzed embryos when using fluorescence in situ hybridization (FISH) to 1% to 2% with array comparative genomic hybridization (aCGH), quantitative polymerase chain reaction, and other modern techniques. Of the few errors that persist, most are probably caused by mosaicism: the presence of more than one distinct cell line within an embryo, each with different chromosome counts (a consequence of chromosome segregation error during mitosis—i.e., after fertilization). Often mosaic embryos do not contain any euploid cells, just a mixture of different aneuploid cell lines. However, in some cases an embryo may be composed of a combination of normal and abnormal cells, and it is these embryos that are at particular risk of misdiagnosis.

The blastocyst has become the preferred stage for PGD-A to be performed, and it is typical for approximately five cells to be biopsied from the trophectoderm and subjected to genetic testing. However, PGD-A is performed the cells removed

FIGURE 1



Implantation rates after transfer of euploid embryos are independent of maternal age. * 2,532 cycles of PGD-A by aCGH with known outcome to 8/2015 from Harton et al. (2) and unpublished data; ** 2013 SART data.

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from the blastocyst are not analyzed individually (unless FISH is used); rather, they are treated as a single sample. Unfortunately, most methods used for PGD-A lack the sensitivity necessary to detect minor cell populations within the biopsy specimen and instead only report the chromosomal status (i.e., normal or aneuploid) of the most common cell line in the sample. Array-CGH, the most widely used PGD-A method, can only begin to detect mosaicism when at least one-third of the cells have a chromosomal makeup distinct from the others, and even then the results may be insufficiently clear for a definitive classification of the embryo. Two other common methods, quantitative polymerase chain reaction and single-nucleotide polymorphism microarrays, have even poorer ability to reliably detect mosaic samples.

It is only with the recent introduction of next-generation sequencing (NGS) techniques that accurate detection of trophectoderm biopsy specimens with low-level mosaicism has become a reality (3). However, it is important to understand that not all NGS techniques are equal. All NGS methods provide accurate diagnosis of aneuploidy, but some have a much greater capacity for detecting mosaicism and subchromosomal (segmental) deletions and duplications than others. The exquisitely sensitive methods, capable of detecting a single aneuploid cell in a trophectoderm biopsy, are referred to as high-resolution NGS (HR-NGS), differentiating them from less subtle methods that assess only limited parts of the genome (low-resolution NGS). The ability of HR-NGS to identify mosaic samples has great scientific value, but is it of clinical relevance?

When faced with treatment cycles in which no euploid embryos are available for replacement as a result of all being classified aneuploid by PGD-A, a few practitioners have chosen to go ahead and transfer embryos that received an abnormal diagnosis. In a few cases, apparently normal babies have been born. This phenomenon could be attributed to misdiagnosis of aneuploidy, natural conception with an oocyte that was left in the ovary and subsequently ovulated, chromosomal mosaicism, or (unproven and much less likely) self-correction. Some critics of PGD-A claim that technical deficiencies mean that errors are being made, leading to viable euploid embryos being discarded. These critics argue that the transfer of embryos classified as monosomic by PGD-A should be considered because full autosomal monosomy is lethal. In the worst case, the diagnosis is correct, and there will be no pregnancy or a very early miscarriage; in the best case the diagnosis is incorrect, and, if the embryo is viable, a healthy pregnancy might be achieved.

Meiotic monosomy and trisomy are mostly caused by premature separation of chromatids, followed by nondisjunction and anaphase lag. However, on some occasions the transfer of monosomic embryos has resulted in trisomic miscarriages. This contradictory observation is explained by mosaicism. A classic mitotic nondisjunction will produce both monosomic and trisomic cells, and both may contribute to the embryo. Detection of a "nonviable" monosomy in the biopsy specimen leaves the embryo at increased risk for the corresponding trisomy. It should be remembered that many trisomies are compatible with pregnancy, although the great majority will end with a miscarriage. If the trisomy is present alongside a population of euploid cells (i.e., mosaicism persists), survival of the embryo to term is even more likely, in some cases producing children with severe congenital abnormalities.

The clinical application of HR-NGS is now beginning to yield important information for the debate between the two opposing strategies of "survival of the fittest" (transfer only those embryos most likely to be chromosomally normal) and "no embryo left behind" (transfer all embryos regardless of genetic status). The ability of HR-NGS to reduce misclassification of embryos with normal and aneuploid cell lines in

their corresponding biopsy sample adds a new dimension to PGD-A, further improving the capacity to differentiate individual embryos in terms of their likelihood of yielding a viable pregnancy. The impact of aneuploidy derived from meiosis, present in every cell of the embryo, is definitive (the vast majority are lethal). Most such abnormalities are a consequence of chromosome segregation errors affecting female meiosis, a phenomenon that greatly increases with advancing female age. Conversely, the incidence of segmental losses/duplications and mosaicism are unaffected by age and have a less clear-cut impact on embryo viability. Nonetheless, the identification of such anomalies is clinically valuable, as we will discuss in detail.

We have found that about 30% of blastocysts are mosaic at the blastocyst stage. This figure is close to that obtained years ago using FISH but has now been confirmed using modern HR-NGS (4). Because these abnormalities have a postmeiotic origin, it is conceivable that inadequacies of embryo culture play a role in the genesis of this problem, increasing the risk of chromosome malsegregation during mitosis. This remains highly speculative at this time, but there is some evidence that blastocyst mosaicism rates may vary between IVF clinics, hinting at procedure-related effects. Although almost one-third of blastocysts are mosaic, the increasing likelihood of meiotic (oocyte-derived) aneuploidy with increasing maternal age means that the proportion of embryos that are euploid/aneuploid mosaics decreases with age, from 26.6% in women <35 years old to 10.5% in >42 year olds (Table 1).

Several laboratories have shown that mosaicism is readily detected using HR-NGS when 20% or more cells in the specimen carry the aneuploidy. Considering that trophectoderm biopsies are typically composed of 5 to 10 cells, abnormal cells should always be detected in small (5-cell) biopsies as they represent at least 20% of the total, but in larger (10-cell) specimens it is not yet clear whether a single aneuploid cell, representing only 10% of the population, will always be detectable. Although HR-NGS is capable of detecting a substantial proportion of the mosaic embryos, there is no consensus about how these data should be interpreted or used in clinical management. In our opinion, the detection of mosaicism is of great clinical value. It should be reported and used to help decide which embryo(s) should be prioritized for transfer. Mosaic embryos can be considered to represent a distinct category in terms of viability, lying in between euploid and fully abnormal embryos, and we will explain the logic for this assertion.

Although miscarriage rates in PGD-A cycles are low (typically around 10% in cases using aCGH), they do sometimes occur. In a recent study, blastocyst biopsy specimens from 46 embryos that had miscarried, which had originally been classified as euploid after aCGH, were reanalyzed using HR-NGS. Remarkably, over 50% were shown to be mosaic (4). These data suggest that mosaicism, undetectable by most PGD-A techniques, plays an important role in pregnancy loss. From these results, it could be argued that miscarriage rates, already greatly reduced after aCGH, could be reduced further still in cycles where non-mosaic euploid embryos are available for transfer.

There is no published proof of a clear correlation between mosaicism in the trophectoderm and the inner cell mass. Thus, in some cases a low level of abnormal cells in the trophectoderm may be associated with a high level in the inner cell mass and vice versa. The detection of mosaicism in a trophectoderm biopsy specimen indicates that the embryo is (or was) mosaic, but the percentage of abnormal cells may be meaningless in terms of the remainder of the blastocyst. Regardless, a 1% to 2% error rate has been reported using other comprehensive chromosome analysis techniques when comparing both tissues. Because the extent of mosaicism in the biopsy specimen may or may not have relevance to the rest of the embryo, the use of a threshold (some laboratories use 50%) to guide whether a mosaic embryo should be considered normal or abnormal has, for now at least, no biological or clinical validity. As such, our recommendation is to report all mosaic embryos detected as mosaic, irrespective of the percentage of abnormal cells.

Further evidence demonstrating the value of detecting mosaicism in terms of embryo selection comes from experiments involving HR-NGS reanalysis of trophectoderm biopsies previously classified euploid by aCGH and where the outcome of embryo transfer was known. In nonselected random transfers in which the biopsy specimen was reanalyzed by NGS, 43 (29%) specimens were revealed to have mosaic chromosome abnormalities that were missed by the original aCGH evaluation. Assessment of outcomes from the transfer of such embryos confirmed that those exhibiting mosaicism do indeed have the potential to produce healthy children. However, the probability for implantation was

TABLE 1

Distribution of embryos by age group and diagnosis.

Age (y)	Euploid (%)	Aneuploid (%)	Mosaic and aneuploid (%)	Mosaic aneuploid/ euploid (%)	Total
Egg donor	61.2	14.8	6.5	17.4	1,972
<35	48.2	18.6	6.6	26.6	2,363
35–37	43.9	26.4	9.3	20.5	1,572
38–40	33.1	35.6	13.5	17.9	1,526
41–42	17.0	51.6	17.5	13.9	689
>42	10.6	57.6	21.2	10.5	436

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significantly reduced (roughly halved) relative to embryos with an entirely normal biopsy result (5). If mosaic embryos are to be replaced, we recommend having an extensive session with a genetic councilor to discuss the potential risks of mosaicism in terms of miscarriage and with respect to the impact on the health of any child produced.

Thus far, the proportion of embryos characterized as euploid using aCGH that can be shown to be mosaic by HR-NGS and additionally go on to produce a term pregnancy is 7% (5), but a larger data set will be needed to determine whether there are differences between mosaics associated with viable pregnancies and those that miscarry. Samples for the HR-NGS studies discussed earlier (4, 5) were analyzed by Illumina MiSeq, with manual workflow, and software VeriSeq PGS 1 (Illumina), MCS 2.5, and BFMv4.1.

For the reasons we have discussed, we now recommend classifying PGD-A biopsy results into three groups: [1] euploid, with the highest implantation potential; [2] mosaic, with an elevated miscarriage rate and reduced (but not null) implantation potential; or [3] aneuploid, with very high probability of implantation failure, a significantly elevated risk of miscarriage, probably close to zero potential for a live birth, and significant fetal and neonatal risk. We also recommend not using the proportion of abnormal cells in a mosaic trophectoderm biopsy as means for classifying embryos euploid or aneuploid because the fraction of abnormal cells in the biopsy specimen is not predictive of the situation in the remainder of the embryo. Artificially forcing mosaic embryos into euploid or aneuploid categories carries a risk of misdiagnosis.

In summary, although the detection of mosaicism has forced a rethink of clinical management strategies and represents a challenge for patient counseling, it should not be viewed as a negative consequence of PGD-A using HR-NGS. The information it contains is clinically important, offering an opportunity to minimize false-positive and false-negative results. The transfer of embryos with an entirely euploid trophectoderm biopsy, in preference to those with evident mosaicism, results in higher implantation rates and a lower risk of miscarriage. In the absence of any fully euploid embryos, the transfer of mosaics, which may have appeared aneuploid using less sensitive methods, will sometimes result in a viable pregnancy. With an improved viability assessment, opposing embryo transfer strategies can be satisfied. The fittest embryo can be transferred, while the risk of leaving behind an embryo with potential for implantation is eliminated. With methods such as HR-NGS, PGD-A as a selection tool is coming of age.

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