Detection of mosaicism at blastocyst stage with the use of high-resolution next-generation sequencing

Santiago Munné, Ph.D.[a] and Dagan Wells, Ph.D.[b,c]

[a] Reprogenetics, Livingston, New Jersey; [b] Reprogenetics UK, Oxford, United Kingdom; and [c] Institute of Reproductive Sciences, Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, United Kingdom

A significant proportion of human preimplantation embryos produced during the course of in vitro fertilization (IVF) treatments contain two or more cytogenetically distinct cell lines. This phenomenon, known as chromosomal mosaicism, can involve the presence of cells with different types of aneuploidy in the absence of any normal cells or a mixture of euploid and abnormal cells. Although a high prevalence of mosaicism at the cleavage and blastocyst stages has been appreciated for two decades, the precise frequency of the phenomenon and its consequences for embryo viability have been difficult to quantify. Recent advances in genetic technologies, such as high-resolution next-generation sequencing, have allowed mosaicism to be detected with much greater sensitivity than earlier methods. The application of these techniques to trophectoderm biopsies, taken from embryos before transfer to the uterus, has provided insight into the clinical impact of mosaicism. Data from recent studies show that blastocysts associated with mosaic trophectoderm biopsy specimens implant less often than embryos with a chromosomally normal biopsy. In addition, the mosaic embryos that succeed in establishing a pregnancy are at a significantly higher risk of miscarriage. Because mosaic embryos are less likely to produce a viable pregnancy than their euploid counterparts, we suggest that they are given a lower priority for transfer to the uterus. However, because these embryos can sometimes produce successful pregnancies, it is important that they can be considered for transfer in the absence of fully euploid embryos and after appropriate patient counseling. Unlike aneuploidy of meiotic origin, mosaicism, which is caused by mitotic errors occurring after fertilization, does not increase with advancing maternal age. There may, however, be clinical, treatment, or patient-related factors that contribute to the risk of mosaicism occurring. This review discusses the validation of methods that permit the detection of chromosomal mosaicism in IVF embryos and findings of clinical relevance. (Fertil Steril® 2017;107:1085–91. ©2017 The Authors. Published by Elsevier Inc. on behalf of the American Society for Reproductive Medicine. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).)

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DETECTION OF MOSAIC BLASTOCYSTS AND THEIR FREQUENCY

Mosaic preimplantation embryos contain two or more cell lines with a different chromosome content, the consequence of errors in chromosome segregation occurring during mitotic divisions. Most studies involving the analysis of mosaic embryos have been performed with the use of fluorescence in situ hybridization (FISH), a method favored because it provides information on the cytogenetic status of each cell. However, the frequency of embryonic mosaicism reported in the literature after FISH varies greatly, ranging from ~30% (1–7) to as high as 90% (8, 9). There are at least four reasons for these differences. One is technical, because FISH requires cell fixation, a technique that is difficult to master and with various alternative protocols available, some of which are associated with significantly higher error rates than others (10). Another, as recently reviewed by Capalbo et al. (11), is the criteria used to classify an embryo as abnormal. Some studies considered an embryo to be mosaic if just one of eight cells appeared to be cytogenetically distinct, whereas others used criteria that were more stringent, and arguably more appropriate, in which an embryo was considered to be mosaic only if it contained several cells with identical abnormalities (e.g., chromosome losses due to anaphase lag), reciprocal aneuploidy (monosomic and trisomic cell lines involving the same chromosome), or polyploidy (which can not be caused by fixation artifacts). The third reason is bias introduced by the type of material tested. Many studies focused on poor-quality material, including arrested embryos, which are more often mosaic

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Reprint requests: Santiago Munné, Ph.D., Reprogenetics, Suite 301, 3 Regent Street, Livingston, NJ 07039 (E-mail: santi@reprogenetics.com).

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than their counterparts of good morphology (3, 6). The fourth explanation for differences in reported mosaicism rates is that mosaicism can be iatrogenic—influenced by culture conditions (temperature, pH, media composition, etc.)—and therefore varies from clinic to clinic (12). The combination of unsuitable fixation techniques, insufficiently stringent criteria for defining mosaicism, and sample populations composed largely of arrested embryos, yields apparent mosaicism rates of 90%, but this is not representative of the biologic reality for most embryos. Studies using appropriate FISH methods provided consistent frequencies of mosaicism, with ~30% of embryos at the cleavage stage affected (4–7) and similar rates observed in blastocysts (5, 13).

Molecular cytogenetic techniques (e.g., array comparative genome hybridization [aCGH], single-nucleotide polymorphism [SNP] array, quantitative polymerase chain reaction [qPCR], next-generation sequencing [NGS]) have the advantage over FISH that they can provide information on the copy number of all 24 types of chromosome. In contrast, FISH studies typically examined only about one-third to one-half of the chromosomes in each cell. Unfortunately, these methods become relatively expensive when many individual cells need to be assessed, and consequently they have rarely been applied to disaggregated embryos as would be required for a definitive study of mosaicism (14, 15). Most research using comprehensive chromosome screening technologies have involved the analysis of blastocyst biopsy specimens, typically composed of ~5 cells, which are not separated but instead are analyzed as a single entity. Although the presence of a mixture of normal and aneuploid cells in the specimen can sometimes be detected with the use of methods such as aCGH, qPCR, and SNP array, they are relatively insensitive for this purpose. If ideal results are obtained, mosaicism associated with proportions of aneuploid cells ranging from 40% to 60% can be detected with a high degree of confidence. However, proportions of abnormal cells outside this range will frequently be indistinguishable from either normality (when there are few abnormal cells) or nonmosaic aneuploidy (when the majority of cells in the sample are aneuploid).

The method with the greatest power to detect mosaic samples is the relatively new technique of high-resolution next-generation sequencing (hr-NGS). Using hr-NGS, one study suggested that 21% of blastocyst biopsy samples contain a mixture of euploid and abnormal cells, and that a further 10% are mosaic for two or more different aneuploid lines. Those embryos found to be mosaic with the use of hr-NGS had proportions of aneuploid cells ranging from 20% to 80% (Liu et al., unpublished data). These results are similar to those of historical FISH studies, which analyzed all cells individually. In contrast to the findings from hr-NGS, a recent study using aCGH reported a mosaicism rate of only 4.8% in blastocyst biopsy specimens, with the proportion of aneuploid cells ranging from 35% to 50% (16). The higher rate of mosaicism detected by hr-NGS is likely explained by a superior sensitivity of this method for detecting minor lines in mixed cell populations compared with aCGH.

Interestingly, unlike aneuploidy of meiotic origin, the incidence of mosaic chromosomal abnormality does not change with advancing years, with ~30% of blastocyst-stage embryos affected across all maternal ages. However, because meiotic errors are more common in the embryos of older mothers, the percentage of blastocysts with biopsy specimens containing only euploid cells declines as a woman ages, falling from 48.2% for women <35 years of age to 10.6% of blastocysts for patients >42 years of age. Over the same period of time, the proportion of embryos with mosaic biopsies that include a normal cell line falls from 26.6% to 10.5%. The mitotic errors, leading to mosaicism, coupled with the advancing risk of meiotic aneuploidy has clinical implications for in vitro fertilization (IVF) treatments, especially those using preimplantation genetic screening for aneuploidy (PGS-A), because it effects the likelihood of detecting an entirely euploid embryo for transfer.

It is important to note that not all NGS strategies deliver the same information. Depending on the depth of sequencing and the specific NGS platform used, the sensitivity for detecting cytogenetically distinct subpopulations of cells varies. Considering that most blastocyst biopsies contain ~5 cells, the ability to detect of <20% abnormal cells (i.e., less than one abnormal cell out of five) or >80% aneuploidy (more than four abnormal cells out of five) is probably not relevant in the context of PGS-A. Nonetheless, it is important that aneuploidy in the 20%–80% range is consistently and reliably detected, because mosaicism in this range has clinical implications (discussed in detail below). Although some NGS methods have been validated for mosaicism detection (17, 18), questions remain as to the ability of other techniques to reliably detect this phenomenon, e.g., copy number variation sequencing (19), EmbryVu, qPCR, and other lower-resolution methods used for PGS-A.

VALIDATION OF MOSAICISM DETECTION WITH THE USE OF HR-NGS

The most widely used highours resolution NGS method is the VeriSeq PGS system (Illumina). This involves sequencing on a benchtop device called a MiSeq, which yields ~24 million short fragments of DNA sequence, known as "reads," per run. Not all of these sequences are necessary for enumeration of chromosome copy number, and, to make the test cheaper, it is usual for several DNA samples to be "barcoded" and analyzed simultaneously during the same run. In general, 60%–70% of reads can be mapped to unique parts of the genome and are therefore suitable for assessing the quantity of DNA from individual chromosomes. Therefore a typical experiment, in which 24 samples are analyzed in parallel, usually provides 600,000–900,000 reads per sample. This is sufficient for the detection of mosaic abnormalities present in 20%–80% of the cells comprising the biopsy sample. The software (BlueFuse Multi v3; Illumina) provides copy number counts for each chromosome pair. A chromosome with two copies is considered to be euploid, a chromosome with one copy monosomic, and a chromosome with three copies trisomic. Values that fall between the thresholds used for assigning one, two, or three chromosome copies may be

considered to be indicative of a mosaic abnormality, provided that the deviation in the values obtained is over and above any background noise. Depending on the exact value for each chromosome presented by the software, samples are classified as mosaic monosomies (1–2 chromosome copies present), mosaic trisomies (2–3 copies), segmental (or partial) mosaics (a piece of a chromosome having 1–2 [partial monosomy] or 2–3 [partial trisomy] copies), or complex mosaics (three or more distinct mosaic chromosomes). The detection of mosaic embryos has been validated by different approaches such as analyzing cell mixtures composed of different ratios of euploid and aneuploid cells and investigation of multiple biopsy specimens taken from mosaic embryos. Some of these experiments are described below.

The conversion of numeric values given by the Bluefuse software into estimates of the percentage of cells affected by a specific aneuploidy has been validated through experiments in which cells from cytogenetically characterized aneuploid cell lines were mixed with euploid cells in defined ratios. Aneuploidies that have been investigated include trisomy 13, trisomy 18, trisomy 21, monosomy 45,X, and 47,XXY (17, 18). Because trophectoderm biopsies usually consist of ~5 cells the aneuploid:euploid cell ratios investigated have been 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5. For each ratio, experiments have been repeated multiple times. To validate detection of segmental mosaicism, DNA from 11 cell lines carrying different partial aneuploidies were mixed with DNA from a chromosomally normal cell line in the same ratios as described above. The quantities of DNA tested were equivalent to ~5 cells (17, 18). Taken together, these experiments show that there was almost no overlap between samples with different ratios of aneuploid and normal cells and therefore it was possible to reliably infer the percentage of abnormal cells from the NGS data. Given that the ability to distinguish proportions of aneuploid cells >80% or <20% has not been subjected to rigorous validation, and considering that for a typical 5-cell biopsy it is not mathematically possible to have aneuploidy rates <20% or >80%, our position, as well as that of Controversies in Preconception, Preimplantation, and Prenatal Genetic Diagnosis (COGEN) and the Preimplantation Genetic Diagnosis International Society (PGDIS), is that trophectoderm biopsies containing <20% aneuploid cells should be classified as euploid and those with >80% abnormal cells should be considered aneuploid.

Other ways of evaluating the ability of a new technique to detect mosaicism include comparing it with established methods and, in the case of human embryos, taking multiple distinct biopsy specimens and assessing diagnostic concordance. The results obtained from the inner cell mass (ICM), which will become the fetus, versus the trophectoderm, from which the placenta and other extraembryonic tissues are derived, are of particular interest. Such studies have been carried out previously during the evaluation of aCGH. These investigations have demonstrated a good correlation, with little impact of mosaicism on diagnostic accuracy (20–23).

Recent studies comparing the abilities of aCGH and hr-NGS to detect aneuploidy have involved embryo biopsy and amplification of the DNA, after which separate aliquots of the amplification product were tested with the use of aCGH and hr-NGS. Embryos were classified by means of hr-NGS as aneuploid (n = 20), euploid (n = 20), or mosaic (n = 20) (17, 24). All embryos categorized as aneuploid or euploid were confirmed as such by means of aCGH (100% specificity and sensitivity). However, mosaic embryos (20%–80% abnormal cells estimated by means of hr-NGS) were not reliably detected with the use of aCGH. Most (80%) were classified as euploid and the remainder were given an abnormal chromosomal assignment. The fact that a majority of mosaics detected by hr-NGS are classified euploid when analyzed using aCGH means that a switch to NGS would result in fewer embryos receiving a "normal" diagnosis after PGS-A. This may have significant clinical ramifications, especially if fertility centers decide that mosaic embryos should not be transferred. Following such a policy, the proportion of cycles with no transfer would inevitably increase. If it is the case that some mosaic embryos are capable of forming viable pregnancies, treatment success rates could be harmed by their exclusion.

An additional validation of hr-NGS has involved re-biopsy of embryos that received either a fully aneuploid diagnosis or were indicated to be mosaic during routine PGS-A. An ICM sample, usually consisting of 5–10 cells, along with two to four trophectoderm samples ranging in size from 10 to ~50 cells, were taken from each embryo and tested with the use of hr-NGS, and the results for each specimen were compared with the original biopsy (Garrisi et al., unpublished data). Abnormal ICMs were observed for all embryos that had nonmosaic aneuploid biopsy specimens. In contrast, when mosaic aneuploidy was detected in a trophectoderm biopsy, it was only indicative of abnormality in the corresponding ICM in ~58% of embryos, although for complex mosaics this increased to 83%.

Another strategy to determine the extent to which mosaicism in a trophectoderm biopsy specimen is predictive of the status of the remainder of the blastocyst is to re-analyze embryos characterized as having nonmosaic aneuploidy for one chromosome and mosaic abnormality for another after routine PGS-A (17). In one study, 14 such embryos were identified and re-biopsied. In 12 of the 14, the full abnormality was confirmed in the subsequent biopsy specimens, and in the other two embryos the aneuploidy was shown to be present but in a mosaic form. However, for the chromosome(s) that PGS-A had shown to be mosaic in the initial biopsy, only three out of 14 displayed evidence of the same abnormality in all additional biopsy specimens (uniform aneuploid or mosaic), four had at least one other specimen with the same chromosome involved either in mosaic of full aneuploidy form, and the rest (50%) did not have any biopsy sample with that chromosome being aneuploid or mosaic, an outcome similar to the findings of Garrisi et al. (unpublished data).

As discussed above, a trophectoderm biopsy specimen, taken for the purpose of PGS-A, might not necessarily be representative of the rest of the embryo in all instances. Although concordance is very good for uniform aneuploidies, it is perhaps unsurprising that mosaic abnormalities are

confirmed with lower frequency. An important question is whether the proportion of abnormal cells in a mosaic biopsy specimen has any value for predicting the status of the remainder of the embryo. In one study, Blazek et al. (unpublished data) re-analyzed whole embryos previously classified as low-grade mosaics (<40% abnormal cells) after PGS-A of a single biopsy specimen with the use of hr-NGS. The conclusion was that, for clinical purposes, the vast majority of such embryos could be considered euploid. Further studies are required to verify this observation, but the suggestion is that low levels of abnormal cells in a mosaic trophectoderm biopsy are seldom indicative of widespread aneuploidy within the embryo.

Although the above studies have provided valuable information on the detection of mosaicism in human blastocysts, they remain insufficient to completely validate hr-NGS for this purpose. Most of the residual uncertainty is related to the possibility of technical artefacts, the impacts of which are yet to be fully quantified. One potential source of technical error is the biopsy itself. It is possible, although currently unproven, that excessive use of the laser or mechanical tearing of cells during the biopsy process could lead to artefactual loss or gain or chromosomes. The existence of dead and/or apoptotic cells in the biopsy specimen, containing genetic material in various stages of degradation might also lead to distortions in the DNA amplification, resulting in overcalling of mosaicism. Another factor that could potentially lead to incorrect assignment of embryos as uniformly aneuploid or entirely normal when they are in fact mosaic is the possibility that abnormal cells are restricted to a single area of the embryo. This would reduce the likelihood that both of the constituent cell lines would be present within a given biopsy specimen. However, evidence so far suggests that abnormal cells tend to be distributed relatively evenly and at random within mosaic embryos (25, 26). This is consistent with the concept that most of the mitotic errors that produce mosaicism occur in the first few divisions after fertilization. Preferential allocation of aneuploid cells to the trophectoderm, which might explain the phenomenon of confined placental mosaicism, does not appear to be present at the blastocyst stage.

In two recent opinion papers, Scott et al. (27) and Capalbo et al. (11) correctly pointed out that molecular cytogenetic methods might fail to detect mosaicism in instances where a trophectoderm biopsy contains an equal number of cells that are trisomic and monosomic for the same chromosome, because the relative excess and deficiency of chromosomal material would balance each other out, giving the appearance of euploidy. Previous FISH studies with appropriate fixation, scoring, and diagnostic criteria showed that mosaicism involving reciprocal trisomy/monosomy, presumably a consequence of classic mitotic nondisjunction, could be observed in 28% (126/455) of mosaic cleavage-stage embryos (28). In a separate study carried out at the blastocyst stage, mosaic embryos were re-biopsied in different areas and only 2/28 were found to contain a mixture of monosomic and trisomic cell lines involving the same chromosome. There is little evidence that one of the two lines produced by a mitotic nondisjunction event tends to be preferentially eliminated during development from the cleavage to the blastocyst stages, although there may be specific instances when this does occur (29, 30). So far, it seems that hr-NGS detects an equal number of monosomic and trisomic mosaics (Reprogenetics, unpublished data).

An alternate approach to determine whether mosaicism detected by hr-NGS is relevant is to consider clinical outcomes rather than technical evaluations. These are discussed below.

CLINICAL IMPLICATIONS OF CHROMOSOMAL MOSAICISM

There is mounting evidence that, in comparison to blastocysts associated with an entirely euploid trophectoderm biopsy specimen, embryos with a mosaic biopsy miscarry more often and implant less frequently, although it is also clear that some affected embryos can produce viable pregnancies. Evidence that mosaic embryos implant less than those that are euploid comes from a recent study in which mosaic embryos, as determined with the use of hr-NGS, resulted in 30.1% initial implantations and 15.4% ongoing pregnancies (18), significantly less than a well matched nonmosaic euploid control group (55.8% implantations, 46.2% ongoing pregnancies). This echoes data from another investigation, obtained with the use of aCGH, in which mosaic embryos were associated with a 38% implantation rate (19). An important difference between these two studies is that hr-NGS detects many more mosaics than aCGH (29% vs. 5%).

Several studies have specifically considered the risk of pregnancy loss following transfer of a mosaic embryo and reported that miscarriage rates are higher than for embryos with a euploid biopsy specimen. The miscarriage rate after transfer of euploid embryos, as classified with the use of NGS, was 6% compared with 13% when aCGH was used (Nisson et al., unpublished data) and 20% with the use of qPCR (31), potentially explained by the inferior ability of aCGH and qPCR to detect mosaicism, resulting in a higher likelihood that affected embryos were transferred. Perhaps more definitively, the study by Fragouli et al. (18) showed a miscarriage rate of 55.6% for blastocysts classified as mosaic, versus 17.2% for euploid control samples. In a separate study, re-analysis with the use of hr-NGS of the leftover DNA from embryos that miscarried despite having received a "euploid" categorization after aCGH indicated that only 46% of biopsy specimens were truly euploid, and the rest were actually mosaic or polyploid (Garrisi et al., unpublished data). Another investigation reported that 50% of biopsy specimens from embryos that miscarried were mosaic after analysis with the use of hr-NGS. This compared to a mosaicism rate of only 9% in samples from embryos associated with ongoing pregnancies ($P=.0062$) (17).

Despite implanting less and miscarrying more, some mosaic blastocysts can reach term (16, 18, 32); for this reason, this type of embryo should not necessarily be placed in the same category as those that are fully aneuploid. As we have argued previously (33), these embryos should be considered as a third group of intermediate potential. The question is: What is the chance that a pregnancy resulting from a mosaic embryo would result in an affected child?

Given their high frequency, it is inevitable that millions of mosaic embryos must have been transferred, unknowingly, since the advent of IVF. Reassuringly, there is no evidence of an increase in the risk of mosaic chromosome abnormality in children born following assisted reproductive treatments. This suggests that abnormal cell lines are either eliminated from the fetus by active mechanisms or they grow so slowly that they end up forming an insignificant population of cells. Alternatively, affected embryos may undergo developmental arrest or they may produce a nonviable pregnancy.

Chorionic villus sampling (CVS) studies show that ~2% of pregnancies originating from IVF are mosaic (34, 35), yet in one study 9% of embryos that succeeded in reaching term were associated with a mosaic biopsy specimen and therefore presumably contained an aneuploid cell line at an early developmental stage (20). There are no detailed studies following up babies resulting from the transfer of mosaic embryos, but to our knowledge more than 100 such babies have been born. So far, there have been no reports of abnormal karyotypes, although it is likely that few pregnancies/children have been subjected to a detailed cytogenetic assessment.

Mosaic embryos can be differentiated according to the percentage of abnormal cells in the biopsy specimen, the chromosomes involved, and the types of abnormalities (full chromosome, segment of a chromosome, single chromosome involved or multiple). A recent study evaluated the pregnancy outcomes after the transfer of different types of mosaic embryos detected with the use of hr-NGS (18). That research indicates that embryos that have several chromosomes affected by mosaic aneuploidy have significantly lower ongoing implantation rates (~6%) than any other class of mosaic embryo. In contrast, embryos with a mosaic segmental abnormality had a capacity to implant that was close to that of embryos with a euploid trophectoderm biopsy (18). Blastocysts with 40%–80% aneuploid cells in the biopsy sample were associated with a pregnancy rate of 22% ongoing pregnancies, and those with <40% abnormal cells resulted in a 56% ongoing pregnancy rate. No other differences were found.

The observation that abnormal cell load may have an impact on viability (32) is supported by older FISH studies (29, 30) and recently by an elegant murine study creating 1:1 chimeras of normal and very abnormal (complex aneuploid) cells at 2-cell and 8-cell stages (26). The mouse model shows that the abnormal cells at blastocyst stage are evenly distributed rather than located in defined clonal patches and are not allocated preferentially to the trophectoderm but to both trophectoderm and ICM. In these experiments, abnormal cell load determined the fate of the embryos, with high proportions of abnormal cells resulting in failure to implant or pregnancy loss and lower levels resulting in viable euploid embryos. These experiments suggest that abnormal cells do not (or very rarely) undergo any form of self-correction, but divide more slowly than normal cells and may ultimately arrest or undergo apoptosis. If the proportion of normal cells is high enough, those soon come to dominate and embryo viability may be preserved. Although these results from the study of Bolton et al. (26) are fascinating, and of potential relevance to embryos containing aneuploid cell lines with multiple abnormal chromosomes, it is unclear to what extent the results are meaningful for embryos with mosaic segmental abnormalities and mosaic trisomies of types that, even in nonmosaic form, are potentially compatible with implantation, pregnancy, and in some cases birth.

It is also important to note that the origin of embryonic mosaicism and placental mosaicism are probably not the same, with the first being progressively eliminated as normal cells take over in low-grade mosaic embryos or by the demise of high-grade mosaic embryos (26), whereas many instances of placental mosaicism appear to originate from cytotrophoblasts acquiring aneuploidies as they differentiate and adopt a more invasive phenotype (36). An origin after differentiation of the ICM and trophectoderm lineages would also offer an explanation as to why placental mosaicism is seldom confirmed in the fetus (37).

Chromosomal mosaicism in pregnancy and at birth can result in congenital abnormalities, as well as problems such as autism and mental retardation. Every chromosome can be associated with an abnormal phenotype when in mosaic form, the spectrum extending from apparently normal to severely affected/lethal. However, as mentioned above, the types of mosaicism observed during preimplantation development and those that affect the fetus or the newborn might represent different phenomena. This is probably one of the most important questions remaining to be answered.

MOSAICISM MAY BE INDUCED OR EXACERBATED BY THE ASSISTED REPRODUCTION PROCESS

Two decades ago, we reported that mosaicism rates can be influenced by specific culture conditions, such as differences in temperature control (12). More recently, variation in culture media have been shown to produce different rates of mitotic chromosome abnormalities (Hickman et al., unpublished data). It is notoriously difficult to compare results from one clinic with another owing to differences in patient populations. However, this difficulty can be mitigated, to some extent, by considering results from oocyte donors only, which represent a somewhat more homogeneous population. Mosaicism rates in the embryos produced by this class of patient have been shown to vary greatly between clinics, ranging from 16% to 44% ($P<.001$; Sachdev et al., unpublished data). This hints at treatment-related influences of the risk of chromosome malsegregation during mitosis (32).

Capalbo et al. (11). have argued that because there is no difference in the prevalence of mosaicism in first-trimester pregnancies conceived either spontaneously or with the use of assisted reproductive technology, treatment conditions are unlikely to result in an increased risk of chromosome malsegregation. However, as hypothesized above, embryonic and fetal mosaicism might be two very different phenomena which are resolved in different ways.

If future studies confirm that variation in chromosome abnormalities do indeed exist between fertility centers, affecting factors such as aneuploidy rate and the incidence of mosaicism and segmental abnormalities, one could

envisage a time when PGS-A might be used as a quality-control measure, assisting in the optimization of embryo culture and treatment conditions. We propose that the genetic optimization of IVF techniques represents a new frontier in the advancement of infertility treatments, which will become an important force for progress in the field in the coming years.

PATIENT MANAGEMENT AND GUIDELINES

As PGS-A began to move away from methods such as qPCR and aCGH and toward more sensitive NGS-based approaches, mosaicism became readily detectable and grew to be a topic of great interest and increasing concern. In response to an urgent need for guidance, professional organizations convened panels of experts and issued recommendations concerning the transfer of mosaic embryos (e.g., COGEN, PGDIS, Besser and Mounts [38]). However, the published guidelines were written at a time when there was still a paucity of published data concerning clinical outcomes. It is likely that some recommendations will need to be reviewed in the light of more recent findings (18). For example, PGDIS suggested that embryos showing mosaic euploid/monosomy are preferable to euploid/trisomy, given that monosomic embryos (excepting 45,X) are not viable. Although limited, current findings do not support this recommendation, because both types of mosaic embryos have been shown to produce the same frequency of ongoing implantation.

Another recommendation is to transfer some mosaic embryos in preference to others, depending on the type of chromosome involved. Mosaics involving chromosomes 14 and 15 are discouraged because of a perceived risk of uniparental disomy; abnormalities in chromosomes 2, 7, and 16 are associated with intrauterine growth retardation; chromosomes 13, 18, and 21 are considered to be problematic because in trisomic form these abnormalities can potentially reach term. However, transfer of embryos with mosaicism affecting each of these chromosomes have produced ongoing pregnancies, with none so far reporting adverse effects. Overall, the initial recommendations may turn out to be overly cautious, but given the scarcity of the data available at the time of their writing, the proposal of a conservative approach to mosaic embryo transfer was entirely understandable and reasonable.

One recommendation that still seems to be justified is that if a pregnancy results from the transfer of a mosaic embryo, genetic counseling should be offered and the pregnancy should receive appropriate monitoring. Prenatal diagnosis should be undertaken, preferably by means of amniocentesis, providing further evidence on the presence/absence of any aneuploid cell line detected at the blastocyst stage. Noninvasive prenatal testing and chorionic villus sampling are also options, but they test placental cells rather than the actual fetus and might produce less definitive results, especially if mosaicism is present.

CONCLUSION AND PENDING QUESTIONS

Currently, there is a lack of uniformity in the way that different genetics laboratories score mosaicism in trophectoderm biopsy samples, some using stricter criteria than others. In our opinion, the use of insensitive methods for the detection of mosaicism and/or the application of diagnostic thresholds that classify most mosaic embryos as either normal or uniformly aneuploid will have negative clinical consequences. Mosaic embryos may be inappropriately categorized as aneuploid, leading to potentially viable embryos being discarded, or as entirely normal, carrying an elevated risk of aneuploid pregnancy. Our initial opinion, and that of PGDIS, was that blastocysts associated with biopsy specimens containing 20%–80% abnormal cells, as determined with the use of validated technologies, should be classified as "mosaic." This represents a third category, distinct from euploid and aneuploid embryos, the constituents of which would receive a lower priority for transfer to the uterus than embryos diagnosed as "euploid" (33). The most recent data available (32) suggests that the majority of embryos with 20%–40% aneuploid cells in their biopsy sample have euploid ICMs and could be considered for transfer if no normal embryos are available. Blastocysts with 40%–80% abnormal cells and those with complex mosaicism should be given the lowest priority for transfer or be excluded. In our opinion, an overly cautious approach to the transfer of mosaic embryos risks an undesired negative impact on cumulative pregnancy rates, because some embryos with the potential to produce babies may be discarded.

The validation experiments outlined in this review demonstrate that hr-NGS succeeds in detecting mosaicism in the vast majority of trophectoderm biopsies in which it is present. The frequency of false positives and negatives appears to be low, but it will require additional studies to be accurately quantified. Other pending questions requiring further research include: 1) How many mosaic embryos are classified as euploid by different techniques used for PGS-A? 2) To what extent does the percentage of abnormal cells in a single biopsy specimen reflect the proportions that exist in other areas of the embryo? 3) What is the frequency of mosaic monosomy versus mosaic trisomy in blastocyst biopsies, and is there any difference in viability of one type of cell line over the other? 4) Do elements of biopsy methodology have the capacity to produce artefactual mosaic results? 5) What is the true frequency of mosaicism, as determined by disaggregation of entire blastocysts and analysis of each cell with the use of comprehensive chromosome analysis methods? 6) Is there a link between mosaicism at the blastocyst stage and that observed later in the pregnancy or at birth, or are these types of mosaicism unrelated and independent? and 7) What are the long-term outcomes for individuals originating from a mosaic embryo transfer, and can any trace of an aneuploid cell line ever be detected in their bodies? It is likely that many of these questions will be answered within the next couple of years, leading to an improved understanding of the causes and consequences of mosaicism and enhanced clinical management.

REFERENCES

1. Delhanty JD, Griffin DK, Handyside AH, Harper J, Atkinson GH, Pieters MH, et al. Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridization (FISH). Hum Mol Genet 1993;2:1183–5.

2. Munné S, Weier HU, Grifo J, Cohen J. Chromosome mosaicism in human embryos. Biol Reprod 1994;51:373–9.
3. Munné S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates and maternal age are correlated with chromosome abnormalities. Fertil Steril 1995;64:382–91.
4. Magli MC, Jones GM, Gras L, Gianaroli L, Korman I, Trouson AO. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Hum Reprod 2000;15:1781–6.
5. Bielanska M, Tan SL, Ao A. Chromosomal mosaicism throughout human Preimplantation development in vitro: incidence, type, and relevance to embryo outcome. Hum Reprod 2002;17:413–9.
6. Munné S. Chromosome abnormalities and their relationship to morphology and development of human embryos. Reprod Biomed Online 2006;12:234–53.
7. Colls P, Escudero T, Cekleniak N, Sadowy S, Cohen J, Munné S. Increased efficiency ofpreimplantation genetic diagnosis forinfertility using "no result rescue". Fertil Steril 2007;88:53–61.
8. Baart E, Martini E, van den Berg I, Macklon N, Galjaard RJ, Fauser BC, Van Opstal D. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. Hum Reprod 2006;21:223–33.
9. Taylor TH, Gitlin SA, Patrick JL, Crain JL, Wilson JM, Griffin DK. The origin, mechanisms, incidence and clinical consequences of chromosomal mosaicism in humans. Hum Reprod Update 2014;20:571–81.
10. Velilla E, Escudero T, Munné S. Blastomere fixation techniques and risk of misdiagnosis for PGD of aneuploidy. Reprod Biomed Online 2002;4:210–7.
11. Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: current challenges and future possibilities. Hum Reprod 2017;32:492–8.
12. Munné S, Magli C, Adler A, Wright G, de Boer K, Mortimer D, et al. Treatment-related chromosome abnormalities in human embryos. Hum Reprod 1997;12:780–4.
13. Fragouli E, Alfarawati S, Daphnis DD, Goodall NN, Mania A, Griffths T, et al. Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: scientific data and technical evaluation. Hum Reprod 2011;26:480–90.
14. Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. Mol Hum Reprod 2000;6:1055–62.
15. Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. Hum Genet 2000;106:210–7.
16. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. N Engl J Med 2015;373:2089–90.
17. Maxwell SM, Collis P, Hodes-Wertz B, McCulloh DH, McCaffrey C, Wells D, et al. Why do euploid embryos miscarry? A case-control study comparing the rate of aneuploidy within presumed euploid embryos that resulted in miscarriage or live birth using next-generation sequencing. Fertil Steril 2016;106:1414–9.
18. Fragouli E, Alfarawati S, Spath K, Tarozzi N, Borini A, Wells D. Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid-aneuploid blastocysts. Hum Genet. In press.
19. Ruttanajit T, Chanchamroen S, Cram DS, Sawakwongpra K, Suksalak W, Leng X, et al. Detection and quantitation of chromosomal mosaicism in human blastocysts using copy number variation sequencing. Prenat Diagn 2016;36:154–62.
20. Capalbo A, Wright G, Elliott T, Ubaldi FM, Rienzi L, Nagy PZ. FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. Hum Reprod 2013;28:2298–307.
21. Colls P, Escudero T, Fischer J, Cekleniak N, Ben-Ozer S, Meyer B, et al. Validation of array comparative genome hybridization for diagnosis of translocations in preimplantation human embryos. Reprod Biomed Online 2012;24:621–9.
22. Kung A, Munné S, Bankowski B, Coates A, Wells D. Validation of next-generation sequencing for comprehensive chromosome screening of embryos. Reprod Biomed Online 2015;31:760–9.
23. Mir P, Mateu E, Mercader A, Herrer R, Rodrigo L, Vera M, et al. Confirmation rates of array-CGH in day-3 embryo and blastocyst biopsies for preimplantation genetic screening. J Assist Reprod Genet 2016;33:59–66.
24. Ribustello L, Escudero T, Lanzas J, Liu E, Munné S. Mosaic embryos, detected by NGS, are mostly classified as euploid by aCGH. Reprod Biomed Online 2016;33:258–9.
25. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. Hum Reprod 2014;29:1173–81.
26. Bolton H, Graham SJL, van der Aa N, Kumar P, Theunis K, Fernandez Gallardo E, et al. Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nat Commun 2016;7:11165.
27. Scott RT, Galliano D. The challenge of embryonic mosaicism in preimplantation genetic screening. Fertil Steril 2016;105:1150–2.
28. Munné S, Sandalinas M, Escudero T, Marquez C, Cohen J. Chromosome mosaicism in cleavage stage human embryos: evidence of a maternal age effect. Reprod Biomed Online 2002;4:223–32.
29. Bielanska M, Tan SL, Ao A. High rate of mixoploidy among human blastocysts cultured in vitro. Fertil Steril 2002;78:1248–53.
30. Sandalinas M, Sadowy S, Alikani M, Calderon G, Cohen J, Munné S. Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. Hum Reprod 2001;16:1954–8.
31. Rodriguez-Purata J, Lee J, Whitehouse M, Duke M, Grunfeld L, Sandler B, et al. Reproductive outcome is optimized by genomic embryo screening, vitrification, and subsequent transfer into a prepared synchronous endometrium. J Assist Reprod Genet 2016;33:401–12.
32. Munné S, Alikani M, Ribustello L, Colls P, Martínez-Ortiz PA, Referring Physician Group, McCulloh DH. Euploidy rates in donor egg cycles significantly differ between fertility centers. Hum Reprod 2017;32:743–9.
33. Munné S, Grifo J, Wells D. Mosaicism: "survival Fittest" Versus "no Embryo Left behind". Fertil Steril 2016;105:1146–9.
34. Kalousek DK, Barrett IJ, Gärtner AB. Spontaneous abortion and confined chromosomal mosaicism. Hum Genet 1992;88:642–6.
35. Huang A, Adusumalli J, Patel S, Liem J, Williams J, Pisarska MD. Prevalence of chromosomal mosaicism in pregnancies from couples with infertility. Fertil Steril 2009;91:2355–60.
36. Weier JF, Weier HU, Jung CJ, Gormley M, Zhou Y, Chu LW, et al. Human cytotrophoblasts acquire aneuploidies as they differentiate to an invasive phenotype. Dev Biol 2005;279:420–32.
37. Malvestiti F, Agrati C, Grimi B, Pompilii E, Izzi C, Martinoni L, et al. Interpreting mosaicism in chorionic villi: results of a monocentric series of 1001 mosaics in chorionic villi with follow-up amniocentesis. Prenat Diagn 2015;35:1117–27.
38. Besser AG, Mounts EL. Counselling considerations for chromosomal mosaicism detected by preimplantation genetic screening. Reprod Biomed Online 2017 [Epub ahead of print].