Diagnosis and clinical management of embryonic mosaicism

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Embryonic mosaicism occurs when two or more cell populations with different genotypes are present within the same embryo. New diagnostic techniques for preimplantation genetic screening (PGS), such as next-generation sequencing, have led to increased reporting of mosaicism. The interpretation of mosaicism is complicated because the transfer of some mosaic embryos has resulted in live births. Mosaic embryos may represent a third category between normal (euploidy) and abnormal (aneuploidy). This category of mosaic embryos may be characterized by decreased implantation and pregnancy potential as well as increased risk of genetic abnormalities and adverse pregnancy outcomes. Euploid embryos should be preferentially transferred over mosaic embryos. Genetic counseling is necessary before the transfer of a mosaic embryo is considered. Certain types of mosaic embryos should be preferentially transferred over others. Transfer of embryos with mosaic trisomies 2, 7, 13, 14, 15, 16, 18, and 21 may pose the most risk of having a child affected with a trisomy syndrome; however, the transfer of embryos with mosaic monosomies or other mosaic trisomies are not devoid of risk. Patients must be counseled about the risk of undetected monosomies or trisomies within a biopsy specimen as well as the risk of intrauterine fetal demise or uniparental disomy with the transfer of mosaic embryos. Until more data are available, patients should be encouraged to undergo another cycle to obtain euploid embryos, when possible, rather than transferring a mosaic embryo. (Fertil Steril® 2017;107:6–11. ©2016 by American Society for Reproductive Medicine.)

Key Words: Preimplantation genetic screening, next-generation sequencing, embryonic mosaicism

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Mosaicism within an embryo is defined as the presence of two or more cell populations with different genotypes. Early studies demonstrated mosaicism within preimplantation human embryos at the cleavage stage with the use of fluorescence in situ hybridization (FISH) of sex chromosomes (1). Embryonic mosaicism was found to result from mitotic errors occurring after fertilization, occasionally in the first cleavage but more commonly in the second or third cleavage (2). Mosaic embryos may be classified as aneuploid mosaic, where two different aneuploid genotypes exist and 100% of the cells within the embryo are abnormal, or diploid-aneuploid mosaic, where one population of the cells is euploid and the other is aneuploid. The percentage of abnormal cells within a diploid-aneuploid mosaic embryo is influenced by the cleavage stage in which the chromosomal segregation error occurs. For example, errors occurring at the time of the second cleavage may result in a greater proportion of abnormal cells than errors occurring during the third cleavage (2).

The early embryo is prone to errors of mitosis because of inactivation of the genome at fertilization. Oocyte mRNA is degraded, and genome stability is dependent on oocyte cytoplasmic transciptomes during the first three cell divisions. Embryonic genome activation does not occur until after the third cleavage stage, and some genes important for cell division are not expressed until the blastocyst stage (3). Mosaicism may develop within a diploid embryo for a variety of reasons, including anaphase lag, mitotic nondisjunction, inadvertent chromosome demolition, and premature cell division before DNA duplication (4, 5). For this reason, the detection of mosaicism among cleavage-stage blastomere biopsies is high (6). Mosaic cleavage-stage embryos left in extended culture have been shown to self-correct to euploid blastocysts in nearly 50% of cases (7). Several mechanisms may be involved in the correction of aneuploidy, including increased apoptosis of aneuploid cells, decreased division of aneuploid cells in relation to euploid cells, or preferential development of euploid cells within the inner cell mass (ICM) (8). Trisomic cell populations may self-correct by losing the extra chromosome via anaphase lag or nondisjunction (9); however, this explanation is less likely, given the low rate of detection of uniparental disomy among blastocysts (10).

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DETECTION OF MOSAICISM AND INTERPRETATION OF MOSAIC RESULTS

The rate of mosaicism within preimplantation embryos not only varies based on the stage of the embryo, but also with the chromosomal detection technique used. Preimplantation genetic screening (PGS) was initially performed with the use of FISH from a single blastomere biopsy. FISH uses fluorescent microscopy to visualize fluorescent probes hybridized, most commonly, to five chromosomes (X, Y, 13, 18, and 21). Aneuploidy detection by means of FISH is limited when probes for more than ten chromosomes are used in one sample. Mosaicism of the remaining autosomes, therefore, could not be detected. Additionally, studies on cleavage-stage embryo mosaicism are limited to discarded embryos because of the risk of embryo damage from the requirement for multiple blastomere biopsies (2). Comprehensive chromosome screening (CCS) with the use of whole genome amplification and comparative genomic hybridization (CGH) to assess all 24 chromosomes emerged as a superior method for the assessment of mosaicism. Findings confirmed that high levels of mosaicism (up to 75%) are seen in cleavage-stage embryos (6).

Given the considerable findings of mosaicism in cleavage embryos, trophectoderm (TE) biopsy of blastocysts with the use of CCS has become widely used in clinical practices worldwide. Blastocyst biopsies contain approximately four to ten TE cells (11), allowing for the detection of mosaicism in a single biopsy. Numerous studies have demonstrated the utility of array CGH (aCGH) for use in PGS (12, 13). It uses whole genome amplification to amplify embryonic DNA and reference DNA, followed by fluorescent labeling of each with two distinct colors. DNA probes, approximately 4,000 DNA markers spaced throughout the genome, are spread out on the microarray. Both sets of DNA then compete for hybridization on the microarray. Computer software analyzes the fluorescent intensities of the hybridized DNA, and calculates the copy number of reference DNA compared with embryonic DNA (14). Array CGH is used to detect whole chromosome aneuploidy, but it is not validated to detect structural chromosomal aberrations in the genome (15).

The rate of mosaicism among blastocysts with the use of aCGH is estimated to be 4.8%–32% (16–18) and may vary based on the aCGH protocols used. The ability of aCGH to detect mosaicism is dependent on the percentage of aneuploid cells within the TE biopsy specimen. Mamas et al. (2012) investigated the detection rate of aCGH on known mixtures of euploid and aneuploid (trisomic) cells. Array CGH was able to pick up mosaicism when >50% of cells were abnormal (defined as log2 ratio >0.3). Confidence intervals of log2 ratios, however, were shown to span from the upper limits of normal (euploid) to abnormal (aneuploid), demonstrating the difficulty in interpreting borderline values (19).

Another study performed by Capalbo et al. (2013) evaluated the concordance of aneuploidy results between aCGH-screened embryos and FISH reanalysis of blastocyst TE biopsy and ICM samples (17). They found that ~2% of embryos studied were diploid-aneuploid mosaic with >40% normal cell lines according to aCGH and FISH. Array CGH failed to detect diploid-aneuploid mosaicism when <25% of cells in the TE biopsy specimens were abnormal. Array CGH accurately detected all cases of mosaicism when >40% of TE biopsy samples were aneuploid. With medium-grade mosaicism (25%–40% abnormal cells), aCGH correctly identified three cases and misdiagnosed two cases. Concordance for all chromosomes was 97% (68/70 blastocysts) between TE and ICM biopsies with the use of aCGH and 100% for chromosomal complement on a per-embryo basis. The distribution of abnormal cells within the tested embryos was uniform, which was consistent with previous findings (17, 20).

Next-generation sequencing (NGS) has emerged as a new technique for PGS with the advantages of high accuracy with increased throughput and decreased cost compared with aCGH (21, 22). Multiple DNA samples may be analyzed at the same time and reports generated within 13–16 hours. The two most common platforms used for PGS are the MiSeq from Illumina and the Personal Genome Machine from Thermo-Fischer Scientific. Whole genome amplification is first performed. DNA is then lysed into fragments, and fragments are fused with an adapter and a barcode. For the MiSeq platform, a bridge polymerase chain reaction (PCR) step is performed, followed by optics-based sequencing by synthesis. After quality assurance metrics are performed, data are then analyzed with the use of BlueFuse software (Illumina). The MiSeq platform is designed to identify whole chromosome aneuploidy and mitochondrial copy number. Illumina's VeriSeq genome analysis on the MiSeq platform is designed to detect whole chromosome aneuploidy and mosaicism of $\geq$50%. The Personal Genome Machine, conversely, involves an emulsion PCR step followed by detection of hydrogen ion release by DNA polymerase during sequencing by DNA synthesis. A sensor detects the change in pH due to the release of hydrogen ions. The Torrent Browser software performs quality assurance metrics, and then data are analyzed with the use of the Ion Reporter Software. The Personal Genome Machine is designed to detect whole chromosome aneuploidy, deletions, or duplications down to a resolution of 800 kb to 1 Mb, mosaicism of $\geq$20%, and mitochondrial copy number. Both NGS platforms can be used to detect single gene mutations (15).

NGS may have a greater ability to detect mosaicism in multicellular samples, owing to its increased dynamic range in comparison to aCGH (23). A randomized blinded study comparing NGS and quantitative PCR for the detection of mosaicism with the use of mixed model aneuploidy cell lines showed that NGS is able to detect mosaicism when as few as 17% of the cells are aneuploid with 100% specificity across variable proportions of aneuploid cell mixtures. The application of custom analysis criteria, however, significantly increased the sensitivity of detecting aneuploid cell lines, but simultaneously increased the false positive rate from 0% to 33% (24). Differences in analysis criteria between laboratories may explain the different reporting rates of mosaicism in blastocyst biopsies.

With increased reporting of mosaicism with the use of NGS, the question of whether a single TE biopsy is indicative of the chromosomal complement of the entire embryo has

again been raised. Garrisi et al. (2016) performed multiple biopsies on 43 embryos diagnosed by means of NGS as mosaic (25). Repeated biopsies of the TE and ICM diagnosed five embryos (11.6%) as normal in all rebiopsy samples, and 18 with normal ICM (41.8%). TE biopsies showing complex mosaicism, however, were consistent with the ICM 83% of the time. The overall predictability of a diagnosis of mosaicism from the TE biopsy was 58.2%. Similarly, Maxwell et al. (2016) performed multiple repeated TE biopsies on 14 aneuploid/mosaic embryos with the use of NGS and confirmed mosaicism in only 48.3% (26). The detection of low-grade mosaicism within an embryo appears to be subject to some degree of sampling error.

Higher rates of reporting of mosaicism with the use of NGS compared with aCGH have led to questions regarding the ongoing pregnancy potential of these embryos and the validity of a diagnosis of mosaicism. Apprehension about transferring mosaic embryos arise from the concern that abnormal pregnancies may result; however, Greco et al. 2015 showed in a small study that mosaic embryo transfers resulted in either healthy live births with normal karyotypes (confirmed by means of chorionic villi sampling), biochemical pregnancies, or a negative pregnancy (16), further suggesting that some mosaic embryos may self-correct. Another explanation may be that some embryos labeled as mosaic may in fact be euploid (false positives). In a retrospective reanalysis of whole genome amplification products from 76 blastocysts determined to be euploid by means of aCGH, 12/38 blastocysts resulting in miscarriage (31.6%) and 6/38 blastocysts resulting in live birth (15.8%) were found to be mosaic by means of NGS (26). This suggests that some mosaic embryos result in live birth, but they may also be at increased risk of early pregnancy loss. The implantation potential of mosaic embryos has yet to be determined, but data suggest that it may be reduced (27).

WHICH PATIENT POPULATION IS AT RISK FOR MOSAICISM?

The association between advanced maternal age and aneuploid embryos is well documented (28, 29); however, its correlation with mosaicism is not yet clear. An earlier study by Daphnis et al. (2005) with the use of FISH analysis on day 5 discarded embryos (not transferred nor suitable for cryopreservation) showed a uniformly high rate of mosaicism (90%) despite a mean maternal age of 34 years, suggesting that mosaicism is not associated with age (30). Turner et al. (2016), performed 24-chromosome FISH with the use of multicolored nuclei to detect blastocyst mosaicism and found no correlation with advancing maternal age (31). In addition, retrospective data from a large reference laboratory using only NGS for PGS screening showed a 33% mosaicism rate among donor oocyte–derived embryos from donors aged 21–30 years, illustrating that post-zygotic errors occur frequently in the young fertile population (32).

Unidentified laboratory factors may contribute to the rates of mosaicism within blastocysts. In a study including 192 donor oocyte cycles from nine different IVF centers, rates of mosaicism detected by NGS performed at a single genetics laboratory ranged from 17% to 47% (33). The effect of laboratory techniques on rates of mosaicism warrants further study.

RESULTS INTERPRETATION AND PATIENT COUNSELING

The increased reporting of mosaicism in embryos has given rise to new challenges in PGS results interpretation and patient counseling. Previously, embryos were diagnosed as either euploid or aneuploid, and in most cases only euploid embryos were considered for transfer. Now, if mosaic embryos are considered to represent a third category of results (34), patient education and counseling strategies must evolve accordingly.

Though early data suggest clinical value in detecting embryonic mosaicism (34), patients may differ in their desire for the potentially uncertain information this provides. Therefore, for patients to make informed decisions about whether or not to pursue PGS, pre-test counseling should include a discussion about the frequency of mosaic results, the challenges associated with interpretation of these results, the possibility of a false positive diagnosis of embryonic mosaicism, and the limited predictive data available. For patients who are uncomfortable with results that are not definitive, this discussion may be a deterrent from pursuing PGS. Patients who elect to move forward with PGS can adjust their expectations regarding the type of information gained from this testing.

Current data support the preferential transfer of euploid embryos; however, many cycles do not produce euploid embryos. Given that some embryos diagnosed as mosaic have the potential to produce healthy children (16), patients who do not receive euploid results and are unable or unwilling to attempt another cycle may consider transfer of a mosaic embryo. These patients must receive thorough genetic counseling about potential pregnancy risks and outcomes. Some patients may not be deterred by data demonstrating significantly lower implantation rates of mosaic embryos (27), but they should be made aware that these embryos may be associated with a higher rate of miscarriage (26). Understanding of this risk and the potentially significant emotional and financial implications of pregnancy loss is an essential component of the discussion.

Embryo Selection: Which Mosaics Are Better to Transfer?

Recently released guidelines delineate specific categories of mosaicism and suggest that some mosaic embryos may be preferential to transfer over others (35). Specifically, these guidelines recommend the preferential transfer of embryos showing mosaic monosomies over mosaic trisomies. Autosomal monosomies are generally not viable, whereas certain trisomies can result in live births with associated physical and cognitive impairments. Chromosomal nondisjunction, which is a common cause of embryonic mosaicism, generates both a monosomic and a trisomic cell population. NGS may fail to detect the presence of monosomic or trisomic cells

when the ratio of monosomy to trisomy is close to 50:50 (36). Counseling should include a discussion about the possibility of undetected trisomic cells in an embryo diagnosed as mosaic monosomy and the potential significance of a trisomic cell line for the chromosome in question.

If a mosaic trisomy is considered for transfer, the Preimplantation Genetic Diagnosis International Society (PGDIS) guidelines suggest that mosaic trisomies 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19, 20, 22, X, and Y are preferred over mosaic trisomies 2, 7, 13, 14, 15, 16, 18, and 21 (35). Although the latter group of chromosomes carry known risks of specific trisomy syndromes (i.e., Down, Edwards, and Patau syndromes), mosaicism involving other chromosomes carries the risk of intrauterine growth restriction (IUGR) or uniparental disomy (UPD). These risks should also be addressed during counseling, recognizing that the lines between "risky" and "safe" aneuploidies may not be straightforward. Although most aneuploidies are not viable in the nonmosaic state, mosaic aneuploidies of nearly every chromosome, including those listed as "preferred" by PGDIS, have been reported in live births, with a wide range of phenotypes that are likely dependent on the proportion of abnormal cells and affected tissue types. Although the same guidelines do recommend that the percentage of mosaicism be considered in embryo selection decisions, it must be recognized that the proportion of aneuploidy in a TE biopsy may not represent the remainder of the TE or the ICM (34). Therefore, patients should be made aware that an outcome associated with a higher proportion of aneuploid cells may be possible.

When mosaicism is the result of a trisomy or monosomy rescue event, UPD may occur. Often, UPD does not pose a risk of an abnormal outcome. However, UPDs of chromosomes 7, 14, and 15 are associated with specific genetic syndromes (i.e., Russell-Silver, Temple, Kagami-Ogata, Prader-Willi, and Angelman syndromes), and UPDs of several other full and partial chromosomes have been associated with variable clinical phenotypes (37). Additionally, UPD may increase the risk of recessively inherited monogenic diseases if a genetic mutation is present on the duplicated chromosome. It is therefore important to consider whether UPD may be a potential risk factor when contemplating transfer of a mosaic embryo.

A euploid pregnancy resulting from an embryo diagnosed as mosaic may be the result of an erroneous PGS result, an ICM that was initially euploid, or the embryo having undergone "self-correction" of a mosaic embryo by apoptosis of aneuploid cells (9). By this mechanism, mosaicism found in a TE biopsy may go on to produce a euploid placenta or a pregnancy with confined placental mosaicism (CPM). CPM of certain chromosomes (particularly 2, 7, 16, and possibly 22) may increase the risk of IUGR and other pregnancy complications, including fetal demise. Therefore, a discussion of the risks associated with CPM is warranted for any patient considering transfer of an embryo diagnosed as mosaic, particularly in cases involving these chromosomes.

There are currently no guidelines regarding the transfer of embryos diagnosed as mosaic for segmental/partial aneuploidies. Interpretation of these results provides additional counseling challenges, because there is little known about the outcomes of these embryos. In contrast to small microdeletions and microduplications, the large segmental aneuploidies that are routinely detected with the use of NGS are generally not viable in the nonmosaic state. However, any segmental aneuploidy can theoretically result in a live birth in the presence of a euploid cell line, and the resulting phenotype is again likely to depend on the proportion of abnormal cells and the affected tissue type.

Given the reasons discussed here, it can be difficult to determine which mosaic aneuploidies may be the "safest" to transfer. Although counseling patients about the risks associated with trisomies 13, 18, and 21 and aneuploidies involving chromosomes 2, 7, 14, 15, and 16 may certainly be more straightforward, it is essential to emphasize that full or partial aneuploidies involving other chromosomes may not necessarily be safer to transfer; there is simply less known about the risks.

Prenatal Testing after Transfer of Mosaic Embryos

In addition to embryo selection considerations, it is essential that patient counseling include a discussion about the benefits and limitations of prenatal testing. Despite advances in noninvasive prenatal screening for aneuploidy, chorionic villus sampling (CVS) and amniocentesis remain the criterion standard for prenatal diagnosis of a chromosome abnormality. CVS can be beneficial for patients seeking diagnostic information in the first trimester; however, it is important to recognize that CVS analyzes placental cells, which originate from the embryonic TE. Therefore, mosaicism detected with the use of CVS may represent CPM and require follow-up amniocentesis to clarify the status of the fetus. This may cause patients unnecessary anxiety. Because amniocentesis analyzes cells derived directly from the fetus, amniotic fluid results are more representative of fetal tissues; however, many patients may find the experience of waiting until the second trimester for prenatal diagnosis equally stressful. It should also be noted that although a normal amniocentesis is certainly reassuring, patients should be aware of its limitations. Amniocentesis may miss low-level mosaicism and can not analyze cells from all fetal tissues. Patients should be encouraged to seek further genetic counseling during pregnancy before prenatal diagnosis, because additional testing options beyond routine karyotyping may be offered. For example, karyotype or FISH analysis of additional cells may improve the detection of low-level mosaicism. In some cases, FISH or microarrays may be indicated to detect segmental aneuploidies, and UPD studies may be considered for chromosomes that carry a known risk of UPD-related phenotypes.

The utility and performance of noninvasive aneuploidy screening methods, particularly cell-free DNA (cfDNA) screening, are not currently known after the transfer of embryos diagnosed as mosaic. Currently, the American College of Medical Genetics and Genomics recommends cfDNA screening only for trisomies 13, 18, and 21. Screening for aneuploidies involving other autosomes or for genome-wide microdeletions and microduplications is not recommended, because the clinical utility is not well established (38). Additionally, it is important to recognize that, as with cells obtained by means of CVS, cfDNA is derived from the

placenta and may not accurately reflect the chromosomal status of the fetus, resulting in false positives and/or negatives.

The interpretation of mosaicism among preimplantation embryos is complicated for both physicians and patients. Physicians should understand that mosaic embryos may represent a third category between normal (euploid) and abnormal (aneuploid) embryos. This category of mosaic embryos may be characterized by decreased implantation and pregnancy potential with increased risk of genetic abnormalities to the fetus and adverse pregnancy outcomes; therefore, euploid embryos should be preferentially transferred over mosaic embryos. Patients should be made aware, ideally before PGS, that aneuploidy screening results are not always straightforward and that the risks associated with embryonic mosaicism are still largely unknown. If transfer of a mosaic embryo is being considered, genetic counseling about potential benefits and risks is of utmost importance to ensure informed decision making by patients.

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