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OPINION

Human embryo mosaicism: did we drop the ball on chromosomal testing?

Navid Esfandiari[1] · **Megan E. Bunnell**[1] · **Robert F. Casper**[2]

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Abstract There are newly recognized challenges presented by the occurrence of mosaicism in the context of trophectoderm (TE) biopsy for pre-implantation genetic screening (PGS) in in vitro fertilization (IVF) embryos. Chromosomal mosaicism, known to be significantly higher in IVF embryos than in later prenatal samples, may contribute to errors in diagnosis. In particular, PGS may result in discarding embryos diagnosed as aneuploid but in which the inner cell mass may be completely or mainly euploid, thus representing a false positive diagnosis. Although less likely, some embryos diagnosed as euploid could be mosaic and contain some aneuploid cells, possibly impacting their implantation potential. The ability of current diagnostic techniques to detect mosaicism is limited by the number and location of TE cells in the biopsy and by the methodology used for chromosomal assessment. The clinical consequences of mosaicism are dependent on the chromosome(s) involved, the developmental stage at which the mosaicism evolved, and whether TE biopsy accurately reflects the status of the inner cell mass that forms the fetus. Consequently, in patients with no euploid embryos identified on PGS, it may be appropriate to consider the transfer of diagnosed aneuploid embryos if the TE biopsy result is a non-viable chromosomal monosomy or triploidy that could not result in a birth. It should be acknowledged in consent forms that mosaicism has the potential to impact test results and that its detection may be below the resolution of the genetic tests being used. This concept represents a major shift in current IVF practice and ought to be considered given the data, or lack thereof, of the impact of mosaicism on IVF/PGS outcomes.

Keywords Mosaicism · Aneuploidy · Pre-implantation genetic screening · Trophectoderm biopsy

Capsule There are newly recognized challenges presented by the occurrence of mosaicism in the context of trophectoderm (TE) biopsy for pre-implantation genetic screening (PGS) in in vitro fertilization (IVF) embryos.

✉ Navid Esfandiari
Navid.esfandiari@hitchcock.org

[1] Division of Reproductive Endocrinology and Infertility, Department of OB-GYN, Dartmouth Hitchcock Medical Center, Geisel School of Medicine, Lebanon, NH, USA

[2] Division of Reproductive Endocrinology and Infertility., Department of OB-GYN, School of Medicine, University of Toronto, Toronto, Canada

Introduction

A successful IVF pregnancy relies first and foremost on successful implantation. This requires interaction between the embryo and endometrium and can fail because of problems with either [1]. For example, a chromosomally abnormal embryo is less likely to implant, and if it does, it is more likely to be lost early in the pregnancy. It is estimated, based upon a systematic review of mosaicism in studies using array CGH (aCGH) or quantitative real-time PCR (qPCR), that at least 40 to 60 % of human embryos are abnormal, increasing to 80 % in women 40 years or older [2]. Such abnormalities result in low implantation rates in women undergoing IVF procedures ranging from 30 % success in women <35 years to less than 10 % in women >40 years [3]. Various methods for identification of the chromosome complement of an embryo prior to transfer have been developed in the past decades, collectively referred to as pre-implantation genetic screening (PGS) and diagnosis (PGD). Early use of this technology relied heavily on the assumption that an embryo that was tested as euploid was, in fact, euploid and that an abnormal result of aneuploidy or mosaic aneuploidy was predictive of an embryo destined to fail implantation or that necessarily would develop into an aneuploid or mosaic fetus [4]. Appreciating these

assumptions and their validity, or lack thereof, requires a comprehensive understanding of the techniques that are used to detect aneuploidy and the stages at which such testing is performed.

Background

New technologies are now available using several different platforms to determine embryo chromosomal number (PGS) to facilitate single normal embryo transfer in older women or in women with repeated spontaneous abortions. Originally, PGS was performed using fluorescence in situ hybridization (FISH) on polar body biopsies from human eggs. This technique was quickly determined to be unsatisfactory since only a few chromosomes out of 22 autosomal chromosome pairs and 2 sex chromosomes could be tested. In addition, only the maternal component of the subsequent embryo could be tested. Attention then moved to FISH to identify aneuploidy for 12 or more chromosomes or array comparative genomic hybridization (a-CGH) on day 3 embryos (biopsy of one or two cells from a six to ten cell embryo). Given a high rate of mosaicism in cleavage stage embryos, biopsy of one or two blastomeres for genetic testing could give false results if the embryo was mosaic. It has also been demonstrated that blastomere biopsy at the cellular stage significantly impairs embryonic implantation by up to 40 % [5]. PGS using day 3 embryos was proved to be ineffective in improving pregnancy rates mainly due to damage to embryo developmental potential, incomplete chromosomal assessment by FISH, and high rate of mosaicism in cleavage stage embryos. Today, almost all PGS is performed on day 5 or 6 embryos where all 23 chromosome pairs are assessed (the new generation of PGS or PGS-2.0 which is the focus of this review). At the blastocyst stage, the embryo is composed of two distinct cell types—the inner cell mass (ICM) and the trophectoderm (TE) [6]. The ICM comprises the mass of cells within the blastocyst cavity that will develop into the fetus, while the TE is composed of trophoblast cells that will form the placenta [7].

The cells of the TE at the 5–6-day stage are numerous. Because these cells are destined to become placenta, a TE biopsy ensures that the developing embryo (cells contained in the ICM) is not disturbed. However, even with a chromosomally abnormal TE biopsy result, a possibility of a chromosomally normal fetus remains. One possible explanation for this observation is embryonic mosaicism leading to biopsied blastomeres that are not representative of the whole embryo [8]. In the presence of mosaicism, it is possible that chromosomes are different among TE cells, or between TE cells and ICM cells. Currently, limited data exist regarding the prevalence and impact of chromosomal mosaicism derived from progressing days 5–6 blastocysts [9].

In general, chromosomal mosaicism is known to be significantly higher in IVF-created embryos than in other prenatal samples [9]. A meta-analysis of human pre-implantation embryos identified mosaicism in 73 % of all embryos [10]. Of note, the cutoffs for calling an embryo mosaic versus diploid varied among studies. Most generally, mosaic embryos are those embryos that contain cell lines with varied chromosome complements. Mosaicism is postulated to originate from improper expression of cell cycle checkpoint genes during the primary mitotic cell divisions in the early embryo when maternal transcripts control the cell cycle [9]. At later cleavage stages, the embryonic genome takes over and may be able to overcome mosaicism by allowing the proliferation of normal cells and the inhibition of mitotic activity in abnormal ones [11–13]. Such a process is supported by data showing that chromosomal mosaicism is far lower at the blastocyst stage (days 5–6) as compared to the cleavage stage (day 3). Therefore, in theory, normal PGS results from TE biopsy, as compared to an earlier cleavage state biopsy, should result in higher pregnancy rates—likely due to decreased rate of mosaicism within the ICM and TE cell populations at the blastocyst stage [7].

Mosaicism

The potential for mosaicism in pregnancy is well documented in natural conception through data on chorionic villus sampling, a prenatal diagnostic technique that samples placental cells from the fetus as a means of ascertaining information about fetal chromosome complement. It has been found that in about 2 % of pregnancies, the fetus had normal chromosomes while the placenta had either a combination of normal and abnormal chromosomes or totally abnormal chromosomes [14–17]. Fetal/placental mosaicism is one type of potential mosaicism. There are four possible types of embryonic mosaicism: (1) embryos which have an aneuploid ICM and euploid and aneuploid TE cells; (2) embryos which have an aneuploid ICM and euploid TE cells; (3) embryos with a euploid ICM and aneuploid TE cells; and (4) embryos with a euploid ICM and both euploid and aneuploid TE cells [18, 19].

Mosaic embryos may be self-correcting, with aneuploid cells becoming apoptotic or locating ultimately in the trophectoderm (embryonic placenta). Mosaicism can manifest in two forms: general and confined. General mosaicism is detected by pre-implantation genetic screening and leads to mosaicism within both the placenta and fetus proper [20]. Just because an embryo is mosaic at the time of early testing does not mean that those cell lines will propagate throughout development. The influence of mosaicism during development may depend on the degree of aneuploidy, the tissues involved, and the particular chromosome complement. Unlike the cleavage stage embryo, the blastocyst contains two distinct parts—a trophectoderm, which will become the placenta, and the inner cell mass, which will develop into the fetus. Like cleavage

stage embryos, high rates of mosaicism may exist in the blastocyst although the proportion of aneuploid cells located in the ICM and TE has not been well characterized, with discordant rates among different studies [19, 21].

If an abnormal cell line segregates to the trophectoderm, a TE biopsy may indicate an abnormal chromosome complement that is not present in the ICM (developing fetus). However, up until now, this concept is not well supported by the data obtained from early PGS platforms. For example, Johnson et al. (2010) and Northrup et al. (2010) found that aneuploid blastocysts displayed no evidence of preferential segregation of abnormalities to the TE, and each blastomere of the early cleavage-stage human embryo could participate in both trophectoderm and inner cell mass formation [8, 22]. Therefore, an abnormal blastomere would have the same chance of ending up in the trophectoderm as in the inner cell mass. This is consistent with early reports from Evsikov and Verlinksy (1998) who, using FISH, had similarly reported the probability of no selection for a euploid inner cell mass, demonstrating an average degree of aneuploidy in the ICM as compared to the overall blastocyst [23]. An early study from Delhanty and Handyside (1995) also concluded that aneuploid cells are not necessarily diverted to the trophectoderm because they believed that a substantial proportion of trisomic fetuses are trisomic due to postzygotic mitotic errors [24].

Testing

It has been determined that about 30 % of blastocysts are mosaic at the blastocyst stage [25, 26]. However, it is important to realize that levels of mosaicism undetectable by current techniques may elevate this statistic and contribute more significantly to pregnancy loss than has been previously understood. It is of obvious importance that a sufficient number of cells be biopsied from the TE compartment to minimize any effects of skewed biopsy sampling [9].

The chromosome complement of an embryo is classically diagnosed at one of the two stages of development. The earliest is the cleavage stage (6–8 cells, 3 days post-fertilization) and later the blastocyst stage (many cells, 5–6 days post-fertilization). When chromosome testing is performed at day 3, a single cell is removed and tested. Whatever the chromosome complement is of that single cell thus classifies the rest of the embryo. For example, if that cell were classified as abnormal, the entire embryo would be discarded as an "abnormal" despite the potential for that abnormal cell line to die or be segregated into the non-fetal tissues. Due to these testing limitations and the potential for greater embryo damage when a biopsy is performed at the cleavage stage, days 5–6 blastocyst testing is now the gold standard [5]. Biopsies at this stage of development permit testing of multiple cells, although these cells are exclusively obtained from the trophectoderm (future placenta) where abnormal cell lines may be more inclined to segregate. In addition, samples taken from different parts of the trophectoderm may not be the same.

Interpretation of the chromosome complement involves first a calibration of the assay using fully characterized cell lines with both euploid and aneuploid specimens. The log2 ratios for individual data points vary and overlap significantly [27]. Once statistical smoothing is applied and weighted averages are calculated, it is possible to discriminate between samples; however, the limits between distributions are still minimal [28]. Despite this, threshold values are typically assigned in an effort to discriminate monosomic, disomic, and trisomic samples. With this methodology, mosaic samples would be expected to fall in the range between these different categories depending on their particular makeup. It has been recently proposed that this result represents a "middle category" which will notably include some disomic samples, resulting in the classification of some normal samples as potentially mosaic [28].

Recent data suggests that TE biopsy for selection of a euploid embryo results in higher pregnancy rates as compared to biopsy at the cleavage stage [7]. Such results may be due to either decreased mosaicism within TE and ICM cells at the blastocyst stage or to an increased likelihood that the ICM is euploid if the mosaic TE contains some euploid cells [18]. Some fetuses with normal chromosomes have a fully abnormal or mosaic chromosome complement within the placenta [15, 16]. It has been estimated that approximately 2 % of viable pregnancies are affected by this type of mosaicism, termed "confined placental mosaicism" [16]. It is also reported that there is a 10 % risk of fetal mosaicism when placental mosaicism is diagnosed, indicating that a substantial number of fetuses with an abnormal or mosaic placenta will still develop into euploid liveborns. Additionally, there exists a concern for the potential of reciprocal errors within the embryo. This type of error is represented by a scenario in which mitotic nondisjunction leads to cells with both monosomy and trisomy for the same chromosome. When the trophectoderm biopsy cells are lysed during the PGD procedure, DNA leaves the cells and creates a mixture. It is therefore possible that such an embryo would be read as normal due to the co-existence of balancing chromosomal errors, particularly if the trisomic/monosomic ratio of cells was close to 50:50 [28].

PGS assumptions

Given this description of various chromosomal abnormalities, PGS is an ostensibly efficient way to improve the chances of a chromosomally normal outcome. However, the utilization of chromosomal identification techniques as part of the IVF process relies on a few critical assumptions. First is the assumption that an embryo determined to be euploid on chromosome screening will have a higher likelihood of implantation than

one with an abnormal chromosome complement. Second is the assumption that the chromosome complement determined during screening will necessarily be the chromosome complement ultimately present in the fetus. Third, the assumption exists that the techniques being used to analyze the chromosome complement are able to reliably and consistently detect chromosome abnormality.

While these assumptions may hold true for the detection of full aneuploidy in an embryo, a gray area of chromosome complement identification exists in the detection of mosaicism in the embryo. With the reported rate of chromosomal mosaicism in embryos at the cleavage stage as high as 50 % or greater, there is a chance that any cells biopsied from the embryo may not be reflective of the true chromosomal status of the fetus [12, 26]. This means that regardless of the technological accuracy of PGS as a technique, its ability to predict the ultimate chromosomal status of the fetal pole is limited [2, 3].

PGS performed at the cleavage (day 3) or blastocyst stage (days 5–6) has the potential to result in a significant number of "false positive" aneuploidy or mosaic diagnoses. These are not all false positives in the sense of an incorrect test, but rather many will be false positive in that the tested embryo would go on to develop into a chromosomally normal fetus. In well-informed patients with lethal aneuploidy/mosaic embryos and no euploid options, transfer of aneuploid or mosaic embryos may be warranted. This view is based upon the present literature, suggesting that a diagnosis of an aneuploid/mosaic "abnormal" embryo is not always correct, or if it is correct, mosaicism detected at the blastocyst stage may not manifest as such in a live born [29–31]. While testing methodology is able to identify definitely normal and grossly abnormal chromosome complements in most cases, there is a significant, not well-delineated, gray zone into which many chromosome-screening results fall, mostly attributable to the problem of mosaicism.

Technological failure

The potential for PGS to improve clinical outcomes is dependent on the ability of screening technology to correctly classify embryos as normal or abnormal according to chromosome complement. This technology can fail for a number of reasons, including failure to detect an abnormality due to technological limitations or failure to accurately classify the chromosome complement due to mosaicism in the embryo. The potential for PGS results to be incorrect is thus a very real concern.

A 2015 comparison of aCGH and rtPCR-based blastocyst screening in 124 aneuploid blastocysts indicated an 18.3 % discordance between the two methods for at least one chromosome with the highest discordant aneuploidy call rate for aCGH [32]. However, on a per-chromosome basis, aCGH and qPCR demonstrated a 99.86 concordance level when the methods were applied on different TE biopsies from the same embryo. The authors attributed the small number of differences between the methods to technological inaccuracies assuming that mosaicism is randomly distributed in all methods. This issue of discordance in chromosome analysis is also well demonstrated by recent publications and case reports. A 2012 study by Shelly et al. compared 30 in-house embryo biopsy results using microarray on TE biopsies provided by a commercial laboratory. This revealed 56.7 % complete concordance and 20 % complete discordance, concluding that the extent of mosaicism was relatively high in the embryo population sampled, and that this was a threat to the ability of PGS to correctly classify embryos as normal or abnormal [31]. A recent study by Orvieto et al. (2016) evaluated the accuracy of trophectoderm biopsies in an effort to addresses the utility of PGS in widespread clinical practice. They performed three trophectoderm biopsies and an ICM biopsy in each of eight blastocysts and used next-generation sequencing (NGS) to analyze the chromosome complement of the TE and of the inner cell mass. They found that over 35 % ($n = 10$) of the 28 biopsies demonstrated mosaicism or inconclusive results [30]. Esfandiari et al. (2014) recently studied the accuracy of different PGS techniques on TE biopsies, using 27 vitrified blastocysts identified as aneuploid, cut into 3 pieces and sent to 3 different laboratories (including the original). The results, presented at the European Society of Human Reproduction and Embryology conference (2014) compared the results of CGH, SNP, and qPCR analysis, showing that 11 out of 24 initially diagnosed "abnormal" embryos were retested as normal. All of these results raise considerable concern over the accuracy and reliability of TE testing for PGS with current laboratory practices [29].

A further limitation of this testing strategy is that the biopsied cells are not typically analyzed individually, but rather as a group, decreasing the sensitivity of detecting an abnormality in the total cell population [26]. The mostly widely used chromosome screening platform at this time, array-CGH, can only detect mosaicism when at least one third of the total biopsy has a distinct chromosomal complement. The advent of NGS is allowing for detection of lower level mosaicism [33]. However, the lack of chromosome-specific cutoffs for predicting aneuploidy from aCGH data remains an issue [26, 32]. While there is no question that mosaicism is detectable using NGS methodologies when >20 % of cells in the specimen are aneuploid, the capabilities of NGS methods to detect, for example, one aneuploid cell in a 10 cell biopsy, are not well-delineated [26].

In addition to data suggesting that common chromosome screening techniques may be inadequate for the detection of mosaicism, a number of recent case reports and series have indicated successful outcomes with the exclusive transfer of mosaic embryos [28]. A recent case series by Greco, Minasi, and Fiorentino detailed the intentional

transfer of mosaic embryos, determined via aCGH, to 18 pre-counseled women. Each woman had one mosaic blastocyst available [34]. Eight pregnancies (positive hCG testing) with six singleton full-term infants resulted in this group and all babies were confirmed to have a normal karyotype. Gleicher et al. (2015) also reported the birth of 3 normal neonates after transfer of "aneuploid embryos" suggesting that abnormal cell lines seen in the blastocyst stage may segregate to the trophectoderm and thus be ultimately absent from the fetus [35]. Of note, the authors did not discuss the platforms on which the determination of aneuploidy was made. They concluded that the use of PGS, particularly in poor prognosis patients, might actually reduce the chance of a normal pregnancy by limiting the transfer of viable embryos based on PGS results. Further complicating the picture of the relevance and impact of mosaicism are case reports like that from Haddad et al. (2013) describing the transfer of a euploid, micro-array tested blastocyst that was later found to be mosaic (47 XX, +21; 46, XX) on prenatal CVS sampling. The pregnancy was continued, and a healthy girl was delivered at 41 weeks [15]. The results of these studies, taken together, point to the potential for mosaicism and abnormal cell line segregation in the trophectoderm to impair the clinical accuracy of PGS results. The potential for such discrepancies calls into question the ability of chromosome screening to truly improve clinical outcomes, especially in women with diminished ovarian reserve.

The clinical consequences of mosaicism may be highly dependent on the chromosome(s) involved and the stage of development at which the mosaicism evolved. When mosaicism occurs during the cleavage state, for example, the mosaicism will be more pronounced (a greater percentage of the total embryo involved) than if the mosaicism occurs later in development. This suggestion is supported by a comparison of the rates of mosaicism seen in prenatal diagnosis (1–2 %) versus the broad range (50–90 %) of mosaicism seen in cleavage stage embryos [10, 36].

Although the link between mosaicism in the ICM versus TE is not well established, it is reasonable to assume that mosaicism detected during a trophectoderm biopsy would raise the consideration of either mosaicism in the ICM as well or of "self-correction" of the mosaic embryo. The extent of this relationship is yet to be determined. Recent literature has suggested that the use of a threshold to guide reporting of a mosaic result has no biological or clinical validity, and any level of mosaicism should be reported as such, irrespective of the fraction of abnormal cells in the sample [26]. Indeed, although mosaicism is recognized as prevalent within IVF-created embryos, the level at which mosaicism switches from problematic to clinically non-relevant is undetermined [20, 26]. Scott and Galliano (2016) have proposed a stratification of PGS diagnoses of disomic and monosomic or trisomic embryos, with an additional category of mosaicism that would ideally represent those embryos at highest risk of having a truly mosaic complement [28].

A recent study by Fragouli et al. (2015) used NGS to reanalyze biopsied material from blastocysts previously tested with aCGH and previously transferred without knowledge of the original PGS results. They found that 43 embryos out of 148 (29 %) were mosaic. Of this group, 62 % did not implant, 12 % miscarried, and 26 % led to ongoing pregnancies. These results were compared to a contemporary control group of 51 diagnosed euploid blastocysts, and it was found that implantation and ongoing pregnancy rates were reduced when mosaic embryos were transferred. Furthermore, their results showed that blastocysts with mosaic whole chromosome aneuploidies had significantly decreased implantation rates compared to those with mosaic segmental abnormalities [37]. The majority of mosaic blastocysts did not implant or led to miscarriage, while a minority produced viable, euploid pregnancies. This data lends support to the idea that mosaic blastocysts should be considered for transfer when euploid embryos are not available. However, any risks associated with mosaic embryo transfer remains to be determined [28].

In conclusion, the biological presence and significance of mosaicism in the IVF process are a poorly understood issue. The prevalence, corrective capabilities and the impact of mosaicism given the exact chromosome complement affected need to be studied. In addition, a focus should be given to validation of present CCS technology as a way to reduce the impact of potential variations in classification of the embryo chromosome complement. That said, given the data that is currently available of absence of significant mosaicism in the newborns resulting from transfer of mosaic embryos, in an individual without normal embryos for transfer, after appropriate counseling, mosaic embryos may present a possibility of having a healthy pregnancy. However, the possibility of a mosaic pregnancy and the uncertain spectrum of outcomes must be taken seriously, and as such, the decision to utilize embryos that are not clearly classified as diploid should involve the input of physicians, genetic counselors, and the patients themselves. One must also bear in mind the very real possibility that much of the present chromosomal screening may fail to detect mosaicism, thereby classifying mosaic embryos as aneuploid or euploid. This will continue to complicate the acquisition of accurate data.

Compliance with ethical standards

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

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