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Review

The Technological Advances in Embryo Selection and Genetic Testing: A Look Back at the Evolution of Aneuploidy Screening and the Prospects of Non-Invasive PGT

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Abstract: Since the birth of the first IVF baby, Louise Brown, in 1978, researchers and clinicians have sought ways to improve pregnancy outcomes through embryo selection. In the 1990s, blastomere biopsy and fluorescence in situ hybridization (FISH) were developed in human embryos for the assessment of aneuploidy and translocations. Limitations in the number of chromosomes that could be assayed with FISH lead to the development of comparative genomic hybridization (CGH); however, pregnancy rates overall were not improved. The later development of trophectoderm biopsy with comprehensive chromosome screening (CCS) technologies, as well as the subsequent development of next-generation sequencing (NGS), have shown much greater promise in improving pregnancy and live birth rates. Recently, many studies are focusing on the utilization of non-invasive preimplantation genetic testing (niPGT) in an effort to assess embryo ploidy without exposing embryos to additional interventions.

Keywords: preimplantation genetic testing; embryo biopsy; in vitro fertilization; assisted reproductive technology; next-generation sequencing; fluorescence in situ hybridization; comparative genomic hybridization

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1. Introduction

Assisted reproductive technologies (ART), including in vitro fertilization (IVF), have become an increasingly common treatment modality for patients suffering from infertility. In 2005, approximately 52,000 children were born following ART cycles [1]; this figure rose to over 81,000 in 2016 [2]. Since the birth of Louise Brown in 1978—the first successful live birth with the use of IVF—researchers and clinicians have continually sought to develop technological advancements to improve pregnancy and live birth rates [3]. It is well-known that a woman's chance of live birth with her own eggs decreases with age. This correlates with increasing rates of aneuploidy, specifically trisomy, which begin to sharply increase at the age of 35 [4]. This plays a major role in the observed decrease in a live birth with advancing maternal age.

One of the main goals of IVF is to try to overcome this known phenomenon with embryo selection. Traditionally this has been approached with morphology-based grading alone in an attempt to evaluate and select "good quality" embryos for transfer. However, the development of preimplantation genetic testing (PGT) provides the additional ability to preferentially select euploid embryos for transfer. Thus, PGT has become one of the principal modalities utilized by fertility clinics for embryo selection. Other technological advancements, such as intracytoplasmic sperm injection (ICSI), improvements in embryo culture techniques, and the advent of embryo vitrification, have undoubtedly improved outcomes as well and have in part allowed for the increasingly successful developments in PGT over the years [5]. Currently, PGT is being used for assessing chromosomal aneuploidy

(PGT-A), structural rearrangements such as translocations (PGT-SR) as well as in assessing embryos for monogenetic disorders (PGT-M). This review will focus on the evolution of PGT-A, and current emerging technologies that may supplement or, ultimately, supplant those currently used.

2. Blastomere Biopsy with FISH

After years of research in mouse models, the first pregnancies with PGT were reported in 1990 [6,7]. Blastomere biopsy of 1–2 cells was performed on day three embryos in conjunction with DNA amplification via polymerase chain reaction (PCR) to detect DNA specific to the Y chromosome, allowing for the identification of gender. This technology was first implemented in couples with an X-linked disorder in order to give them the capability of identifying and subsequently transferring unaffected embryos. This development was used to prevent couples from having to face a dreadful dilemma of whether or not to terminate a pregnancy if their fetus was diagnosed with an X-linked disorder in pregnancy. A few years later, the same group took this technology one step further by using fluorescence in situ hybridization (FISH) to simultaneously screen X and Y chromosomes from day three embryos in couples with X-linked disorders [8]. At the same time, a separate group performed PGT using FISH with eight directly labeled probes for both autosomal and sex chromosomes [9]. Thus, this technology could now be used to assess for aneuploidy in addition to evaluating for sex-linked disorders.

FISH was the first technique used for PGT and utilizes the hybridization of specific fluorochrome-tagged DNA probes to target DNA [8,9]. Fluorescent microscopy can then be used to determine the copy numbers as well as large chromosomal abnormalities such as deletions, duplications and translocations. It was classically performed on day three embryos and took only hours to perform. Consequently, biopsy results returned quickly, allowing couples to select which embryos they desired to use for fresh transfer on day five. The main limitation of FISH is the fact that it could only be performed on eight chromosomes [10]. In addition, if one desires to test more than six specific DNA probes, repeat hybridization is necessary. Another concern with this technology is the inherent risk of inaccuracies due to signal artifacts related to the assessment of interphase nuclei, which increases the rate of false negatives and false positives [11].

In 2007, a large multicenter double-blinded randomized control trial (RCT) compared the use of PGT with FISH to non-PGT controls in women aged 35 to 41 years old. The ongoing pregnancy rate at 12 weeks was 25% in the PGT-FISH group compared to 37% in the control group, with a rate ratio of 0.69 (95% CI 0.51–0.93) [12]. Not only did the authors conclude that PGT did not increase live birth rates, they found that it actually decreased pregnancy and live birth rates in women over 35 years old. A meta-analysis of 3 RCTs showed similar findings with a live birth rate of 32% in the PGT-FISH group compared to 42% in the non-PGT control group, risk difference of 0.10 (95% CI 0.2–0.01) [13]. Although one of the main goals of PGT is to help improve live birth rates in older women by selecting euploid embryos, in an attempt to overcome the increasing rate of aneuploidy that occurs with advancing maternal age, these studies demonstrated that FISH did not improve live birth rates in women greater than 35. Three main reasons were thought to be responsible for the lack of improvement seen with PGT with FISH: (1) FISH does not have the ability to screen all 24 chromosomes for aneuploidy, and (2) the technology was used with day 3 embryo biopsy, which was thought to be potentially harmful to the embryo and (3) the potential for embryo mosaicism and self-correction between day 3 and day 5. Due to the fact that only one cell is biopsied on day three, and the day three embryo is pluripotent, there is potential for embryos to self-correct with abnormal cells preferentially being extruded or sent to trophectoderm, which would result in a normal inner cell mass by day five [14,15].

3. Development of CGH

Due to the aforementioned limitations of FISH, a technology with the ability to comprehensively screen all chromosomes was desired. In 1999, two separate groups first reported on the use of new technology, comparative genomic hybridization (CGH), to screen for chromosomal aneuploidy of all 24 chromosomes from a single cell [16,17]. The first successful live birth with the application of CGH was reported shortly thereafter in 2001 [18]. CGH requires whole-genome amplification of DNA from lysed cells using oligonucleotide-primed PCR. The product is then labeled with a colored fluorescent tag and hybridized with normal reference DNA, which is also fluorescently labeled. The ratio of patient to reference DNA produces the fluorescent assay [10,19]. Despite the widespread use of CGH that ensued over the following years, CGH is limited by its inability to detect triploidy/tetraploidy or balanced structural rearrangements, such as balanced translocations or inversions [10,19,20].

4. Development of Trophectoderm Biopsy

When initial applications of PGT for aneuploidy screening did not produce favorable results, one plausible explanation was the potential negative impact of the embryo biopsy, which at that time was routinely performed on cleavage stage (day three) embryos. It was hypothesized that perhaps the detrimental effect of removing one or two cells (blastomeres) from the 8-cell cleavage embryo negated the potential positive impact of selecting a euploid embryo [21]. Furthermore, it was thought that performing an embryo biopsy at a later stage may be less impactful on its subsequent development. In 2009, a prospective cohort study examined the effect of day three biopsy by comparing the following groups: (1) one blastomere biopsied on day three, (2) two blastomeres biopsied on day three and finally (3) no biopsy performed [22]. Results demonstrated that removing two blastomeres from an eight-cell day three embryo impaired blastocyst development and implantation potential. It also was associated with a reduction in live birth outcomes compared to the one blastomere biopsy and the no biopsy groups. Although removing one blastomere did not impair development, utilization of PGT did not improve live birth rates in either biopsy group compared to non-biopsy.

An RCT from 2013 that compared day three cleavage-stage biopsy to day five trophectoderm biopsy demonstrated that trophectoderm biopsy had no measurable impact on reproductive outcomes [14]. Cleavage stage biopsy resulted in a significant reduction in sustained implantation rates compared to non-biopsied cleavage stage embryos (30% versus 50%, relative reduction 39%). On the other hand, sustained implantation rates were similar for blastocyst (day 5) embryos that underwent trophectoderm biopsy compared to non-biopsied blastocyst embryos (51 vs. 54%). Based on these findings, the authors concluded that day three biopsy was detrimental to embryo developmental potential, but this negative effect was not seen with day five trophectoderm biopsy. Downsides of trophectoderm biopsy include the need for extended culture and the inability to do fresh transfers in cycles in which PGT-A is performed for most centers due to the need to freeze embryos while awaiting biopsy results. However, due to both the data surrounding the potential negative consequences of day three biopsy as well as the improvements in both extended embryo culture and vitrification technology [14,15], day five trophectoderm biopsy has become the accepted standard for performing PGT.

5. Advancements of Comprehensive Chromosome Screening

Since the development of CGH as the first comprehensive chromosome screening (CCS) method, several other advancements have been introduced to try and further improve pregnancy outcomes. Single nucleotide polymorphisms (SNPs), another CCS tool, can be utilized to create an array that looks for the presence/absence of alleles for multiple genes on each chromosome. Whole-genome amplification is performed, then embryo DNA is hybridized to an array with labeled SNP alleles, and the relative color intensity of the two alleles at each genetic loci can be distinguished. Unlike CGH, SNP can be used

to detect triploidy/tetraploidy and uniparental disomy [10,19]. The high cost and time needed to perform SNP are its major disadvantages. However, recent developments in this technology include improvements in cost as well as the ability to perform testing for polygenic disorders (referred to at PGT-P), such as type 1 diabetes [23,24]. Array CGH can be performed faster than SNP array, 12 to 15 h compared to 30–40 h, respectively [10,19]. CCS via quantitative PCR (qPCR) was developed as a quicker alternative to SNP testing. With qPCR, at least two sequences from each arm of every chromosome are amplified and analyzed. DNA probes can be used to detect single-gene disorders. qPCR does not require whole-genome amplification, and assay time is only 4–12 h. Its use is limited by the fact that it cannot detect triploidy/tetraploidy, balanced translocations or inversions [10,19]. Next-generation sequencing, also known as massively parallel sequencing, is another CCS method that sequences the entire genome through repetitive sequencing of DNA fragments followed by hybridization to an array. It has the unique ability to evaluate multiple genetic loci as well as multiple samples at the same time. Strengths include its ability to detect balanced translocations, small deletions and mosaic embryos [10,19]. There are a wide variety of NGS platforms, and the full capabilities of the testing are determined by the specific technology used; for example, some platforms are able to detect triploidy/tetraploidy, while others are not [25]. NGS technologies generally have a high level of accuracy and are low cost to perform, and their use is now widespread in modern PGT-A testing [10,19].

With the development of these various CCS tools, questions arose in regard to whether one method was superior to the others in terms of the ability to accurately diagnose aneuploidy as well as if one resulted in improved pregnancy and live birth rates. A retrospective cohort study of over 900 cycles evaluated the use of NGS compared to microarray CGH for PGT in single euploid frozen embryo transfers. A significantly increased ongoing pregnancy/live birth rate with NGS was noted after logistic regression analysis [26]. Over the years, NGS has become the most popular CCS method due to its short testing time, cost-effectiveness, and high level of consistency.

6. Current State-of-the-Art Screening with NGS/Karyomapping

In 2010, the concept of karyomapping was first described. High-density SNP genotyping is used for genome-wide parental haplotyping. Karyomapping is used to identify genetic loci for each of the four parental haplotypes across chromosomes. This is then used to map the inheritance of these haplotypes in order to determine which alleles of specific loci were inherited from each parent. It can also be used to detect the position of any crossovers in the event of translocation [27]. This new technology can be used at the time of PGT-A in order to identify embryos carrying the normal chromosomal copies as opposed to those with abnormal copies. This is especially helpful for couples with a genetic condition or known balanced translocation.

7. Current Controversies in PGT

7.1. Is PGT-A Successful?

As the use of PGT has become more widespread, several clinicians have sought to determine if IVF with PGT-A improves pregnancy/live birth rates compared to IVF and embryo selection based on morphology alone. In an RCT from 2013, PGT-A with the use of qPCR (n = 72) was compared to the use of morphology-based embryo selection in blastocysts embryos (n = 83) [15]. Statistical increases were noted in both implantation and sustained implantation rates with the use of PGT-A, which were the primary outcomes of this study. There was no significant difference noted between the two groups for the live birth rate. This study was limited by the fact that it included only good prognosis patients with good quality embryos; thus, results are not necessarily generalizable to all couples that are seeking IVF. In 2016, a large case–control study compared PGT-A (n = 274,) which was performed with either SNPs or microarray CGH, to IVF with morphology-based grading alone (n = 863). [28] The authors of this study found that there was no benefit of PGT-A in patients <37 years old in regard to either live birth or clinical pregnancy. By

contrast, the improvement was noted in the live birth rate for patients >37 years old per single or double embryo transfer. However, when the secondary analysis was performed per retrieval in this age group instead of per embryo transfer, an improvement in a live birth was no longer observed.

A recent large multicenter RCT from 2019, known as the Single embryo TrAnsfeR of euploid embryo (STAR) trial, analyzed PGT-A compared to morphology alone in the selection of a single embryo for frozen embryo transfer in good prognosis patients [29]. The study included 661 women in total, ages 25–40 years old. In the analysis of all participants, no difference in ongoing pregnancy rate at 20 weeks was observed between the PGT-A arm compared to morphology alone when analyzed per embryo transfer (50% vs. 46%) or per intention to treat at the time of randomization (41.8% vs. 43.5%). A secondary subgroup analysis of women aged 35–40 years old showed a significant increase in ongoing pregnancy rate if the patient had an embryo available for transfer (51% vs. 37%). However, no improvement from PGT-A was observed when this group was analyzed per intention to treat at randomization. Other small studies, as well as a 2015 meta-analysis, have shown an improvement in ongoing pregnancy or sustained implantation rates using PGT-A, though the study designs are heterogeneous and thus difficult to generalize [30,31].

The primary goal of PGT-A is to improve the live birth rate by selecting a euploid embryo. Other benefits of PGT-A include potential decreased time to pregnancy due to the decreased number of transfers required to achieve pregnancy, in addition to decreased miscarriage rates [30,32]. By using PGT-A to confirm euploid status, the hope is that clinicians can more confidently select the single best embryo for transfer, which will subsequently decrease the number of double embryo transfers performed as well as the rate of multiple gestations. It is well established that twin and triplet gestations are higher risk pregnancies than singleton gestations due to an increased rate of pregnancy co-morbidities (hypertensive disorders of pregnancy, gestational diabetes, pregnancy loss, preterm labor), worse postnatal outcomes due to preterm birth, and higher neonatal intensive care costs.

The combined results of these studies do not support the use of PGT-A for all couples that undergo IVF. Even in women who are 35–40 years old, who theoretically have the most to gain from PGT-A due to increased rates of aneuploidy in women with advancing maternal age, improved live birth rates with PGT-A have not been consistently reported. Rather than viewing PGT-A as a modality that should be used for all couples seeking IVF in order to improve pregnancy outcomes, it likely is best to view PGT-A as an adjunct to IVF that should be discussed on an individualized basis in order to help patients decide whether or not it would likely be beneficial to them. This shared decision between provider and patient should be based on several factors that include, but are not limited to, age, ovarian reserve, prior cycle outcomes with IVF, laboratory protocols and expertise.

7.2. Mosaic Embryos: The Dilemma to Transfer or Not?

Throughout the years, technological advancements to CCS modalities have led to the detection of mosaic embryos. Mosaic embryos are defined as the presence of more than one chromosomally distinct cell line, where both normal and abnormal cell lines are detected within the same embryo [33]. Consequently, whether or not clinicians should transfer mosaic embryos has become a controversial topic. It is presumed that mosaic embryos have a negative impact on implantation. Additionally, there is concern for increased miscarriage rates or the development of aneuploid fetuses.

The ability to detect mosaicism depends on the type of CCS technology that is used to perform PGT-A. Most methods used for PGT-A lack the sensitivity needed to detect minor cell populations within the biopsy and instead only report the chromosomal status based on the most common cell line that is present in the sample. Array CGH, one of the most popular methods used for PGT-A, can detect mosaicism when at least one-third of the cells have a chromosomal make-up that is distinct from the others [34]. NGS has increased sensitivity and can detect mosaicism when <20% of the cells are chromosomally distinct; exact rates are dependent on the specific type of NGS platform that is used [35].

Pregnancy outcomes after the transfer of mosaic embryos have been reported in the literature. A study from 2017 compared miscarriage, implantation and ongoing implantation rates between euploid and mosaic embryos as detected by NGS [36]. As expected, implantation rates and ongoing implantation rates were both significantly increased, while miscarriage rates were lower after the transfer of euploid embryos compared to mosaic. Additionally, there was a tendency for mosaic embryos with 40–80% abnormal cells to have a lower ongoing implantation rate than those with <40% (22% vs. 56%). No difference was found between monosomic vs. trisomic mosaic embryos or between entire chromosome mosaicism vs. segmental mosaicism.

In another study from 2018, higher rates of implantation, clinical pregnancy and live birth were seen with embryos with <50% mosaicism compared to embryos with >50% mosaicism [37]. Furthermore, contrary to what many people believed was possible in regard to mosaic embryos, this study demonstrated that mosaic embryos could develop into healthy, euploid newborns. Similar to other studies, the authors concluded that the extent of mosaicism influences and was predictive of reproductive success. However, another study from 2018 reported that no difference was observed in ongoing pregnancy or miscarriage rates among mosaic embryo transfer at any threshold of aneuploidy [38]. Thus, they concluded that the degree of mosaicism noted on trophectoderm biopsy was a poor predictor of ongoing pregnancy and miscarriage. Although it is clear from the literature that euploid embryos lead to better pregnancy outcomes compared to mosaic embryos, it is unclear at this time whether the degree of mosaicism can predict outcomes consistently.

According to the American Society of Reproductive Medicine's (ASRM) recent committee opinion highlighting the clinical management of mosaic embryos, approximately 100 live births had been documented in the literature after the transfer of mosaic embryos as of 2019 [33]. These reports have further muddied the water in regards to what should be done with mosaic embryos. Additionally, a recent study from 2019 has called into question the ability of PGT-A to accurately diagnose mosaic embryos. In this study, PGT-A with NGS was performed, then embryos were placed in an extended vitro culture until 8 or 12 days post-fertilization and re-tested. A high proportion (58%) of embryos that were originally diagnosed as a mosaic by PGT-A remained viable at 12 days post-fertilization. Based on validation data, there was an 18.5% false positive error rate when diagnosing mosaicism, which resulted in an 80% diagnostic accuracy of PGT-A in this study [39]. This study brings to light the concern that it is possible for euploid embryos to be misdiagnosed as mosaic or unsuitable for transfer at the time of trophectoderm biopsy, that ultimately will "self-correct," continue developing as a normal embryo/fetus and possibly lead to live birth. Alternatively, it is possible that at least a portion of the embryos labeled as mosaic are not true mosaics and that this inaccuracy in diagnosis is due to a technological artifact [40,41].

7.3. Ethical Considerations

Given the ability to determine embryo sex, it is understandable that some patients would want to utilize this technology for sex-selection or family balancing. However, utilizing IVF with PGT-A in patients who do not have a diagnosis of infertility or other clinical indication (such as concern about a sex-linked disorder) for the sole purpose of sex selection can present an ethical dilemma for clinicians. There may be cultural, religious or personal values/beliefs motivating patients to pursue this, and on the other hand, providing this treatment would be in support of the principles of patient autonomy and reproductive liberty. It is difficult to overstate the importance of upholding these ethical principles in patient care. On the other hand, there are several concerns regarding utilizing PGT-A for this indication, including the perception of misuse of what could be considered limited medical resources; concerns about exposing women to invasive treatments that are not deemed medically necessary, or the potential risks to offspring associated with ART; and the potential for discrimination on the resulting offspring, or their siblings [42]. Ultimately,

the decision of whether or not to offer IVF with PGT-A for sex selection without a medical indication for IVF should be made at the discretion of the physician and their practice.

8. Future Directions—Non-Invasive PGT

Given the controversial data regarding the efficacy of PGT-A and its inherently invasive nature, many clinicians and scientists have begun to investigate the potential of less invasive or non-invasive PGT (ni-PGT) modalities as diagnostic alternatives to trophectoderm biopsy. A 2013 study was able to identify genomic DNA in the blastocele fluid of blastocysts prior to vitrification [43]. In 2014, Gianaroli et al. compared the ploidy status of samples collected from blastocele fluid with those collected from trophectoderm cells, whole embryos, polar bodies, and blastomeres [44]. In this pilot study, they concluded that blastocele fluid could potentially be used as an alternative aneuploidy screening technique. Another study from 2017 found that cell free DNA could reliably be amplified from embryo culture media and that the accuracy/concordance with trophectoderm biopsy results was significantly impacted by the stage of embryo development when the fluid was collected [45]. However, these testing modalities did not show equivalency to current PGT-A testing with traditional methods. A more recent study from 2018 evaluated the accuracy of combined blastocele fluid and culture media in assessing for aneuploidy [46]. Compared with trophectoderm biopsy and/or whole embryo biopsy using NGS, their combination method was found to have a high level of concordance for whole chromosome copy number. Additional studies are needed to compare these aneuploidy screening techniques to current traditional methods in terms of fidelity of results as well as pregnancy and live birth rates. niPGT may represent a less invasive and ultimately less expensive/labor-intensive testing modality, should it be found to be equivalent or superior to trophectoderm biopsy.

9. Conclusions

Preimplantation genetic testing has evolved significantly since its early inception, from a method to detect X-linked recessive diseases to a modern technology capable of assessing aneuploidy through next-generation sequencing of only a small number of trophectoderm cells of the preimplantation embryo. The significant improvements in technology have only further deepened the questions as to the utility of PGT-A as a method of improving live birth outcomes in IVF. Although PGT-A clearly has a role in embryo selection, its general applicability to all women is uncertain, given the data from randomized controlled trials. However, perinatal outcomes are significantly improved with the more widespread adoption of elective single embryo transfer, and PGT-A has helped women in every age group have high success rates with single embryo transfer. The question of embryonic mosaicism will need to be addressed to ensure testing accuracy and potentially improve overall live birth outcomes with PGT-A. Additionally, non-invasive PGT testing has the potential to provide the benefits of PGT without the potential downsides of embryo biopsy. With continued improvements in PGT-A technology and non-invasive testing, embryo selection will likely continue to improve to provide optimal success and outcomes for IVF patients.

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