Technique to ‘Map’ Chromosomal Mosaicism at the Blastocyst Stage

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Key Words

Aneuploidy · Blastocyst · Comprehensive chromosome screening · Mosaicism · Preimplantation genetic screening

Abstract

The purpose of this study was to identify a technique that allows for comprehensive chromosome screening (CCS) of individual cells within human blastocysts along with the approximation of their location in the trophectoderm relative to the inner cell mass (ICM). This proof-of-concept study will allow for a greater understanding of chromosomal mosaicism at the blastocyst stage and the mechanisms by which mosaicism arises. One blastocyst was held by a holding pipette and the ICM was removed. While still being held, the blastocyst was further biopsied into quadrants. To separate the individual cells from the biopsied sections, the sections were placed in calcium/magnesium-free medium with serum for 20 min. A holding pipette was used to aspirate the sections until individual cells were isolated. Individual cells from each section were placed into PCR tubes and prepped for aCGH. A total of 18 cells were used for analysis, of which 15 (83.3%) amplified and provided a result and 3 (16.7%) did not. Fifteen cells were isolated from the trophectoderm; 13 (86.7%) provided an aCGH result, while 2 (13.3%) did not amplify. Twelve cells were euploid (46,XY), while 1 was complex

abnormal (44,XY), presenting with monosomy 7, 10, 11, 13, and 19, and trisomy 14, 15, and 21. A total of 3 cells were isolated from the ICM; 2 were euploid (46,XY) and 1 did not amplify. Here, we expand on a previously published technique which disassociates biopsied sections of the blastocyst into individual cells. Since the blastocyst sections were biopsied in regard to the position of the ICM, it was possible to reconstruct a virtual image of the blastocyst while presenting each cell's individual CCS results.

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The presence of 2 or more distinct cell lines, commonly referred to as chromosomal mosaicism, is one of the potential pitfalls when analyzing embryos by comprehensive chromosome screening (CCS). The ability to detect mosaicism accurately is determined by the technology used, number of chromosomes examined, and number of cells analyzed [Taylor et al., 2014a]. Even if mosaicism is present, the impact on subsequent development varies, depending upon which chromosome is involved and at what stage the chromosomal abnormality occurs [Taylor et al., 2014a].

CCS requires that the cells be pipetted into a PCR tube for analysis rather than fixed on a slide as previously performed by FISH studies [Magli et al., 2000]. To examine

Fig. 1. **A** The whole blastocyst with the quadrants and inner cell mass (ICM) marked prior to biopsy. **B** Blastocyst undergoing ICM removal; the quadrants are marked. **C** The blastocyst during the biopsy of the 'B' quadrant. The 'A' quadrant has already been biopsied. **D** The blastocyst after the biopsy of the ICM, quadrant 'A', and quadrant 'B'. **E** Quadrant 'B' of the blastocyst prior to separation into single cells. **F** Quadrant 'B' being pipetted through the holding pipette. **G** Individual cells of quadrant 'B' prior to placement into the PCR tube.

individual cells, each cell needs to be pipetted into a PCR tube, and each tube must undergo the CCS procedure. This makes the process labor intensive and expensive compared to FISH.

Although multiple studies have examined mosaicism at the blastocyst stage with CCS, these studies all involved biopsied sections with multiple cells in each section, perhaps masking the true extent of mosaicism [Fragouli and Wells, 2011; Liu et al., 2012; Capalbo et al., 2013]. The examination of individual cells at the blastocyst stage is particularly important to gain insight into possible ori-

gins and mechanisms of mosaicism, such as non-disjunction, endoreduplication, anaphase lagging, uniparental disomy, and their prevalence during preimplantation development [Taylor et al., 2014a]. Indeed, mosaicism could be responsible both for false negative and false positive preimplantation genetic screening (PGS) diagnoses [Haddad et al., 2013; Wener et al., 2014].

In this present study, we expand upon a novel technique by which individual cells of a blastocyst could be isolated and a virtual image of the blastocyst with CCS results could be created [Taylor et al., 2016]. Unfortu-

nately, the previous study did not perform CCS. With this report, we have successfully isolated individual cells from the blastocyst, mapped their location in reference to the inner cell mass (ICM), and successfully performed CCS on the individual cells. This proof-of-concept study could allow insights into the mechanism through which mosaicism arises in the blastocyst.

Methods

One blastocyst, donated to research from a 33-year-old patient, that did not initially have assisted hatching, underwent the following procedure. The whole blastocyst was placed into a 20- μ l drop of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium (Cooper/Sage, Trumbull, Conn., USA) with 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, Calif., USA) and overlaid by oil (Irvine Scientific). The blastocyst was held with a holding pipette (Origio, Denmark), positioning the ICM at the 9 o'clock position (fig. 1A). A laser was used to create a hole in the trophoctoderm at the 3 o'clock position. A biopsy pipette was inserted into the blastocyst, and the ICM was removed with gentle suction and isolated (fig. 1B). The ICM was removed from the drop and placed into another drop of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium with 10% SSS. Using a similar method, Capalbo et al. [2013] demonstrated a 2% trophoctoderm contamination rate when removing the ICM.

The blastocyst underwent 4 further biopsies, thereby separating the blastocyst into quadrants (fig. 1C, D). After each biopsy, the biopsy needle was changed and the biopsied piece was pipetted out of the biopsy drop and into an individual drop of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium + 10% SSS for 20 min (fig. 1E). This process was repeated after each section, so there was no cross-contamination or mislabeling of sections during the procedure. After 20 min, a holding pipette was used to gently aspirate the sections of the blastocyst (fig. 1F). Doing so allowed the sections of the blastocyst to break apart into smaller pieces. Therefore, multiple individual cells were obtained from each quadrant (fig. 1G).

The cells of the blastocyst were identified under a dissecting scope. Cells were rinsed in wash solution and prepped for aCGH, which was performed as previously described [Harton et al., 2013].

Results

A total of 18 cells were used for aCGH. Of the 15 cells isolated from the trophoctoderm, 13 (86.7%) provided a result, while 2 (13.3%) did not amplify. Twelve were euploid (46,XY) and 1 was complex abnormal (44,XY), presenting with monosomy 7, 10, 11, 13, and 19, and trisomy 14, 15, and 21. The complex aneuploid cell was located in region '3', which is from the polar trophoctoderm adjacent to the ICM (fig. 2).

A total of 3 cells were isolated from the ICM, 2 (66.7%) were euploid and 1 did not amplify (fig. 2).

Fig. 2. Reconstructed trophoctoderm and ICM with the location and the CCS results of individual cells within the blastocyst.

Discussion

We herein describe a novel approach that we believe to be the first to combine isolation of individual blastocyst cells with the utilization of CCS. This powerful approach can be used to determine the extent of mosaicism in the human blastocyst. Moreover, by examining the CCS results of individual cells within the blastocyst, the mechanisms of mosaicism can be determined (e.g., non-disjunction, uniparental disomy, endoreduplication, or anaphase lagging) [Taylor et al., 2014a].

Multiple studies have attempted to determine mosaicism at the blastocyst stage with mosaicism rates ranging from 16–70% [Northrop et al., 2010; Fragouli and Wells, 2011; Liu et al., 2012]. All these studies examined mosaicism in 2–3 sections of the trophoctoderm, each containing several cells. Examining this large amount of sections would not allow the chromosome constitution of individual cells within the blastocyst to be determined and thus, the true rate of mosaicism may be masked by the presence of multiple cells. In order to minimize the impact of multiple cells on the rate of mosaicism, the chromosome content for individual cells must be examined.

As previously mentioned, the detection of mosaicism depends on how many cells are analyzed. All of these aforementioned studies examined mosaicism in large sections which contained multiple cells. In our study, the blastocyst was mosaic, but this mosaicism would not have been detected had we not analyzed individual cells. Eight

individual aneuploidies were detected in the trophoctoderm. In a background of otherwise euploid cells we would infer that each was an individual post-zygotic error. In the absence of a reciprocal pattern for each (i.e., a corresponding trisomy and monosomy of the same chromosome) we would infer that the +14, +15, and +21 aneuploidies arose via independent chromosome gain (perhaps some mechanism involving endoreduplication) and the monosomies -7, -10, -11, -13, and -19 by an independent chromosome loss (anaphase lag). Utilizing FISH, Delhanty et al. [1997] and Ioannou et al. [2012] demonstrated a lack of mitotic non-disjunction (3 + 1 pattern), suggesting that it is a rare mechanism for post-zygotic aneuploidy in human development. More recent data utilizing CCS support the notion that non-disjunction is a uncommon event, demonstrating that chromosome losses occur at a 4-fold higher rate than chromosome gains [McCoy et al., 2015]. We did not test individual cells, and therefore it is possible we ‘missed’ the corresponding reciprocal aneuploidies. Further studies are certainly warranted to improve upon our technique.

A meiotic error should be present in the entire, or at least a majority, of cells analyzed. In our proof-of-concept study, only 1 cell contained aneuploidies, while the remaining cells were euploid. This would suggest that the error arose during mitosis and not meiosis. Previous research has demonstrated that ~25% of polar bodies are aneuploid [Salvaggio et al., 2014], while ~50% of blastocysts are aneuploid [Taylor et al., 2014b, c]. The higher incidence of aneuploidy at the blastocyst stage suggests that a majority of aneuploidy may be mitotic in origin. The approach described in this study will allow us to test the hypothesis that post-zygotic aneuploidy of individual cells is commonplace in the trophoblast during human development but less so in the ICM.

Unfortunately, in our study we were only able to detect 1 aneuploid cell. It cannot be overlooked that this aneuploid cell could be due to an error in the CCS test. Capalbo et al. [2015] demonstrated that aCGH overcalls aneuploidy. However, they also demonstrated that on a per-chromosome-basis the accuracy of aCGH is >98%. Another source of error could be due to ‘noise’ within the plot of the CCS result. Some next-generation sequencing (NGS) protocols minimize ‘noise’ and produce cleaner CCS plots, reducing the chance of misdiagnosis. NGS was not used in this study because it had not been validated on single cells when this study occurred, whereas aCGH had [Gutierrez-Mateo et al., 2011]. Moreover, Fiorentino et al. [2014] reran 192 aCGH samples with NGS and found that 191 (99.5%) were concordant. Nonetheless, future studies

should utilize NGS to reduce the chances of misdiagnosis. Due to the high concordance of NGS to aCGH, the accuracy of aCGH on a per-chromosome-basis and the fact that our study had 8 different chromosomes from 1 cell diagnosed as aneuploid suggests that this diagnosis is indeed biological and not an artifact.

Ozawa and Hansen [2011] were able to desegregate individual bovine blastocysts by exposure to trypsin and pipetting the blastocysts through a small glass-pulled pipette. Similarly, we utilized a holding pipette designed for holding the oocyte or embryo during micromanipulation procedures. This pipette had a very small bore size and assisted in the separation of cells from the trophoctoderm. Our technique could also prove valuable for human embryonic stem cells (hESC). These cells are often in clumps and clusters, and the isolation of single hESC may be desired for hESC culture. Prowse et al. [2009] performed a similar process by which clumps of hESC were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ medium. After the wash, they added trypsin to help in the dissociation of cells. Similarly, Hasegawa et al. [2006] also disassociated clumps of hESC into individual cells utilizing trypsin. We did not add trypsin to our cells, and it is unknown if this would have aided in our separation. In these studies, trypsin was used on hESC whereas our study dealt with trophoctoderm cells and trypsin may not separate trophoctoderm cells as easily as hESC cells. We utilized $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium because it was readily available and has been used in conjunction with CCS tests and embryo biopsy for years, and its influence on CCS results would be minimal [Orris et al., 2010]. Another problem is the difficulty in the visualization of the cells after isolation. One suggestion could be the addition of a hypotonic solution to the isolated cells, thereby allowing them to swell and become more easily distinguishable under a microscope [Drey et al., 2003]. Another technique referred to as optical tweezing allows for the control of small particles and possibly could be used to isolate individual cells [Grier, 2003; Prada et al., 2016]. However, this technique would require an expensive piece of equipment and training, neither of which our technique requires.

Given our success with this proof-of-concept study, larger studies are certainly warranted, despite the cost of CCS. Even increasing the number of blastocysts to 10 in our study would utilize ~200–250 CCS tests, and patients may present with different rates of mosaicism, thereby making a well-designed, high-powered study difficult and costly. Our findings stress the need to perform a similar study on a greater number of embryos with the ultimate aim of both improving diagnosis for PGS families and better understanding the nature of our own early development.

Statement of Ethics

This study was approved by an institutional review board (WIRB #1138244) and utilized blastocysts deemed not viable and destined for discard. The University of Kent Research Ethics Advisory Group also approved this study.

Disclosure Statement

The authors have no conflicts of interest to declare.

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