Preimplantation genetic screening: a reappraisal
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Citation for published version (APA):

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Chapter 3

Extensive chromosomal mosaicism in human preimplantation embryos: theoretical implications for PGS.

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Submitted
Abstract

Background
Preimplantation genetic screening (PGS) decreases pregnancy chances in women of advanced maternal age undergoing in vitro fertilization (IVF). We assessed whether chromosomal mosaicism, i.e. the fact that not all cells in the preimplantation embryo have the same chromosomal makeup, is underlying this decrease in pregnancy chance.

Methods
Fluorescence in situ hybridisation (FISH) analysis for chromosomes 1, 13, 16, 17, 18, 21, X and Y was used to study the chromosomal constitution of 360 donated spare human cleavage stage preimplantation embryos of women of advanced maternal age. The chromosomal makeup of these embryos was used to construct a model to estimate the effect of chromosomal mosaicism on pregnancy rates after PGS.

Results
Fifty-five percent of all embryos contained both diploid and aneuploid blastomeres and forty-six percent of the blastomeres of these diploid-aneuploid mosaic embryos were diploid. A model based on this high degree of diploid-aneuploid mosaicism showed that diploid-aneuploid mosaicism provides a plausible explanation for the reduced pregnancy chances after PGS.

Conclusions
The first mitotic divisions of human preimplantation embryos are error prone, and as a result most cleavage stage embryos are mosaic. The high prevalence of diploid-aneuploid mosaicism precludes the determination of the chromosomal makeup of a human embryo by analyzing a single cell during cleavage stage development.
Aneuploidies in human preimplantation embryos were first detected in 1983 (Angell et al., 1983). Since the frequency of aneuploidies in clinically recognized pregnancies increases exponentially with advancing maternal age (Hassold and Hunt, 2001), they are presumed to be central in the sharp decline in pregnancy chances of women aged 35 years and up, both in normal conception and after in vitro fertilization (IVF) (Baird et al., 2005; Lintsen et al., 2007). Therefore, preimplantation genetic screening (PGS), i.e. the removal of a single blastomere from preimplantation embryos, analysis of its chromosomal content, and subsequent transfer of euploid embryos, was thought to increase pregnancy chances in older women undergoing IVF (Wilton, 2002). In contrast with this hypothesis, several randomized controlled trials were not able to demonstrate an increase in pregnancy rate after PGS using FISH in cleavage-stage embryos as compared to standard IVF treatment (Staessen et al., 2004; Mastenbroek et al., 2007; Hardarson et al., 2008; Debrock et al., 2010). On the contrary, meta-analysis of published randomized controlled trials shows a significant decrease in pregnancy rates with a reported odds ratio of 0.56 (95% CI: 0.42-0.76) when comparing ongoing pregnancy per cycle of IVF with PGS to IVF without PGS (Mastenbroek et al., 2008).

Several mechanisms have been suggested to explain the compromised pregnancy rates after PGS. Firstly, the biopsy itself may be harmful for the potential of an embryo to successfully implant, but the effect of biopsy alone on pregnancy rates has never been studied (De Vos and Van Steirteghem, 2001). Secondly, the FISH analysis that is generally used for PGS is limited by the number of chromosomes that can be analyzed. Finally, chromosomal mosaicism may cause the chromosomal constitution of the analyzed blastomere not to be representative for the entire embryo. Clinical studies on the effect of mosaicism on PGS are scarce and commonly focus on ‘misdiagnosis’ rates, i.e. false-negative and false-positive-rates of FISH analysis, rather than on the impact of mosaicism on pregnancy rates (Los et al., 2004; Staessen et al., 2004).

The aims of this study were to first determine the prevalence of diploid-aneuploid mosaicism among human cleavage stage embryos and then to use this frequency to construct a model to estimate the effect of mosaicism on PGS pregnancy rates. In addition, we aimed at elucidating the origin of mosaicism and to see whether there is natural selection against embryos with a high percentage of aneuploid blastomeres. To do so, we performed a detailed analysis the chromosomal makeup of human cleavage stage embryos from both arms of a double blind randomized controlled trial on PGS.

**Methods**

Donated spare human preimplantation embryos obtained from a randomized controlled trial (RCT) on PGS (Mastenbroek et al., 2007), were fixed on glass slides four days after insemination, using polyoxy-ethylene(20)sorbitanmonolaurate (Tween20) (0,1% in 0,01N HCl) and methanol acetic acid (3:1) (Dozortsev and McGinnis, 2001). Excessive cytoplasm was removed using pepsin if necessary. Fluorescence in situ hybridization (FISH) analysis was performed in two rounds for a total of eight chromosomes. First, all available nuclei were
analyzed for chromosomes 1, 16, 17 (chromosome enumeration probes, Vysis) and the next day (following overnight hybridization) the same nuclei were analyzed for chromosomes 13, 18, 21, X and Y (MultiVysion PGT, Vysis). In case of unclear FISH signals, nuclei were washed free of FISH probes using phosphate buffered detergent and subsequently rehybridized with the same probe set.

Embryos were then classified as diploid, aneuploid or mosaic according to the predetermined criteria listed in Table 1. The relationship between the origin of the embryos (embryos from IVF cycles without PGS, embryos diagnosed diploid after PGS, embryos diagnosed aneuploid after PGS) and the percentage of diploid-aneuploid mosaicism was tested with a Chi-square test.

Table 1. Classification criteria for the chromosomal makeup of human preimplantation embryos.

<table>
<thead>
<tr>
<th>Chromosomal makeup</th>
<th>Criteria*</th>
<th>FISH examples for X,Y and 18**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>All blastomeres contain two chromosomes for each chromosome pair tested</td>
<td>XX,1818[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XY,1818[7]</td>
</tr>
<tr>
<td>Aneuploid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haploid</td>
<td>All blastomeres contain one chromosome for each chromosome pair tested</td>
<td>X,18[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y,18[7]</td>
</tr>
<tr>
<td>Polyploid</td>
<td>All blastomeres contain more than two chromosomes for each chromosome pair tested</td>
<td>XXX,181818[7]</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>All blastomeres contain the same abnormality for one chromosome pair tested</td>
<td>XX,18[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XX,1818[7]</td>
</tr>
<tr>
<td>Complex abnormal</td>
<td>All blastomeres contain the same abnormalities for multiple chromosome pairs tested</td>
<td>X,181818[7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XYY,18[7]</td>
</tr>
<tr>
<td>Mosaic</td>
<td>Not all blastomeres contain the same chromosomal makeup</td>
<td></td>
</tr>
<tr>
<td>Aneuploid-mosaic</td>
<td>A mosaic embryo without one or more diploid blastomeres</td>
<td>XX,181818[3]/XXX,181818[4]</td>
</tr>
<tr>
<td>Diploid-aneuploid mosaic</td>
<td>A mosaic embryos with one or more diploid blastomeres</td>
<td>XX,1818[5]/XX,18[2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XY,1818[3]/XY,181818[4]</td>
</tr>
</tbody>
</table>

* Embryo should have at least three cells.

Based upon this classification, we estimated the implantation potential by multiplying the percentage of transferred embryos with the percentage of diploid blastomeres in those embryos.

To see whether mosaicism originates at all developmental stages or at a specific stage before time of biopsy, we plotted the percentages of diploid blastomeres against the cumulative number of embryos. Finally, we studied the presence of natural selection against embryos with a high percentage of aneuploid blastomeres by plotting the total number of blastomeres against the percentage of diploid blastomeres to detect any correlation between these two variables. Analysis was performed using Spearman correlation coefficient as these two variables were not normally distributed.

The study protocol was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO) and written informed consent was obtained from all couples.
Results

Three-hundred-and-sixty normally fertilized human cleavage stage embryos, donated for research because they were either of insufficient quality for transfer to the uterus or cryopreservation, were analyzed. The embryos were donated by 124 women (mean age: 38.0 ±1.5 years) in 162 cycles of IVF/ICSI (93 IVF and 69 ICSI). In total 2290 blastomeres were studied. One-hundred-and-sixteen embryos were from the RCT control arm, 44 embryos from the PGS arm diagnosed as diploid and 200 embryos from the PGS arm diagnosed as aneuploid (Table 2).

We constructed a model to estimate the consequences of diploid-aneuploid mosaicism on the implantation potential of embryos after PGS (Fig. 1). Based upon the facts that 55% of all embryos were diploid-aneuploid mosaic and that 46% of the blastomeres in such embryos were diploid, this model showed that the overall chance to aspirate a diploid blastomere from a diploid-aneuploid mosaic embryo is 25% (calculation: 0.55*0.46=0.25). In these cases, since embryos at the time of PGS biopsy consist of a mean number of six blastomeres (Mastenbroek et al., 2007), the removal of one diploid blastomere from such diploid-aneuploid mosaic embryos lowers the mean percentage of diploid blastomeres in these embryos from 46% to 35% (calculation: ((6*0.46)-1)/5=0.35). These embryos are judged suitable for transfer.

Table 2. Chromosomal constitution of day four human cleavage stage embryos.

<table>
<thead>
<tr>
<th>Chromosomal makeup</th>
<th>All embryos (n=360)</th>
<th>Embryos without PGS (n=116)</th>
<th>Diploid after PGS (n=44)</th>
<th>Aneuploid after PGS (n=200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>29 8%</td>
<td>10 9%</td>
<td>6 14%</td>
<td>13 7%</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>11 3%</td>
<td>0 0%</td>
<td>1 2%</td>
<td>10 5%</td>
</tr>
<tr>
<td>Haploid</td>
<td>1 0%</td>
<td>0 0%</td>
<td>0 0%</td>
<td>1 1%</td>
</tr>
<tr>
<td>Polyploid</td>
<td>0 0%</td>
<td>0 0%</td>
<td>0 0%</td>
<td>0 0%</td>
</tr>
<tr>
<td>Aneuploid-mosaic</td>
<td>6 2%</td>
<td>0 0%</td>
<td>1 2%</td>
<td>5 3%</td>
</tr>
<tr>
<td>Complex</td>
<td>4 1%</td>
<td>0 0%</td>
<td>0 0%</td>
<td>4 2%</td>
</tr>
<tr>
<td>Abnormal mosaic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid-aneuploid mosaic</td>
<td>320 89%</td>
<td>106 91%</td>
<td>37 84%</td>
<td>177 89%</td>
</tr>
<tr>
<td>Aneuploid-mosaic</td>
<td>122 34%</td>
<td>43 37%</td>
<td>8 18%</td>
<td>71 36%</td>
</tr>
<tr>
<td>Diploid-aneuploid mosaic</td>
<td>198 55%</td>
<td>63 54%</td>
<td>29 66%</td>
<td>106 53%</td>
</tr>
</tbody>
</table>
Conversely, based upon the facts that 55% of all embryos were diploid-aneuploid mosaic and 54% of the blastomeres in such embryos were aneuploid, the model showed that the overall chance to aspirate an aneuploid blastomere from a diploid-aneuploid mosaic embryo is 30% during PGS (calculation: 0.55*0.54=0.30). The removal of one aneuploid blastomere from a six-cell diploid-aneuploid mosaic embryo, increases the percentage of diploid blastomeres in these embryos from 46% to 55% (calculation: (6*0.46)-0)/5=0.55). These potentially viable embryos are not transferred, but discarded. Assuming that the implantation potential of a human preimplantation embryo correlates directly with the percentage or number of diploid cells, diploid-aneuploid mosaicism leads to an overall reduction in implantation potential of 49% in PGS (3330-->1675; Fig. 1).

Every possible combination of diploid and aneuploid blastomeres was found in the plot of the percentages of diploid blastomeres against the cumulative number of embryos (Fig. 2). There was no correlation between the number of blastomeres and the percentage of diploid blastomeres (correlation-coefficient: -0.13) (Fig. 3).

**Discussion**

We studied spare human cleavage stage embryos and found a high percentage of diploid-aneuploid mosaicism: 55% of all embryos analyzed consisted of both diploid and aneuploid
blastomeres. Our model based on this high degree of diploid-aneuploid mosaicism showed that the implantation potential of the cohort of embryos in an IVF cycle is potentially diminished by 49% with the use of PGS.

Our model is for obvious reasons limited by the fact that only spare embryos, i.e. embryos that were not transferred or cryopreserved, were analyzed. These embryos may contain more chromosomal abnormalities than embryos that are transferred or cryopreserved, since they are in general of less morphological quality (Magli et al., 2007). The fact that the calculated reduction in implantation potential (49%) is larger than the observed reduction in ongoing pregnancy rates in the RCT these embryos originated from (32%) indicates that this might indeed be the case (Mastenbroek et al., 2007). On the other hand, FISH analysis of embryos that originated from randomly selected donated oocytes that were inseminated with donor sperm showed a similar diploid-aneuploid-mosaicism rate of 55% (57 out of 103 embryos), suggesting that spare embryos and embryos selected for transfer may not differ that much (Ziebe et al., 2003). Our observation of the absence of a correlation between the degree of diploid-aneuploid mosaicism and the origin of the embryos we studied (i.e. embryos from IVF cycles without PGS, embryos diagnosed diploid after PGS, embryos diagnosed aneuploid after PGS) also supports this view.

Figure 2. Percentages of diploid blastomeres in human preimplantation embryos. Each dot in the graph represents a diploid-mosaic embryo on the fourth day after insemination (N=198). The embryos are sorted based on the percentage of diploid blastomeres in each embryo.
Although in view of these observations the validity of our model is not proven beyond any
doubt and needs further fine-tuning, it seems likely that the discarding of diploid-aneuploid
mosaic embryos seriously undermines the efficacy of PGS using cleavage stage biopsy and
FISH.

Several observations support the idea that these discarded diploid-aneuploid mosaic embryos
are viable. First, experiments using tetraploid embryo complementation, a technique in
which mice originate from ES cells that are injected in tetraploid blastocysts, have shown
that even the injection of donor ES cells of which only a small percentage are diploid (20%
diploid cells combined with 80% cells with chromosomal abnormalities) results in fully diploid
normal adult mice (Eggan et al., 2002). Second, frozen-thawed embryos that have lost nearly
half of their blastomeres due to the cryopreservation procedure are still able to result in live
births, implying that not all blastomeres of human preimplantation embryos are necessary
for proper development into a child (Munne et al., 1995). Third, embryos are suggested
to contain rescue mechanisms that allow discarding of aneuploidies once the embryonic
genome has been activated, such as preferential cell proliferation of diploid cells, preferential
allocation of diploid cells to the inner cell mass or embryo proper, or aneuploidy rescue by
anaphase lagging or chromosome demolition (Los et al., 1998). Interestingly, in humans the
embryonic genome is not activated before the third day of development which is exactly the
time when PGS is generally performed (Braude et al., 1988).

**Figure 3. No selection against aneuploid blastomeres during first cleavages.** The percentage of diploid
blastomeres in each diploid-aneuploid mosaic embryo analyzed (N=198) is plotted against the total
number of blastomeres of that embryo.
We found every possible combination of diploid and aneuploid blastomeres. This indicates that the first cleavages of human development, up to the fourth day after insemination, are all prone to mitotic errors. The fact that there was no correlation between the total number of blastomeres and the percentage of diploid blastomeres indicates that there is probably no or only limited selection in terms of growth arrest against embryos with a high percentage of aneuploid cells during these first cleavages up to the fourth day after insemination.

Since we studied day four embryos only, our results do not necessarily translate to other stages of preimplantation development. The degree of mosaicism in blastocysts has for example been suggested to be different when compared to cleavage stage embryos (Bielanska et al., 2002).

Chromosomal mosaicism could be an intrinsic phenomenon of human preimplantation development, although the molecular basis for this remains elusive. Gene expression does not occur in the first postzygotic cleavages in humans, making these cleavages fully dependent on maternally derived proteins and gene transcripts, stored within the oocyte since prenatal gonad development (Braude et al., 1988; Wells et al., 2005). The quality of human oocytes and their proteins and gene transcripts could diminish over time by the accumulation of radiation or toxic agents, oxidative stress (Tarin, 1996), compromised mitochondria (Keefe et al., 1995), or telomere shortening (Keefe et al., 2006). This compromised environment could then result in absence or reduced stringency of cell cycle checkpoint mechanisms during the earliest stages of human preimplantation development, thereby facilitating chromosome segregation errors, especially in women of advanced maternal age (Harrison et al., 2000; Delhanty, 2005; Steuerwald, 2005).

Alternatively, chromosomal mosaicism might be attributed to, or become aggravated by, the artificial ovarian hyperstimulation and/or in vitro culture of human preimplantation embryos that are intrinsic parts of IVF treatments. This explanation is corroborated by data that showed an increase in the number of mosaic embryos with increasing intensity of ovarian hyperstimulation, suggesting that the extra oocytes obtained after (intense) ovarian hyperstimulation show compromised mitotic segregation (Baart et al., 2007). In addition, mouse model studies showed that slight changes in oxygen tension during embryo culture drastically effect chromosomal mosaicism rates (Bean et al., 2002).

In conclusion, the first mitotic divisions of human embryos are error prone, and as a result most human cleavage stage embryos are mosaic. This could be an intrinsic phenomenon of human preimplantation development or, alternatively, be attributed to or become aggravated by the in vitro fertilisation technique. Although the validity of our model is not proven beyond any doubt and needs further fine-tuning, diploid-aneuploid mosaicism most likely contributes to the compromised pregnancy rates after cleavage stage PGS using FISH.
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