

Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol

D.S. Johnson^{1,8}, G. Gemelos¹, J. Baner^{1,2}, A. Ryan¹, C. Cinnioglu¹,
M. Banjevic¹, R. Ross³, M. Alper⁴, B. Barrett⁴, J. Frederick⁵,
D. Potter^{1,5}, B. Behr⁶, and M. Rabinowitz^{1,7}

¹Gene Security Network, Inc., 2686 Middlefield Road, Suite C, Redwood City, CA 94063, USA ²Genome Technology Center, Stanford University, 318 Campus Drive, Stanford, CA 94305, USA ³La Jolla IVF, 9850 Genesee Avenue No. 610, La Jolla, CA 92037, USA ⁴Boston IVF, 130 Second Avenue, Waltham, MA 02451, USA ⁵Huntington Reproductive Center, 23961 Calle de la Magdalena, Suite 503, Laguna Hills, CA 92653, USA ⁶Obstetrics and Gynecology, Stanford University Medical Center, 900 Welch Road, Palo Alto, CA 94304, USA ⁷School of Engineering, Aeronautics and Astronautics, Stanford University, Stanford, CA 94305, USA

⁸Correspondence address. E-mail: djohnson@genesecurity.net

BACKGROUND: Preimplantation genetic screening (PGS) has been used in an attempt to determine embryonic aneuploidy. Techniques that use new molecular methods to determine the karyotype of an embryo are expanding the scope of PGS.

METHODS: We introduce a new method for PGS, termed 'parental support', which leverages microarray measurements from parental DNA to 'clean' single-cell microarray measurements on embryonic cells and explicitly computes confidence in each copy number call. The method distinguishes mitotic and meiotic copy errors and determines parental source of aneuploidy.

RESULTS: Validation with 459 single cells of known karyotype indicated that per-cell false-positive and false-negative rates are roughly equivalent to the 'gold standard' metaphase karyotype. The majority of the cells were run in parallel with a clinical commercial PGS service. Computed confidences were conservative and roughly concordant with accuracy. To examine ploidy in human embryos, the method was then applied to 26 disaggregated, cryopreserved, cleavage-stage embryos for a total of 134 single blastomeres. Only 23.1% of the embryos were euploid, though 46.2% of embryos were mosaic euploid. Mosaicism affected 57.7% of the embryos. Counts of mitotic and meiotic errors were roughly equivalent. Maternal meiotic trisomy predominated over paternal trisomy, and maternal meiotic trisomies were negatively predictive of mosaic euploid embryos.

CONCLUSIONS: We have performed a major preclinical validation of a new method for PGS and found that the technology performs approximately as well as a metaphase karyotype. We also directly measured the mechanism of aneuploidy in cleavage-stage human embryos and found high rates and distinct patterns of mitotic and meiotic aneuploidy.

Key words: microarray / aneuploidy / preimplantation genetic screening / *in vitro* fertilization / segmental imbalance

Introduction

Roughly 138 198 *in vitro* fertilization (IVF) cycles were performed in the USA in 2006 (Centers for Disease Control and Prevention, 2008). Unfortunately, ~31% of embryos that are transferred to the uterus during IVF survive to live birth (Centers for Disease Control and Prevention, 2008). Therefore, in each IVF cycle, it is critical to make a well-informed decision regarding which embryo(s) to transfer (Sermon *et al.*, 2004; Thornhill *et al.*, 2005; Verlinsky *et al.*, 2005). Embryo transfer choices are frequently made by examining gross

morphological features. Though there is evidence to support the correlation between morphology and implantation rates (Hardarson *et al.*, 2001; Borini *et al.*, 2005; Nomura *et al.*, 2007), recent studies report that 30–40% of morphologically normal embryos have chromosomal abnormalities such as aneuploidy (Munné, 2003; Baltaci *et al.*, 2006). Transfer of aneuploid embryos leads to universally undesired outcomes such as failed embryo implantation (Munné *et al.*, 2003), miscarriage (Warburton *et al.*, 1986; Munné *et al.*, 1998; Sarosi *et al.*, 1998; Vidal *et al.*, 1998; Pellicer *et al.*, 1999) or birth of trisomic offspring (Kuliev *et al.*, 2003).

As a result, reproductive specialists have frequently used preimplantation genetic screening (PGS) in hopes of increasing the probability that they will select embryos with the best chances for implantation and development into a healthy child (Delhanty *et al.*, 1993; Munné *et al.*, 1993; Colls *et al.*, 2007). In the process of PGS, single cells (blastomeres) are often biopsied from cultured embryos on Day 3 and analyzed for chromosome copy numbers. Embryos diagnosed as normal (euploid) on the basis of the single- or double-cell diagnosis are then considered for transfer into the uterus, typically between Days 4 and 6. Currently, the majority of PGS uses fluorescent *in situ* hybridization (FISH), which typically tests 12 or fewer chromosomes in 24 h (Delhanty *et al.*, 1993; Munné *et al.*, 1993; Staessen *et al.*, 2004, 2008; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Meyer *et al.*, 2008; Debrock *et al.*, 2009). A number of studies suggest the positive effect of PGS on clinical outcomes, with an increase implantation rates (Munné *et al.*, 2003; Wilton *et al.*, 2003), lower miscarriage rates (Gianaroli *et al.*, 1999) and increased live birth rates (Verlinsky *et al.*, 2005). However, an overwhelming number of recent, randomized, controlled clinical studies have suggested that PGS with FISH does not improve implantation rates or live birth rates (Staessen *et al.*, 2004, 2008; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Meyer *et al.*, 2008; Debrock *et al.*, 2009).

However, all prior clinical trials using PGS for IVF have used FISH, which screens for a minority of chromosomes. The limitations of the FISH technology could have compromised the results of these studies. Recent advances in molecular technology, such as comparative genomic hybridization (CGH) and DNA microarrays, have increased the scope of PGS (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Wilton *et al.*, 2003; Hu *et al.*, 2004; Le Caignec *et al.*, 2006; Sher *et al.*, 2007; Hellani *et al.*, 2008; Handyside *et al.*, 2009; Vanneste *et al.*, 2009; Wells *et al.*, 2008). One challenge of molecular technology for PGS is that the amplification of DNA from a single cell can result in errors. This is because single-cell measurements suffer from allele dropout (ADO), or the random loss of alleles that can result in ambiguous copy number calls, and also suffer from over-amplification and under-amplification of certain loci or entire chromosomes (Walsh *et al.*, 1992; Findlay *et al.*, 1995; Piyamongkol *et al.*, 2003; Handyside *et al.*, 2004; Renwick *et al.*, 2006; Spits *et al.*, 2006; Glentis *et al.*, 2009).

As a result of the measurement noise caused by whole-genome amplification, we have found that standard commercial software packages (i.e. Illumina BeadStudio) underperform when calling copy number on single-cell data (Supplementary Material, Fig. S1). Therefore, we developed a novel method that 'cleans' the error-prone single-cell microarray data using parental genetic information. The key insight of the algorithm, termed 'parental support' (PS), is that copy number determination in embryonic DNA is enhanced if supplemented with parental genetic data because all embryonic chromosomes are derived from parental chromosomes (Rabinowitz *et al.*, 2007; Johnson *et al.*, 2008; Rabinowitz *et al.*, 2008). The method allows IVF physicians to screen a single cleavage-stage blastomere for chromosome copy number across all 24 chromosomes, delivering results in time to inform decisions on Day 5 regarding embryo transfer to the uterus.

No major preclinical validation studies have been performed for any PGS method, including FISH. Here, we describe a major preclinical validation of the new PS microarray method on 459 cells with known karyotype. The data demonstrate that the preclinical accuracy

of the PS microarray method is roughly consistent with the 'gold standard' traditional metaphase karyotyping. We also applied the technology to determine chromosome copy number on 134 single-blastomeres derived from 26 cryopreserved embryos from six unrelated couples. In this cohort of embryos, we found high rates and distinct patterns of mitotic and meiotic aneuploidy, as well as a high rate of segmental chromosome imbalances.

Materials and Methods

Single-cell isolation, DNA amplification and genotyping

Single cells were isolated from buccal swabs, semen samples, adult blood, immortalized cell lines and Day 3 cryopreserved embryos. Single-tissue culture (lymphocytes) and buccal cells were isolated using a sterile stripper tip (Midatlantic Diagnostic, Mt Laurel, NJ, USA) affixed to a pipette (Drummond Scientific, Broomall, PA, USA) and a stereoscope (Leica, Wetzlar, Germany). Embryos were thawed, and then individual blastomeres were separated using a micromanipulator (Transferman NK2-Eppendorf, Westbury, NY, USA) after zona pellucida drilling using acidified Tyrode's solution. Sperm cells were manually isolated using a micromanipulator (Transferman NK2-Eppendorf). Aside from sperm, single cells were washed sequentially four times with wash buffer (5.6 mg/ml KCl, 6 mg/ml bovine serum albumin). Two different lysis/amplification protocols were used in the analysis: (i) Rubicon whole-genome amplification (Ann Arbor, MI, USA) and (ii) multiple displacement amplification (MDA) with proteinase K buffer (PKB). Protocol (i) was performed according to the manufacturer's instructions. For protocol (ii), cells were placed in 5- μ l PKB (Arcturus PicoPure Lysis Buffer, 50 mM DTT), incubated at 56°C for 1 h and then heat-inactivated at 95°C for 10 min. MDA reactions were incubated at 30°C for 2.5 h and then 65°C for 5 min. Genomic DNA from bulk tissue (Epicentre MasterAmp Buccal Swabs, Madison, WI, USA) was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). No template controls (buffer blanks) were performed for each amplification method. All buffer blanks produced intensities equivalent to the noise floor (an intensity of 1000 in the 95th percentile on the green detection channel).

Amplified single cells and bulk parental tissue were genotyped using the Illumina (San Diego, CA, USA) Infinium II genome-wide genotyping single-nucleotide polymorphism (SNP) microarrays (HapMap CNV370Quad or CytoSNP-12 chips). For bulk tissue (i.e. parent samples), the standard Infinium II protocol (www.illumina.com) was used and required call rates of >99% using standard BeadStudio allele calling. All single cells were genotyped using a modified Infinium II genotyping protocol such that the entire protocol, from single-cell lysis through array scanning, was completed in <24 h. The microarray protocol was performed as per the manufacturer's instructions, except that the duration of the amplification and hybridization steps was reduced to 7 and 6 h, respectively. Single-cell array measurements that fell below the noise floor (an intensity of 1000 in the 95th percentile on the green detection channel) were removed from downstream analysis. For the MDA and Rubicon protocols, this represented ~1.6 and 1.3% of all amplifications, respectively. All microarray data have been archived by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo/) under accession identifier GSE19247.

Copy number determination and haplotype phasing

We determined that commercial software packages (i.e. ChromoZone or Homozygosity Detector in Illumina BeadStudio) underperform when

calling copy number on noisy single-cell data. This is because these methods generally use heterozygosity to determine copy number, and high rates of ADO and preferential amplification in single-cell measurements result in unpredictable heterozygosity, regardless of chromosome copy number (Supplementary Material, Table S1 and Fig. S1). Allele drop-in also occurs, but with much lower frequency (typically <0.1%). With such issues in mind, we implemented a chromosome copy number classification algorithm in MATLAB (MathWorks, Natick, MA, USA) that makes use of parental genotypes and the observed distribution of unprocessed single-cell microarray channel intensities grouped by parental context (Rabinowitz et al., 2007, 2008; Johnson et al., 2008). As an illustrative example, one can think of the ADO rate as one piece of information contained in the channel intensity distributions. Furthermore, the ADO rate is correlated to the chromosome copy number in the sense that it decreases as the chromosome copy number increases. Hence, we use ADO rates as a measure of the chromosome copy number. Though in this example we focused on a single aspect of the microarray channel intensity distributions (i.e. ADO rate), in our algorithm we make use of the entire distribution. One can think of this as a finer scale observation containing much more information than any single statistic such as ADO rates.

Consider a generalized example where possible alleles at one locus are A and B. If both parents have homozygous BB states, then the embryo will never have AB or AA states (Supplementary Material, Fig. S2). In this case, measurements on the A allele will, on average, have low intensities with a distribution determined by background noise. Conversely, if both parents have homozygous AA states, then the embryo will never have AB or BB states, and measurements on the A allele will, on average, have very high intensities with a distribution subject to the ADO rate and other measurement noise characteristics.

Notice that this analysis is done without *a priori* knowledge of chromosome copy number. In general, the statistical behavior of each parental context group will differ on the basis of the underlying chromosome copy number of the embryo. These changes are predictable and based on the additional allelic content that is contributed or missing from each of the parents. First, let us define the 'AB|BB' parental context as the set of all genetic loci for which the mother is AB and the father is BB, and the 'BB|AA' parental context as the set of all genetic loci for which the mother is BB and the father is AA. We would not expect the contexts AB|BB and BB|AA to be statistically similar for a disomy, but we would expect statistical similarity for a maternal trisomy (Supplementary Material, Fig. S2). Our chromosome copy number calling algorithm is an extension of this concept. In particular, we examine rank statistics for each parental context and compare them against expected orderings under the various chromosome copy number possibilities. We then examine the probability that each parental context could have swapped rank by random chance to determine copy number calls and calculate confidences (Rabinowitz et al., 2007, 2008).

Note that algorithmic calls of 'trisomy' are in fact three or more chromosomes where there is an imbalance between the number of chromosomes contributed from the mother and father, since the technique does not attempt to distinguish that case from trisomy. The algorithm will call tetrasomy with equal contribution from both parents, so long as the entire cell is not tetraploid, in which case the algorithm will call the cell euploid. However, if any balanced tetrasomy contains unmatched chromosomes from the parents, which is the more frequent case, this tetrasomy will be detected by the component of the algorithm that leverages phasing (Rabinowitz et al., 2007, 2008).

Many chromosome copy number errors are meiotic, and therefore will include three unmatched haplotypes. Detection of three unmatched haplotypes adds additional confidence to a trisomy call. Therefore, we also use parental information, high-confidence disomic single-cell measurements on children and recombination probabilities (genome.ucsc.edu) to

determine the phase of the parental chromosomes. For example, if two neighboring loci both correspond to the AB|BB context, we observe high A allele channel intensities in the children, and there is a low probability of crossover between these loci, we can predict that one parent has two genetically linked loci with A alleles. A maximum likelihood estimator (MLE) algorithm is then used to phase full chromosomes for all parental genotype contexts. We then look for each of the possible haplotypes in single-cell measurements to detect meiotic trisomies.

To detect segmental copy imbalances, each chromosome is divided into five segments, and the algorithm is applied to each segment independently. If any of the segments differ in copy number with high confidence, the chromosome is flagged with a segmental chromosome imbalance. Note that the reported copy number for chromosomes with a segmental imbalance is reflective of the call on the majority of the chromosome, even if part of the chromosome shows loss or gain. Thus, depending on size, segmental copy imbalances may depress the overall confidence of the full chromosome call. However, confidences on chromosomes with segmental imbalances may remain high if the deletion is relatively small and/or the remainder of the chromosome is called with very high confidence.

As reference against the PS algorithm, we developed a novel statistical method in MATLAB, Probe Intensity Means (PIM), that uses the per-chromosome means and standard deviations of normalized microarray probe intensities to call chromosome copy number (Rabinowitz et al., 2007). For each single-cell measurement, a training set of single-cell amplification microarray measurements was used to normalize probe intensities across each chromosome. Then, an MLE algorithm was used to compute the most likely chromosome state for all of the single-cell amplification microarray data.

Results

Accuracy of PS technology is roughly concordant with traditional metaphase karyotyping

Four hundred and fifty-nine cells of known karyotype were analyzed with the 24-h microarray genotyping protocol (Table I; Supplementary Material, Tables SII and SIII). The majority of the cells were run in parallel with a commercial clinical PGS service. We used cells with trisomy 21 to estimate false-negative rate, and euploid cells to estimate false-positive rate. Two no-template-control replicates (buffer blanks) were run for each amplification protocol, and the results were equivalent to background microarray intensities. All samples were labeled with random identifiers so that laboratory technicians were blind to the ploidy states of the cells. Results were then compared with known karyotypes to determine accuracy.

Of the 330 known trisomy 21 cells, 7 were called either monosomy 21 or disomy 21, for a false-negative proportion of 2.1% (Supplementary Material, Table SII). Metaphase karyotypes of 100 trisomy 21 cells by an independent reference laboratory indicated one cell that was disomy 21, for a false-negative proportion of 1% (Table II). Thus, the difference between the false-negative rate of the PS microarray assay and the gold standard metaphase karyotype was no different than expected by chance (χ^2 , $P = 0.77$). Similarly, microarray analysis indicated five aneuploid calls among 129 single euploid cells, for a false-positive proportion of 3.9% (Supplementary Material, Table SIII). The difference between the false-positive proportion of the PS microarray assay and the gold standard metaphase karyotype of a euploid cell line was no different than expected by chance (χ^2 , $P = 0.74$). Across all

Table I Molecular karyotyping with microarrays and parental support

Cell type	Number cells	Parental support errors	Parental support false detection rate	Probe intensity means errors	Probe intensity means false detection rate
Aneuploid	330	7	2.1%	21*	6.4%
Euploid	129	5	3.9%	124*	96.1%
Haploid	26	0	0%	26*	100%

Performance statistics for molecular karyotype using microarray data and two analysis algorithms.

*Significantly more errors using the probe intensity means analysis method (χ^2 , $P < 0.05$).

Table II Traditional metaphase karyotyping

Cell type	Total cells	Total errors	False detection rate
Aneuploid	100	1 [#]	1.0%
Euploid	100	4 [#]	4.0%

Performance statistics for metaphase karyotype by an independent laboratory.

[#]Unable to detect significant difference in errors between microarray testing with PS and metaphase karyotype (χ^2 , $P > 0.05$).

the 459 cells, gender was always called correctly, significantly better than at least one prior report (Hu *et al.*, 2004; χ^2 , $P < 0.0001$). In addition to the cells of known karyotype, 29 single sperm and were analyzed using PS (Table I; Supplementary Material, Table SIV). The expectation for the sperm cells would be that all chromosomes are paternal monosomies. With this assumption, we achieved an accuracy of 100% on the single sperm data. Fourteen of the sperm cells were OX and 15 of the sperm cells were OY, a ratio not significantly different than expected by chance (χ^2 , $P = 0.84$).

Next, we analyzed the same single-cell microarray data using a computerized algorithm (PIM) that does not make use of parental information (Table I; Supplementary Material, Table SV). PIM uses relative chromosome intensities measured on amplified single cells to determine a molecular karyotype. The method had a significantly higher false-negative rate than PS, with a 6.4% of trisomy 21 cells called disomy 21 and 0% of sperm called haploid (χ^2 , $P = 0.012$ and $P < 0.0001$, respectively). Additionally, the false-positive rate was significantly higher using the chromosome means analysis method, with 96.1% of euploid cells called aneuploid on at least one chromosome (χ^2 , $P < 0.0001$). Though other intensity-only methods might perform better than PIM, the additional information supplied by parental measurements significantly improves accuracy in the present data set.

Because the PS algorithm computes explicit confidences for copy number calls, we expect that higher error rates will occur among groups of chromosomes that are assigned lower confidences, and vice versa. For example, the expectation is that 50% confidence would produce 1 error out of 2 measurements, 95% confidence would produce 1 error out of 20 measurements and so on. To examine the relationship between confidence and accuracy, we sorted chromosome copy calls into eight confidence ranges and then calculated accuracy for each confidence range (Table III). This analysis shows that computed confidences are generally conservative, because accuracy exceeds confidence for all confidence ranges $< 90\%$

(χ^2 , $P < 0.05$). For confidences in the 90–100% range, accuracy was not significantly different than confidence, suggesting that confidences in this range reflect true accuracy (χ^2 , $P > 0.05$). This finding is particularly important for measurements on single blastomeres, for which the true karyotype is unknown and for which multiple measurements are not possible.

High incidence of aneuploidy and mosaicism in cleavage-stage embryos

Genotyping microarray measurements were made on 26 disaggregated, cryopreserved, cleavage-stage embryos from 7 unrelated couples, for a total of 134 single blastomeres and 3082 chromosomes (Supplementary Material, Table SVI). The PS algorithm was then applied to these measurements. The average donor age for the cohort of embryos was 38.8, the youngest woman was 32 and the oldest woman was 44. The average number of cells successfully biopsied per embryo was 5.2, and the average embryo grade was 1.5 (Grade 1 was the highest quality, Grade 4 was the lowest quality). In general, our cohort of embryos was of high quality and from women of advanced maternal age.

To determine the accuracy of any test, blind diagnoses are first made on cells with known karyotype. The proportion of cells with a correct diagnosis is defined as accuracy. Unfortunately, embryos commonly suffer from mosaicism (Los *et al.*, 1998, 2004; Bielanska *et al.*, 2005; Baart *et al.*, 2004, 2006; Barbash-Hazan *et al.*, 2008; Daphnis *et al.*, 2008; Frumkin *et al.*, 2008), so the underlying ploidy state of any particular blastomere is unknown. Therefore, it is not possible to compute accuracy on blastomere data. However, because chromosome confidences computed by PS are indicative of the probability of correct calls (Table III), confidences can be used as a proxy to assess the quality of the measurements. Across the molecular karyotypes of 134 blastomeres, 90.1% of the chromosomes were reported at $> 95\%$ confidence, and the mean confidence was 97.9% (Supplementary Material, Table SVI). Notably, chromosomes with segmental imbalances had significantly lower confidences than chromosomes without segmental imbalances (one-sided *t*-test, $P = 0.018$), suggesting that segmental imbalances are a common cause of lower confidences in this cohort of embryos. In summary, we did not find a significant difference between the mean confidence proportion of blastomeres and the various other cell types, so we conclude that the blastomere data performed roughly equivalent to other cell types.

The majority of embryos and blastomeres were aneuploid, and most of the embryos were mosaic (Table IV). More specifically, 47.8% of the blastomeres were euploid, 32.1% of the blastomeres

Table III Confidence ranges compared with accuracy

Confidence range	Number chromosomes	Expected errors	Actual errors	PS accuracy	P-value
50–60%	20	9	1	95.0%	0.0084*
60–70%	37	13	4	89.2%	0.0271*
70–80%	62	15	2	96.8%	0.0017*
80–90%	142	21	6	95.8%	0.0046*
90–95%	172	13	7	95.9%	0.2493 [#]
95–99%	526	2	3	99.4%	1 [#]
99–99.9%	700	4	6	99.1%	0.751 [#]
99.9–100%	1638	8	9	99.5%	1 [#]

Computed confidence ranges and accuracy for chromosome measurements using microarrays with PS.

*Significantly better accuracy than expected from the computed confidence (χ^2 , $P < 0.05$).

[#]Unable to detect difference between accuracy and computed confidence (χ^2 , $P > 0.05$).

Table IV Blastomere ploidy states

Category	Proportion
Euploid	64/134
Aneuploid	43/134
Complex aneuploid	27/134
Maternal only	42/134*
Paternal only	8/134
Meiotic trisomy	19/134*
Segmental error	16/134

Proportion of 134 blastomeres falling in various ploidy categories. *Significantly more maternal errors than paternal errors (χ^2 , $P < 0.0001$).

were simple aneuploid (one or two non-disomic chromosomes) and 20.1% of the cells were complex aneuploid (more than two non-disomic chromosomes). We found no significant difference between the numbers of male (XY) and female (XX) blastomeres (χ^2 , $P = 0.66$), as expected assuming random segregation of chromosomes during meiosis. When the data are examined per embryo, 23.1% of embryos were euploid (all blastomeres euploid) and 46.2% of embryos were mosaic euploid (at least one euploid blastomere; Table V). Mosaicism was common in the embryos, with 57.7% of embryos showing some discordance between at least two blastomeres. Three of the embryos (11.5%) were chaotic aneuploid, i.e. all blastomeres were aneuploid and no two blastomeres had matching ploidy. Segmental imbalances occurred in 11.9% of the blastomeres and 38.5% of the embryos. Though segmental imbalances were common among blastomeres and embryos, they were relatively rare among chromosomes, accounting for only 0.7% of all chromosomes measured. The difference between the per-embryo segmental imbalance in our cohort and a prior report is not significantly more than expected by chance (Vanneste et al., 2009) (χ^2 , $P = 0.18$). Finally, euploid embryos did not have better morphology than chaotic aneuploid embryos (one-sided t-test, $P = 0.43$), suggesting that cleavage-stage morphology does not predict underlying genetic state. This finding corresponds to at least one prior report (Munné et al.,

Table V Embryo ploidy states

Category	Proportion	Embryo grade
Euploid	6/26	1.5 [#]
Mosaic euploid	12/26	1.9
Chaotic aneuploid	3/26	1.7
Paternal meiotic aneuploid	1/26	3
Maternal meiotic aneuploid	8/26*	2.1
Segmental error	10/26	1.4

Proportion of 26 embryos falling in various ploidy categories, and average embryo grade within each category.

*Significantly more maternal errors than paternal errors (χ^2 , $P < 0.0001$).

[#]Unable to detect significant difference from the distribution of all embryos combined (t-test, $P > 0.05$).

2004) but contradicts at least one other report (Magli et al., 2007), suggesting the need for further research. However, our cohort of embryos was small and of consistently high quality, so a larger cohort with more variable embryo quality might yield different results.

Chromosome copy errors occurred on all chromosomes, highlighting the utility of screening for errors on all 24 chromosomes. Though embryos and blastomeres showed high rates of aneuploidy, most chromosomes (83.9%) were disomic (Table VI). Monosomies were more common than trisomies (χ^2 , $P < 0.0001$), concordant with larger studies using FISH (Munné et al., 2004; Thornhill and Handyside, 2009). All chromosomes showed at least one monosomy or trisomy in at least one blastomere, demonstrating that no chromosome escapes copy number errors in human cleavage-stage embryos. We found no per-chromosome bias in copy number errors, i.e. no chromosome had a particular propensity for aneuploidy. This contradicts some prior reports that aneuploidy occurs more frequently on certain chromosomes (Munné et al., 2004, 2007). We found no chromosomes with uniparental disomy, which is not significantly different than expected given a prior report (Vanneste et al., 2009; χ^2 , $P = 0.42$). Notably, if applied to these embryos, FISH for Chromosomes 1, 13, 16, 17, 18, 21, X and Y (e.g. Mastenbroek et al., 2007) would have failed to detect 27.2% of all aneuploid blastomeres.

Table VI Per-chromosome blastomere copy number state

Blastomere copy number state		Disomy	XX	XY	(-mat)mit	(-pat)mit	(-mat)mei	(-pat)mei	(+mat)mit	(+pat)mit	(+mat)mei	X, (-pat)mit	X, (-mat)mit	Y	XXY, (+mat)mit	XXY, (+mat)mei	XXX, (+mat)mit	(-mat), (-pat)	Segmental imbalance
Chromosome	1	121			1	4			1		4							3	3
	2	117			3	7			2	1	2							2	
	3	120			5	5			2		2								1
	4	119			6	4			3		2								
	5	116			5	5			3	1	2							2	6
	6	118			5	4			2	1	2							2	
	7	118			4	7			2	1	2								3
	8	120			4	4			2	1	2							1	
	9	119			3	4			3		2							3	2
	10	120			4	5			2		2							1	
	11	119			4	5			2	1	2							1	1
	12	112			4	5			3		8							2	
	13	119			1	2			4		2							4	
	14	116			4	7		2	1		2								
	15	119			4	4			1	3	2							1	2
	16	111			7	2			2		8							4	
	17	119			4	4			3		2							2	
	18	109			6	5	4		5		5								2
	19	121			4	4			1		2							2	
	20	121			4	3			2	1	2							1	1
	21	119			4	2			4		2							3	
	22	113			4	5	8		1		2							1	
sex			69	52								1	1	2	2	2	2	3	
Sum	2586	69 [#]	52 [#]	90	97	12*	2	51	14	61*		1	1	2	2	2	2	38	21

Number of chromosome calls falling into various copy number states. The disomy column indicates disomic chromosomes measurements, and XX and XY indicate disomic female and male measurements, respectively. The (-pat)mit and the (-mat)mit columns indicate maternal and paternal mitotic monosomy, respectively. The Y column indicates a monosomy on the sex chromosomes. The XXY, (+mat)mit column indicates an additional mitotic X chromosome from the mother, the XXY, (+mat)mei column indicates an additional meiotic X chromosome from the mother. The XXX, (+mat)mit column indicates maternal mitotic trisomy X. The (+mat)mit and (+pat)mit columns indicate maternal and paternal mitotic trisomies, respectively. The (-mat)mei and (-pat)mei columns indicate maternal and paternal meiotic monosomies, respectively. The (+mat)mei column indicates maternal meiotic trisomy. The (-mat), (-pat) column indicates a null chromosome measurement. Finally, the segmented column indicates a chromosome that showed segmental errors.

Additionally, if FISH for 8-chromosomes had been used, 22.9% of the blastomeres diagnosed as 'euploid' would have actually been aneuploid. Thus, clinical application of 8-chromosome FISH to our cohort of embryos would have resulted in the transfer of some aneuploid embryos.

Meiotic errors are predictive of fully aneuploid embryos

Aneuploidies arise from mitotic or meiotic non-disjunction (Los et al., 1998, 2004; Mantzouratou et al., 2007; Frumkin et al., 2008). Parental source of aneuploidy can be a useful proxy for the detection of meiotic errors, because maternal meiotic errors are expected to predominate over paternal meiotic errors (Fragouli et al., 2006a, b; Frumkin et al., 2008). Across the 134 blastomeres in this study, significantly more trisomies (84.8%) were maternal in origin than were paternal in origin (Table VI; χ^2 , $P < 0.0001$). This is despite the fact that there was no significant difference between the numbers of maternal and paternal monosomies across the 134 blastomeres (χ^2 , $P = 0.96$). This skewed distribution suggests that most monosomies are mitotic, whereas most trisomies are maternal meiotic.

However, better conclusions can be drawn regarding mechanism of non-disjunction if individual monosomies and trisomies are specifically diagnosed as mitotic or meiotic. Thus, we identified meiotic trisomies by looking for three distinct parental haplotypes for a particular chromosome, and meiotic monosomies by looking for monosomy concordant in the same chromosome across all blastomeres in an embryo. Across our data set, 30.8% of embryos harbored meiotic aneuploidy and 14.2% of blastomeres harbored meiotic trisomies. However, a high frequency of maternal meiotic errors alone did not explain the dominance of maternal trisomies, as the incidences of mitotic and meiotic trisomies were roughly equivalent (χ^2 , $P = 0.99$). We postulate that this unexpected result is due to the failure of our algorithm to accurately distinguish certain meiotic errors from mitotic errors, since the method is dependent on computational phasing, which requires meiotic crossovers to produce highly rearranged chromosomes. The difference between counts of maternal and paternal meiotic monosomies was not significantly greater than expected by chance (χ^2 , $P = 0.59$). Notably, however, significantly more meiotic trisomies were maternal than paternal, as zero paternal meiotic trisomies were detected (χ^2 , $P < 0.0001$). Thus, our data set suggests that, though mitotic and meiotic errors were both common in this set of human cleavage-stage embryos, most meiotic trisomies were maternal in origin.

If necessary, a clinician might consider transfer of a mosaic euploid embryo instead of a fully aneuploid embryo. Any evidence that suggests a fully aneuploid embryo could help inform transfer decisions. In our study, 79.0% of blastomeres that contained meiotic trisomies belonged to embryos that were fully aneuploid, i.e. all of the blastomeres in the embryo were aneuploid. In contrast, only 30.4% of blastomeres that had only mitotic trisomies belonged to embryos that were fully aneuploid. Thus, blastomeres with meiotic trisomies had a significantly higher probability of belonging to a fully aneuploid embryo (χ^2 , $P = 0.005$). In summary, our analysis of aneuploidy mechanisms demonstrates that most trisomies are maternal meiotic errors, and that the presence of maternal meiotic errors in a single blastomere is positively predictive of a fully aneuploid embryo.

Discussion

We have performed a large preclinical validation study to determine the accuracy of a new microarray method for molecular karyotyping. With over 200 cells measured by metaphase karyotype and 459 cells measured by the PS microarray method, we found that the per-cell accuracies of the methods are roughly in line. However, any validation of a method for single-cell karyotyping suffers from possible karyotypic mosaicism inherent to tissues. Thus, the true accuracy of both traditional metaphase karyotyping and the new microarray method might be even higher than reported here, because many of the 'errors' may reflect accurate measurements on a mosaic population of cells.

Confidence is essentially the probability that the copy number call is correct, i.e. a 90% confidence means that 9 out of 10 calls will be correct. Medical practitioners use the confidence of a particular chromosome call to help make informed clinical decisions, i.e. if two blastomere measurements are euploid, but one has lower confidence, it should be preferable to transfer the embryo with higher confidence. Our preclinical validation data set on 459 cells of known karyotype indicated that confidences were conservative, with accuracy rates generally exceeding confidences.

The PS microarray method also achieved high rates of accuracy on single sperm. As shown in our separate analysis of these same data, intensity-only algorithms necessarily make assumptions regarding the underlying ploidy state of the cell, i.e. haploid, diploid or triploid. In the case of sperm measurements, the proper ploidy using the mean chromosome intensity was actually a haploid, but the PIM algorithm was not able to distinguish this from diploid mean chromosome intensity. Similar problems would presumably occur with triploid genome measurements. Here, parental information has proven useful to overcome this challenge.

We applied PS to a cohort of 26 early-cleavage-stage (Day 3) embryos for initial validation and to gain a better understanding of embryo mosaicism and aneuploidy rates when all 24 chromosomes are tested. Our results indicate that 23.1% of embryos were euploid and 57.7% were mosaic. Studies using FISH reported mosaicism rates of 18.1–57% and euploidy rates of 19–36% (Bielanska et al., 2005; Baart et al., 2004, 2007; Munné et al., 2007; Barbash-Hazan et al., 2008). Prior studies using alternative 24-chromosome screening methods indicated whole-chromosome imbalances in at least one blastomere in 75–83% of embryos, and mosaicism rates of 66–87% (Wells and Delhanty, 2000; Vanneste et al., 2009). Rates of per-embryo segmental chromosome imbalances were also not significantly different from prior reports (Vanneste et al., 2009; χ^2 , $P > 0.05$). Thus, proportions of chromosome imbalances and mosaicism in our present study are consistent with expectations based on prior data and add to the increasing body of evidence demonstrating the extreme karyotypic instability of human embryos generated from oocytes collected after artificial stimulation and then cultured *in vitro*.

We were able to determine whether aneuploidies were meiotic or mitotic, and the parental source of aneuploidy, without data from polar body biopsies. These data are evidence that aneuploidies commonly arise due to both zygotic and meiotic non-disjunction. However, monosomies are mostly zygotic (i.e. anaphase lag; Kalousek et al., 1991) and trisomies are mostly maternal meiotic. In concordance with our data on maternal meiotic errors, prior data suggest

that 12–41.8% of human oocytes are aneuploid (Kuliev *et al.*, 2003; Pellestor *et al.*, 2003; Kuliev and Verlinsky, 2004; Frumkin *et al.*, 2008), and that ~80% of children born with aneuploidy harbor maternal trisomies (Nicolaidis and Peterson, 1998). At least one study measured aneuploidy in polar bodies using CGH and found that oocytes confer a higher risk of trisomy than monosomy (Keskin-tepe *et al.*, 2007), whereas other studies have suggested that fertilized oocytes are equally at risk for monosomy and trisomy (Fragouli *et al.*, 2006a, b). Measurements should be made on larger sets of embryos in order to further clarify the issue. Regardless, one might speculate that ovarian stimulation is disruptive to meiosis, or that *in vitro* culture is disruptive to zygotic mitosis. In the future, IVF clinicians may be able to adjust stimulation protocols and *in vitro* culture conditions to reduce the possibility of certain types of aneuploidy during IVF.

Paradoxically, high rates of aneuploidy and chromosomal imbalances suggest the importance of PGS in selecting healthy embryos, whereas mosaicism complicates the meaningfulness of the diagnosis delivered (e.g. Vanneste *et al.*, 2009). Thus, information that helps to predict the ploidy state of the remainder of an embryo given single-blastomere measurements could be a powerful addition to PGS. There is already some evidence that mosaic euploid embryos are more likely to ‘self-correct’ by blastocyst stage (Baart *et al.*, 2004; Munné *et al.*, 2005; Barbash-Hazan *et al.*, 2008). Several mechanisms have been proposed to explain the self-correction phenomenon such as chromosome demolition (Los *et al.*, 1998), anaphase-lag correction (Kalousek *et al.*, 1991) or non-disjunction (Tarin *et al.*, 1992). Self-correction of a mosaic embryo might also occur as a result of aneuploid cells undergoing apoptosis and/or better proliferative capacity of euploid cells (Lightfoot *et al.*, 2006). We are currently conducting studies to further examine the relationship between meiotic aneuploidy at the cleavage stage and the potential to form a euploid blastocyst, and may, in the future, add this information to clinical PGS reports.

Recent, randomized, controlled studies have indicated that PGS does not improve implantation rates or live birth rates in IVF (Staessen *et al.*, 2004, 2008; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Meyer *et al.*, 2008; Debrock *et al.*, 2009). This might be because these studies used first-generation PGS technology that does not screen all 24 chromosomes, and therefore misses many aneuploid blastomeres (Wilton *et al.*, 2003; Keskin-tepe *et al.*, 2007). In the present data set, we found that 8-chromosome FISH would have had a high false-negative rate because many blastomeres had aneuploidies on chromosomes not typically tested in FISH. Therefore, it is likely that nearly one-quarter of all ‘euploid’ embryos transferred in prior randomized, controlled clinical studies were actually aneuploid. Additionally, the FISH studies have unknown false-positive rates, i.e. euploid cells might be diagnosed aneuploid due to poor technique (Cohen and Grifo, 2007). Future studies that use more comprehensive technology with known accuracy rates might therefore significantly improve both implantation rates and live birth rates.

The microarray technology described here has been used since October 2008 in PGS clinical practice by IVF clinics for a variety of clinical indications using both cleavage-stage and blastocyst biopsies. Given the broad applicability of the PS microarray technology, the method can be easily extended to other areas where DNA material is limited, such as non-invasive prenatal diagnostics, studies on early embryonic stem cell passages and early tumor detection.

Supplementary data

Supplementary data are available at humrep.oxfordjournals.org.

Acknowledgements

We would like to thank the staff at Boston IVF and Huntington Reproductive Center for their kind assistance with clinical sample collection. Professor Ron W. Davis of the Stanford University Department of Biochemistry kindly provided materials and advice on molecular biology. Thanks to Drs Joe Leigh Simpson, Keith Blauer and Athena Cherry, as well as two anonymous reviewers, for their critical reviews of the manuscript.

Funding

This work was partially funded by grant 2R44HD054958 from the United States National Institute of Child Health and Human Development (NICHD). The Swedish Society for Medical Research provided postdoctoral funding to J.B.

References

- Baart EB, Van Opstal D, Los FJ, Fauser BC, Martini E. Fluorescence in situ hybridization analysis of two blastomeres from day 3 frozen-thawed embryos followed by analysis of the remaining embryo on day 5. *Hum Reprod* 2004;**19**:685–693.
- Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, Van Opstal D. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod* 2006;**21**:223–233.
- Baart EB, van den Berg I, Martini E, Eussen HJ, Fauser BC, Van Opstal D. FISH analysis of 15 chromosomes in human day 4 and day 5 preimplantation embryos: the added value of extended aneuploidy detection. *Prenat Diagn* 2007;**27**:55–63.
- Baltaci V, Satioglu H, Kabukcu C, Unsal E, Aydinuraz B, Uner O, Aktas Y, Cetinkaya E, Turhan F, Aktan A. Relationship between embryo quality and aneuploidies. *Reprod BioMed Online* 2006;**12**:77–82.
- Barbash-Hazan S, Frumkin T, Malcov M, Yaron Y, Cohen T, Azem F, Amit A, Ben-Yosef D. Preimplantation aneuploid embryos undergo self-correction in correlation with their developmental potential. *Fertil Steril* 2008;**92**:890–896.
- Bielanska M, Jin S, Bernier M, Tan SL, Ao A. Diploid–aneuploid mosaicism in human embryos cultured to the blastocyst stage. *Fertil Steril* 2005;**84**:336–342.
- Borini A, Lagalla C, Cattoli M, Sereni E, Sciajno R, Flamigni C, Coticchio G. Predictive factors for embryo implantation potential. *Reprod BioMed Online* 2005;**10**:653–668.
- Centers for Disease Control and Prevention, American Society for Reproductive Medicine. *2006 Assisted Reproductive Technology Success Rates: National Summary and Fertility Clinic Reports*. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, 2008.
- Cohen J, Grifo JA. Multicentre trial of preimplantation genetic screening reported in the New England Journal of Medicine: an in-depth look at the findings. *Reprod BioMed Online* 2007;**15**:365–366.
- Colls P, Escudero T, Cekleniak N, Sadowy S, Cohen J, Munné S. Increased efficiency of preimplantation genetic diagnosis for infertility using “no result rescue”. *Fertil Steril* 2007;**88**:53–61.

- Daphnis DD, Fragouli E, Economou K, Jerkovic S, Craft IL, Delhanty JD, Harper JC. Analysis of the evolution of chromosome abnormalities in human embryos from Day 3 to 5 using CGH and FISH. *Mol Hum Reprod* 2008;**14**:117–125.
- Debrock S, Melotte C, Spiessens C, Peeraer K, Vanneste E, Meeuwis L, Meuleman C, Frijns JP, Vermeesch JR, D'Hooghe TM. Preimplantation genetic screening for aneuploidy of embryos after in vitro fertilization in women aged at least 35 years: a prospective randomized trial. *Fertil Steril*. 2009; epub ahead of print.
- Delhanty JD, Griffin DK, Handyside AH, Harper J, Atkinson GH, Pieters MH, Winston RM. Detection of aneuploidy and chromosomal mosaicism during preimplantation sex determination by FISH. *Hum Genet* 1993;**2**:1183–1185.
- Findlay I, Ray P, Quirke P, Rutherford A, Lilford R. Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation genetic diagnosis of sex and cystic fibrosis. *Hum Reprod* 1995;**10**:1609–1618.
- Fragouli E, Wells D, Whalley KM, Mills JA, Faed MJ, Delhanty JD. Increased susceptibility to maternal aneuploidy demonstrated by comparative genomic hybridization analysis of human MII oocytes and first polar bodies. *Cytogenet Genome Res* 2006a;**114**:30–38.
- Fragouli E, Wells D, Thornhill A, Serhal P, Faed MJ, Harper JC, Delhanty JD. Comparative genomic hybridization analysis of human oocytes and polar bodies. *Hum Reprod* 2006b;**21**:2319–2328.
- Frumkin T, Malcov M, Yaron Y, Ben-Yosef D. Elucidating the origin of chromosomal aberrations in IVF embryos by preimplantation genetic analysis. *Mol Cell Endocrinol* 2008;**282**:112–119.
- Gianaroli L, Magli MC, Ferraretti AP, Munné S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro fertilization with a poor prognosis: identification of the categories for which it should be proposed. *Fertil Steril* 1999;**72**:837–844.
- Glentis S, SenGupta S, Thornhill A, Wang R, Craft I, Harper JC. Molecular comparison of single cell MDA products derived from different cell types. *Reprod Biomed Online* 2009;**19**:89–98.
- Handyside AH, Robinson MD, Simpson RJ, Omar MB, Shaw MA, Grudzinskas JG, Rutherford A. Isothermal whole genome amplification from single cells and small numbers of cells: a new era for preimplantation genetic diagnosis of inherited disease. *Mol Hum Reprod* 2004;**10**:767–772.
- Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara NA, Shaw MA, Griffin DK. Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet* 2009; [Epub ahead of print 25 October].
- Hardarson T, Hanson C, Sjogren A, Lundin K. Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Hum Reprod* 2001;**16**:313–318.
- Hardarson T, Hanson C, Lundin K, Hillensjö T, Nilsson L, Stevic J, Reismer E, Borg K, Wikland M, Bergh C. Preimplantation genetic screening in women of advanced maternal age caused a decrease in clinical pregnancy rate: a randomized controlled trial. *Hum Reprod* 2008;**23**:2806–2812.
- Hellani A, Abu-Amro K, Azouri J, El-Akoum S. Successful pregnancies after application of array-comparative genomic hybridization in PGS-aneuploidy screening. *Reprod Biomed Online* 2008;**17**:841–847.
- Hu DG, Webb G, Hussey N. Aneuploidy detection in single cells using DNA array-based comparative genomic hybridization. *Mol Hum Reprod* 2004;**10**:283–289.
- Johnson DS, Rabinowitz M, Banjevic M, Singer J, Cinnioglu C, Baner J, Keller J, Sheena J, Behr B. Leveraging parental genotypes to increase confidence in genotype calls on single cells. *Hum Reprod* 2008;**23**(Suppl. 1):i67.
- Kalousek DK, Howard-Peebles PN, Olsen SB. Confirmation of CVS mosaicism in term placentae and high frequency of intrauterine growth retardation association with confined placental mosaicism. *Prenat Diagn* 1991;**11**:743–750.
- Keskintepe L, Sher G, Keskintepe M. Reproductive oocyte/embryo genetic analysis: comparison between fluorescence in-situ hybridization and comparative genomic hybridization. *Reprod Biomed Online* 2007;**15**:303–309.
- Kuliev A, Cieslak J, Ilkevitch Y, Verlinsky Y. Chromosomal abnormalities in a series of 6733 human oocytes in preimplantation diagnosis for age-related aneuploidies. *Reprod BioMed Online* 2003;**6**:54–59.
- Kuliev A, Verlinsky Y. Meiotic and mitotic nondisjunction: lessons from preimplantation genetic diagnosis. *Hum Reprod Update* 2004;**10**(5):401–407.
- Le Caignec C, Spits C, Sermon K, De Rycke M, Thienpont B, Debrock S, Staessen C, Moreau Y, Fryns JP, Van Steirteghem A et al. Single-cell chromosomal imbalances detected by array CGH. *Nucleic Acids Res* 2006;**34**:e68.
- Lightfoot DA, Kouznetsova A, Mahdy E, Wilbertz J, Höög C. The fate of mosaic aneuploid embryos during mouse development. *Dev Biol* 2006;**289**:384–394.
- Los FJ, van Opstal D, van den Berg C, Braat AP, Verhoef S, Wesby-van Swaay E, van den Ouweland AM, Halley DJ. Uniparental disomy with and without confined placental mosaicism: a model for trisomic zygote rescue. *Prenat Diagn* 1998;**18**:659–668.
- Los FJ, Van Opstal D, van den Berg C. The development of cytogenetically normal, abnormal and mosaic embryos: a theoretical model. *Hum Reprod Update* 2004;**10**:79–94.
- Magli MC, Gianaroli L, Ferraretti AP, Lappi M, Ruberti A, Farfalli V. Embryo morphology and development are dependent on chromosomal complement. *Fertil Steril* 2007;**87**:534–541.
- Mantzouratou A, Mania A, Fragouli E, Xanthopoulou L, Tashkandi S, Fordham K, Ranieri DM, Doshi A, Nuttall S, Harper JC et al. Variable aneuploidy mechanisms in embryos from couples with poor reproductive histories undergoing preimplantation genetic screening. *Hum Reprod* 2007;**22**:1844–1853.
- Masterbroek S, Twisk M, van Echten-Arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, Vogel NE, Arts EG, de Vries JW, Bossuyt PM et al. In vitro fertilization with preimplantation genetic screening. *N Engl J Med*. 2007;**357**:9–17.
- Meyer LR, Klipstein S, Hazlett WD, Nasta T, Mangan P, Karande VC. A prospective randomized controlled trial of preimplantation genetic screening in the “good prognosis” patient. *Fertil Steril* 2008;**91**:1731–1738.
- Munné S, Lee A, Rosenwaks Z, Grifo J, Cohen J. Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum Reprod* 1993;**8**:2185–2191.
- Munné S, Magli C, Bahçe M. Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births: XY, 13, 14, 15, 16, 18, 21, 22. *Prenat Diagn* 1998;**18**:1459–1466.
- Munné S. Preimplantation genetic diagnosis and human implantation—a review. *Placenta* 2003;**24**(Suppl. B):S70–S76.
- Munné S, Sandalinas M, Escudero T, Velilla E, Walmsley R, Sadowy S, Cohen J, Sable D. Improved implantation after preimplantation genetic diagnosis of aneuploidy. *Reprod BioMed Online* 2003;**7**:91–97.
- Munné S, Bahçe M, Sandalinas M, Escudero T, Márquez C, Velilla E, Colls P, Oter M, Alikani M, Cohen J. Differences in chromosome susceptibility to aneuploidy and survival to first trimester. *Reprod BioMed Online* 2004;**8**:81–90.
- Munné S, Velilla E, Colls P, Garcia Bermudez M, Vemuri MC, Steuerwald N, Garrisi J, Cohen J. Self-correction of chromosomally

- abnormal embryos in culture and implications for stem cell production. *Fertil Steril* 2005;**84**:1328–1334.
- Munné S, Chen S, Colls P, Garrisi J, Zheng X, Cekleniak N, Lenzi M, Hughes P, Fischer J, Garrisi M, Tomkin G, Cohen J. Maternal age, morphology, development, and chromosome abnormalities in over 6000 cleavage-stage embryos. *Reprod BioMed Online* 2007;**14**:628–634.
- Nicolaidis P, Peterson MB. Origin and mechanisms of non-disjunction in human autosomal trisomies. *Hum Reprod* 1998;**13**:313–319.
- Nomura M, Iwase A, Furui K, Kitagawa T, Matsui Y, Yoshikawa M, Kikkawa F. Preferable correlation to blastocyst development and pregnancy rates with a new embryo grading system specific for day 3 embryos. *J Assist Reprod Genet* 2007;**24**:23–28.
- Pellicer A, Rubio C, Vidal F, Mínguez Y, Giménez C, Egozcue J, Remohí J, Simón C. In vitro fertilization plus preimplantation genetic diagnosis in patients with recurrent miscarriage: an analysis of chromosome abnormalities in human preimplantation embryos. *Fertil Steril* 1999;**71**:1033–1039.
- Pellestor F, Andréo B, Arnal F, Humeau C, Demaille J. Maternal ageing and chromosomal abnormalities: new data drawn from in vitro unfertilized human oocytes. *Hum Genet* 2003;**112**:195–203.
- Piyamongkol W, Bermudez MG, Harper JC, Wells D. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. *Mol Hum Reprod* 2003;**9**:411–420.
- Rabinowitz M, Sweetkind-Singer J, Banjevic M, Johnson DS, Kijacic D, Petrov D, Xu J, Demko Z. System and method for cleaning noisy genetic data from target individuals using genetic data from genetically related individuals, 2007; U.S. Patent 2007018467A1, 9 August 2007.
- Rabinowitz M, Banjevic M, Demko ZP, Johnson DS. System and method for cleaning noisy genetic data and determining chromosome copy number. World Intellectual Property Organization, 2008. WO2008/115497, 25 September 2008.
- Renwick PJ, Trussler J, Ostad-Saffari E, Fassihi H, Black C, Braude P, Ogilvie CM, Abbs S. Proof of principle and first cases using preimplantation genetic haplotyping—a paradigm shift for embryo diagnosis. *Reprod Biomed Online* 2006;**13**:110–119.
- Sarosi C, Schwartz LB, Lublin J. Chromosomal analysis of early fetal losses in relation to transvaginal ultrasonographic detection of fetal heart motion after infertility. *Fertil Steril* 1998;**69**:274–277.
- Sermon K, Van Steirteghem A, Liebaers I. Preimplantation genetic diagnosis. *Lancet* 2004;**363**:1633–1641.
- Sher G, Keskinetepe L, Keskinetepe M, Ginsburg M, Maassarani G, Yakut T, Baltaci V, Kotze D, Unsal E. Oocyte karyotyping by comparative genomic hybridization provides a highly reliable method for selecting “competent” embryos, markedly improving in vitro fertilization outcome: a multiphase study. *Fertil Steril* 2007;**85**:1033–1040.
- Society for Assisted Reproductive Technology, the American Society for Reproductive Medicine. Assisted reproductive technology in the United States: 2000 results generated from the American Society for Reproductive Medicine/Society for Reproductive Technology registry. *Fertil Steril* 2004;**81**:1207–1220.
- Spits C, Le Caignec C, De Rycke M, Van Haute L, Van Steirteghem A, Liebaers I, Sermon K. Whole-genome multiple displacement amplification from single cells. *Nat Protoc* 2006;**1**:1965–1970.
- Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M, Devroey P, Liebaers I, Van Steirteghem A. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. *Hum Reprod* 2004;**19**:2849–2858.
- Staessen C, Verpoest W, Donoso P, Haentjens P, Van der Elst J, Liebaers I, Devroey P. Preimplantation genetic screening does not improve delivery rate in women under the age of 36 following single-embryo transfer. *Hum Reprod* 2008;**23**:2818–2825.
- Tarin JJ, Conaghan J, Winston RML. Human embryo biopsy on the 2nd day after insemination for preimplantation diagnosis: removal of a quarter of embryo retards cleavage. *Fertil Steril* 1992;**5**:970–976.
- Thornhill AR, deDie-Smulders CE, Geraedts JP, Harper JC, Harton GL, Lavery SA, Moutou C, Robinson MD, Schmutzler AG, Scriven PN et al. ESHRE PGD Consortium ‘Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)’. *Hum Reprod* 2005;**20**:35–48.
- Thornhill AR, Handyside AH. The role of aneuploidy screening in human preimplantation embryos. *Biennial Rev Infertil* 2009;**1**:277–288.
- Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F et al. Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009;**15**:577–583.
- Verlinsky Y, Tur-Kaspa I, Cieslak J, Bernal A, Morris R, Taranissi M, Kaplan B, Kuliev A. Preimplantation testing for chromosomal disorders improves reproductive outcome of poor-prognosis patients. *Reprod BioMed Online* 2005;**11**:219–225.
- Vidal F, Giménez C, Rubio C, Simón C, Pellicer A, Santaló J, Egozcue J. FISH preimplantation diagnosis of chromosome aneuploidy in recurrent pregnancy wastage. *J Assist Reprod Genet* 1998;**15**:310–313.
- Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet* 2000;**106**:210–217.
- Walsh SP, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: mechanisms and solutions. *PCR Methods Appl* 1992;**2**:241–250.
- Warburton D, Kline J, Stein Z. Cytogenetic abnormalities in spontaneous abortions of recognized conceptions. *Perinatal Genetics: Diagnosis and Treatment*. New York: Academic Press, 1986.
- Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod* 2000;**6**:1055–1062.
- Wells D, Alfarawati S, Fragouli E. Use of comprehensive chromosomal screening for embryo assessment: microarrays and CGH. *Mol Hum Reprod* 2008;**14**:703–710.
- Wilton L, Voullaire L, Sargeant P, Williamson R, McBain J. Preimplantation aneuploidy screening using comparative genomic hybridization or in situ hybridization of embryos from patients with recurrent implantation failure. *Fertil Steril* 2003;**80**:860–868.

Submitted on August 24, 2008; resubmitted on November 19, 2009; accepted on November 23, 2009