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Preimplantation Genetic Diagnosis for Monogenic Disorders and Chromosomal Rearrangements – The German Perspective

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Preimplantation Genetic Diagnosis for Monogenic Disorders and Chromosomal Rearrangements – The German Perspective

U. Koehler, U. Schoen, V. Mayer, E. Holinski-Feder

Since its dawn in the late 1980s, preimplantation genetic diagnosis (PGD, or Präimplantationsdiagnostik, PID) has evolved into a well-established technique, which can be offered to couples at risk of transmitting a mutation or a chromosomal aberration to their offspring. Polar bodies as well as day 3 blastomeres and day 5 blastocysts (trophectoderm) can be employed for the detection of a specific gene mutation or unbalanced karyotypes. For the latter, array comparative genomic hybridisation (array CGH) has replaced fluorescence in situ hybridisation (FISH) approaches. Furthermore, as blastocysts seem to exhibit less mosaicism compared to blastomeres, current PGD protocols focus on the analysis of blastocysts, however polar body testing is still applied for maternally derived conditions. In November 2011, the German embryo protection law (ESchG) has been supplemented by §3a, which defines the conditions for the legal implementation of PGD (PräimpG) in Germany. **J Reprodukionsmed Endokrinol 2013; 10 (Special Issue 1): 38–44.**

Key words: reimplantation genetic diagnosis, PGD, polar body diagnosis, blastocyst, trophectoderm biopsy, monogenic disease, reciprocal translocation, Robertsonian translocation, mosaicism, polymerase chain reaction (PCR), array CGH

■ Introduction

Preimplantation genetic diagnosis is the genetic analysis of polar bodies (PB), blastomeres or blastocysts before transferring the embryo into the uterus within an in-vitro-fertilisation (IVF) cycle and intracytoplasmic sperm injection (ICSI). Different PGD approaches are applied depending on legal regulations and technical preferences throughout reproductive and genetic centers worldwide (see also <http://www.drze.de/in-focus/preimplantation-genetic-diagnosis/modules/legal-regulation-of-pgd>). In the majority of countries, PGD is applied primarily to polar bodies and totipotent blastomeres (day 3 cleavage stage embryos), however, a switch to pluripotent trophectoderm (TE) samples from blastocysts (day 5) can be observed, as these cells are considered to yield more reliable results. The legal situation in Germany is ambiguous even though a “Präimplantationsdiagnostikgesetz” (PräimpG) [1] was published in November 2011 as a supplement of the Embryo Protection Act (ESchG) [2]. In contrast to other countries, preimplantation genetic diagnosis of totipotent day 3 blastomeres remains prohibited in Germany, whereas genetic testing of pluripotent trophectoderm samples after biopsy of day 5 blastocysts will be in accordance with the PräimpG. However, guidelines regulating the conditions under which a PGD

can be performed in licensed centers after genetic and social counselling and a positive vote from a clinical ethics committee, is still lacking as of May 2012.

A prerequisite for PGD is an intracytoplasmic sperm injection within the setting of an assisted reproductive technology (ART). Sir **Robert Edwards**, a pioneer in the field, settled the foundation for ART in the 1960s and 1970s. PGD then was first performed in 1989, when Handyside et al. [3] successfully determined the sex of an embryo in couples at risk for recessive X-linked diseases; this was followed by single cell diagnosis for the monogenic disease cystic fibrosis [4]. First pregnancies after PGD were reported by Handyside et al. in 1990 [5]. Since then, PGD has evolved rapidly and is applied for more than 200 single gene disorders and chromosomal abnormalities [6]. Furthermore, applying improved molecular genetic techniques, PGD protocols for every gene causing a monogenic inherited disease are imaginable.

■ Indications for PGD

PGD is in accordance with German law, when (1.) a genetic condition in the female or the male partner can lead to a severe disease in the offspring or (2.) when a severe genetic impairment of the embryo which leads to a miscarriage can be avoided. The underlying genetic aber-

ration can be a single gene mutation or a chromosome rearrangement (translocation, inversion). Furthermore, employing array CGH, embryos carrying de novo chromosome aneuploidies can be detected, thus preventing pregnancies which would lead to a miscarriage.

■ Sample Options

Maternally derived gene mutations or chromosomal translocations can be analysed through preimplantation genetic diagnosis of polar bodies [7], blastomeres [8], or blastocysts [9], whereas paternally derived genetic conditions can only be detected in blastomeres or blastocysts. Polar bodies I and II (PB I, PB II) are haploid cells, which can be aspirated from the oocyte. Blastomeres are diploid totipotent embryonic cells (day 3 cleavage stage embryo). Blastocysts are day 5–6 embryos after the inner cell mass (ICM, embryonic cells) has separated from the trophectoderm (trophoblast cells).

■ Polar Body Biopsy

Polar bodies accumulate during meiosis as by-products of the mature oocyte. Polar body I is present before fertilisation as a product of meiosis I, whereas polar body II forms after fertilisation (meiosis II). PB I reflects the genetic configuration of the oocyte in a mirror

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image fashion, whereas the genome of PB II is identical to the oocyte, provided that no genetic recombination occurred during the first meiotic division. Thus, analyzing the polar bodies can detect mutations and chromosomal aberrations present in the oocyte [7]. The disadvantage of polar body analysis is that it provides an insight only into the maternal genome, which in the case of a recessive disease can lead to the rejection of heterozygously mutated oocytes which may otherwise have developed into healthy embryos. Additionally, no prospective rating of the blastocyst development can be made at the time of polar body diagnosis.

■ Blastomere Biopsy

Blastomeres are embryonic cells of cleavage stage embryos. A biopsy at day three after fertilisation removes one (or two cells) from the totipotent 8 cell embryo. The biopsy of two blastomeres in the early days of PGD revealed an inappropriate decrease in viability of the embryo, so that current approaches employ the biopsy of a single blastomere. These samples still are the preferred ones for PGD in the majority of genetic centers, even though they exhibit a higher rate of chromosomal instability compared to blastocysts [10, 11]. Therefore, a change in PGD protocols to a trophectoderm biopsy of blastocysts is about to occur.

■ Blastocyst Biopsy

At day 5 after fertilisation, the blastocyst has differentiated into the embryoblast (inner cell mass, ICM) and the trophoblast (trophectoderm, TE). Trophoblast cells are no longer totipotent, but pluripotent. After implantation into the uterus, the trophoblast cells constitute the chorionic villi and the placenta, whereas the fetus evolves from the embryoblast. Trophoblast cells are genetically identical to the genome of the embryoblast and thus represent an ideal sample with which to test the embryonic genome without affecting the embryonic cells themselves. Schoolcraft et al. could show that analysis of day 5 blastocysts is as reliable as analysis of day 3 cleavage stage embryos [12]. Compared to day 3 blastomeres, blastocysts tend to present more homogeneous karyotypes and a less mosaicism. Although PGD following blastocyst biopsy most probably re-

veals less aneuploidies due to the self-selection processes in the embryonic cells [13], it has yet to be shown in a larger cohort of samples that mosaicism in trophoblast cells and the embryoblast does not lead to misdiagnoses; Johnson et al. showed a high concordance of PGD results between the trophectoderm and the inner cell mass [14]. As a consequence, trophectoderm cells seem to be the ideal sample for PGD. However, blastocyst biopsy is more challenging than blastomere biopsy, and only some centers have changed their protocols so far (Fig. 1).

■ Diagnostic Approaches

Different technical approaches are employed in PGD: PCR based techniques

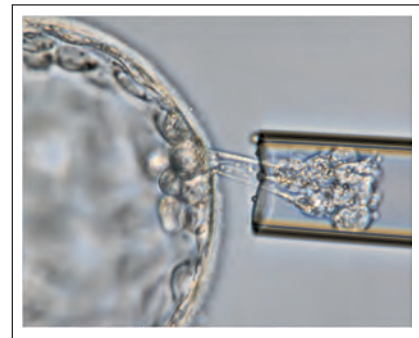


Figure 1. Trophectoderm biopsy. Courtesy of Kinderwunschzentrum Regensburg, Professor Dr. med. Bernd Seifert and Bernd Paulmann.

[15, 16], fluorescence *in situ* hybridisation (FISH) [17, 18], and comparative genomic hybridisation techniques (CGH) [19–21]. Within the PGD procedure, microarray based comparative genomic

Table 1. Selection of single gene disorders for which PGD protocols for polar bodies and day 5 blastocysts are established in Germany

Disease	Gene
Adenylosuccinase deficiency	ADSL
Andermann syndrome	SLC12A6
Aromatic L-amino acid decarboxylase deficiency	DDC
Ceroid lipofuscinosis	CLN3
Charcot-Marie-Tooth disease, X-linked (CMTX1)	CX32 (GJB1)
Cystic fibrosis	CFTR
Desbuquois syndrome	CANT1
Familial adenomatous polyposis 1	APC
Fragile X syndrome	FMR1
Freeman Sheldon syndrome	MYH3
Gorlin Goltz syndrome	PTCH1
Haemophilia A	F8
Huntington disease	HTT
Hydrocephalus, X-linked	L1CAM
Hypophosphatasia, perinatal lethal	ALPL
Joubert syndrome	INPP5E
Krabbe disease	GALC
Leigh syndrome	SURF1
Marfan syndrome	FBN1
Mitochondriopathy, Lactat acidosis	BOLA3
Mucopolysaccharidosis type IIIC	GNPTAB
Mucopolysaccharidosis type IIIA	SGSH
Muscular dystrophy type Duchenne	DMD
Myotonic dystrophy	DMPK
Neurofibromatosis type 1	NF1
Nonketotic hyperglycinemia	GLDC
Norrie syndrome	NDP
Ornithine transcarbamylase deficiency	OTC
Peters-Plus syndrome	B3GALT1
Retinoblastoma	RB1
Simson-Golabi-Beihmel syndrome	GPC3
Spinal muscular atrophy	SMN1
Spinocerebellar ataxia 1	SCA1
Surfactant deficiency	SFTPB
Tuberous sklerosis, type 1 and 2	TSC1/TSC2
Van der Woude syndrome	IRF6
Walker Warburg syndrome	POMT1
Zellweger syndrome	PEX1, PEX6

hybridisation (array CGH) is replacing FISH and the time-consuming conventional or metaphase comparative genomic hybridisation [22–25]. Mutation analyses as well as array CGH protocols are optimized to meet the narrow time slot of a maximum of 24 hours between the trophectoderm biopsy at day 5 and the embryo transfer in the same cycle on day 6.

■ Monogenic Diseases

A mendelian gene disorder (monogenic disease) originates from a mutation of a single gene. In the case of an X-chromosomal recessive disorder, as a rule, boys are more severely affected than girls; female carriers of the mutation may be healthy or only moderately affected. In autosomal dominant disorders the recurrence risk is 50% independent of gender. In the case of an autosomal recessively inherited disorder, the recurrence risk is 25%, again independent of gender. The setup of a PGD for a monogenic disorder is a very individual procedure, as the distinct mutation as well as genetic markers need to be tested in this setting. A protocol of an established PGD procedure is therefore of limited use for couples having different mutations even in the same gene. The setup requires approximately 3–6 months and the incorporation of genetic information from the parents and, wherever applicable, the index patient and, in some cases, additional family members. All new assays have to be verified in a single cell test system (e.g. single lymphocyte cells, buccal cells). The major challenges of mutation analysis within a PGD setup are the limited amount of DNA, allelic dropout (ADO), contamination, and the short time slot between biopsy and reporting the result. If detection of the mutation by sequencing or fragment length analysis is not applicable, a selection of informative microsatellite markers enclosing the mutated part of the gene can be employed for an indirect diagnosis [26]. The segregation analysis (haplotyping) of family members then enables the detection of the affected oocytes or embryos without detecting the mutation itself. The polymorphic markers are also used to exclude contamination and to diminish the effects of allelic dropout which could lead to misdiagnosis if one allele is preferentially amplified in the PCR (Tab. 1).

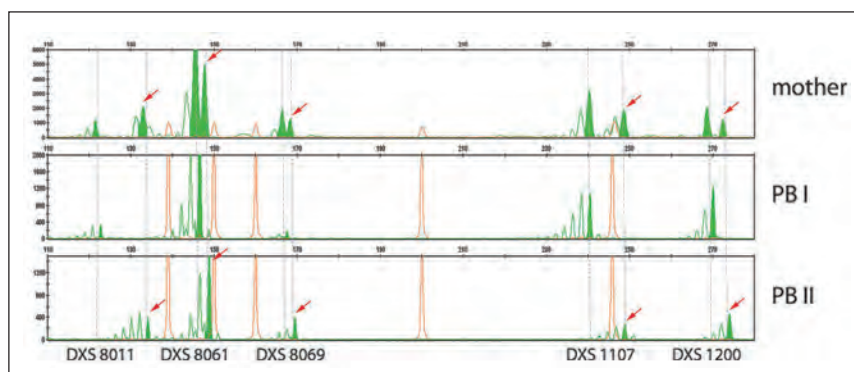


Figure 2. Polar body diagnosis for haemophilia A: fragment length analysis of amplified polymorphic markers of the carrier mother and of PB I and PB II. Arrows indicate polymorphic marker alleles linked to the mutation. The detection of the wild type haplotype in PB I and the haplotype linked to the mutation in PB II indicates the presence of the mutation in the oocyte.

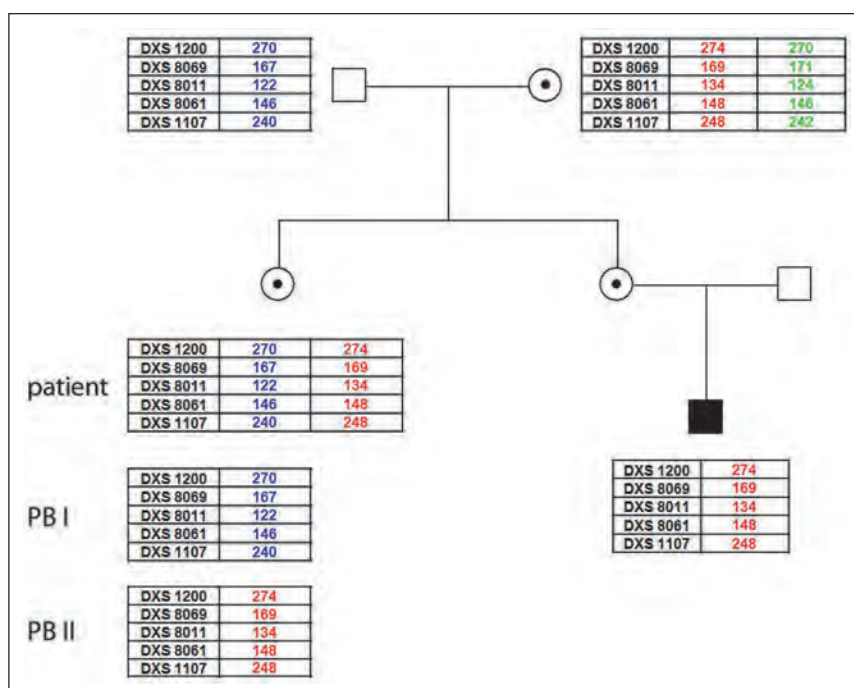


Figure 3. Polar body diagnosis of haemophilia A: The pedigree shows PCR product sizes (in bp) of five polymorphic markers linked to the F8 gene. Red PCR products are linked to the mutation, green and blue ones are linked to the wild type. As an example, the result of the fragment length analysis is shown for an affected oocyte indicated by the result of PB I (blue) and PB II (red).

■ Example 1: PGD for Haemophilia A

Haemophilia A (OMIM *306700) is a severe X-linked recessive disorder which affects blood coagulation. The disease is caused by mutations in the gene for coagulation factor VIII (F8, OMIM *300841), resulting in low or no activity of this protein. One of the most common mutations in severe haemophilia A is a large genomic inversion in intron 22 of F8 which leads to a reduced protein activity of less than 1%. This inversion can only be detected by an indirect mutation analysis employing amplification of

polymorphic microsatellite marker sequences within and close to the putative mutation of the F8 gene, which determines the status of the cell. Figure 2 illustrates the result of fragment length analysis, Figure 3 shows the corresponding pedigree with transmission of affected and unaffected alleles.

■ Example 2: PGD for Spinal Muscular Atrophy (SMA)

Spinal muscular atrophy type 1 (SMA1), an autosomal recessive disorder, is caused by the degeneration of spinal cord neurons. The incidence of SMA1 is

about 1 in 10000 newborns. About 95% of SMA1 patients show a homozygous deletion of exon 7 within the survival motor neuron 1 gene (SMN1, OMIM *600354). SMN2 (OMIM *601627) is a highly homologous gene copy to SMN1 differing in only a very few nucleotides. The loss of SMN1 activity is mainly caused by deletions in SMN1 or by gene conversion from SMN1 to SMN2. Only one nucleotide discriminates SMN1 from SMN2 in exon 7. Dreesen et al. were the first to describe a PGD for SMA in 1998 [27]. Figures 4–6 illustrate the results of fragment length and sequence analysis of exon 7 in two trophoctoderm biopsies (TE1, TE2). The carrier parents display both nucleotides (C and T) in exon 7, whereas only the T-nucleotide was detected in the affected son, indicating a homozygous deletion of the SMN1 gene.

■ Chromosomal Rearrangements and Aneuploidies

Approximately 1 in 500 individuals carries a balanced reciprocal translocation. In this type of translocation, terminal segments of any size from different chromosomes are exchanged. By definition, no loss (deletion) or gain (duplication) of chromosomal material occurs in these balanced chromosomal alterations. A Robertsonian translocation, which occurs in approximately 1 in 1000 individuals is the exchange of whole arms of the acrocentric chromosomes 13, 14, 15, 21 and 22. Carriers of these translocations are healthy, however, offspring of either a Robertsonian or a reciprocal translocation carrier may exhibit an unbalanced chromosomal constitution. Affected, unbalanced embryos can be identified within a PGD setting by performing an array CGH, which also is capable to detect aneuploidies of all other chromosomes.

Fluorescence *in situ* Hybridisation (FISH)

FISH employs fluorescently labelled DNA fragments to detect homologous chromosomal regions. Selected probes designed for the detection of chromosomal translocations verify the presence or absence of an unbalanced karyotype in polar bodies or embryos [28]. FISH has limited value for aneuploidy testing, as only a maximum of 12 different chromosome probes can be administered in a single PGD setup.

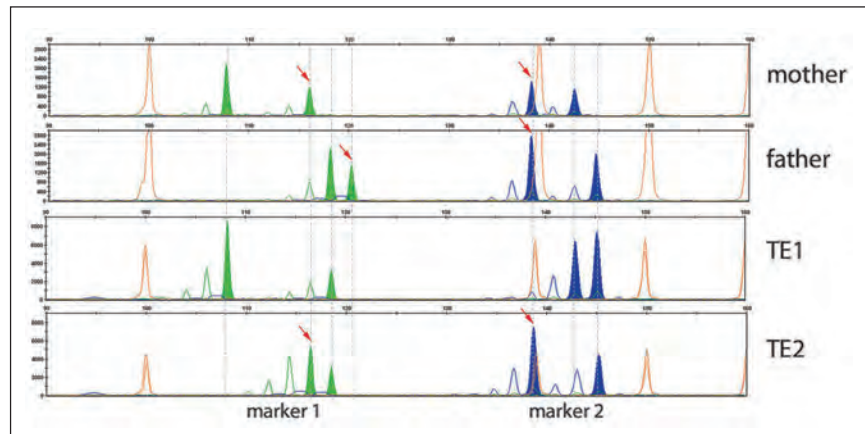


Figure 4. PGD for Spinal Muscular Atrophy, SMA. Fragment length analysis for multiplex-PCR products of two polymorphic markers generated from DNA of two trophoctoderm biopsies (TE1, TE2). Arrows indicate the alleles linked to the mutation. The TE1 sample shows no alleles linked to the mutation, therefore the corresponding embryo is unaffected. TE2 shows one allele linked to the mutation and one allele linked to the wild type, respectively. This embryo is a heterozygous carrier of the mutation.

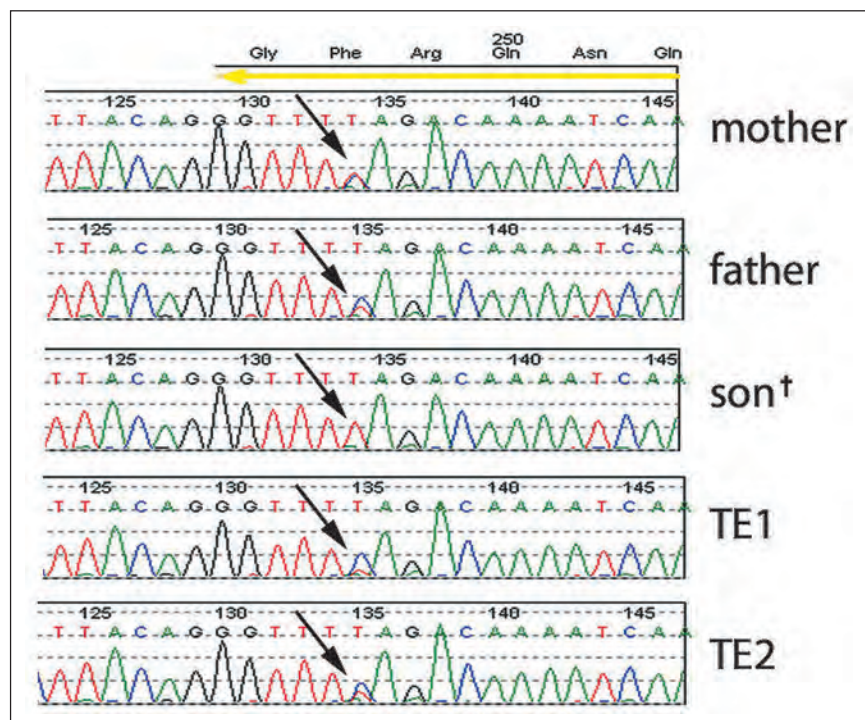


Figure 5. PGD for Spinal Muscular Atrophy, SMA. DNA sequence analysis for amplification products of exon 7 in mother, father, affected son and two trophoctoderm biopsies (TE1, TE2). The mother and father are heterozygous for the nucleotide (C or T) in exon 7 corresponding to the presence of gene copies of SMN1 and SMN2. The son shows only the T-nucleotide of the SMN2 gene and thus carries a homozygous deletion in SMN1. Both TE samples show both nucleotides at this base position, indicating that the blastocysts are either wild type or heterozygous carriers of the deletion.

Array Comparative Genomic Hybridisation (array CGH)

Comparative genomic hybridisation enables the detection of unbalanced chromosomal rearrangements and aneuploidies of all 24 chromosomes with a single approach. Wells et al. demonstrated the feasibility of conventional or metaphase CGH for single cells and thus proved the reliability of CGH for PGD [19, 20]. Array CGH, which employs a microarray

instead of metaphase chromosomes is a well-established technique in postnatal as well as in prenatal genetic diagnostics for the detection of unbalanced copy number variations (CNVs). In PGD, array CGH has the unquestionable benefit of not only detecting imbalances caused by a chromosome rearrangement, but also revealing aneuploidies of all chromosomes simultaneously [22–25]. Array CGH protocols for bacterial artificial

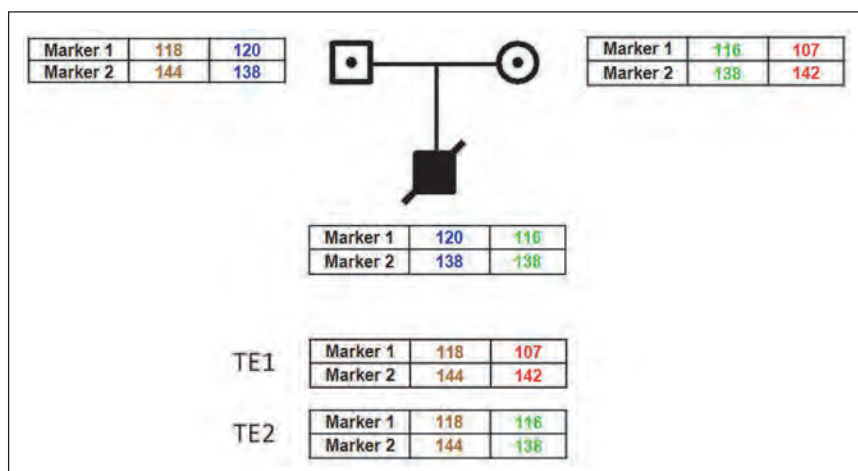


Figure 6. PGD for Spinal Muscular Atrophy, SMA. Pedigree illustrating allele sizes of two microsatellite markers linked to SMN1 and SMN2. Blue and green alleles are linked to the mutation, the orange and red ones to the wild type.

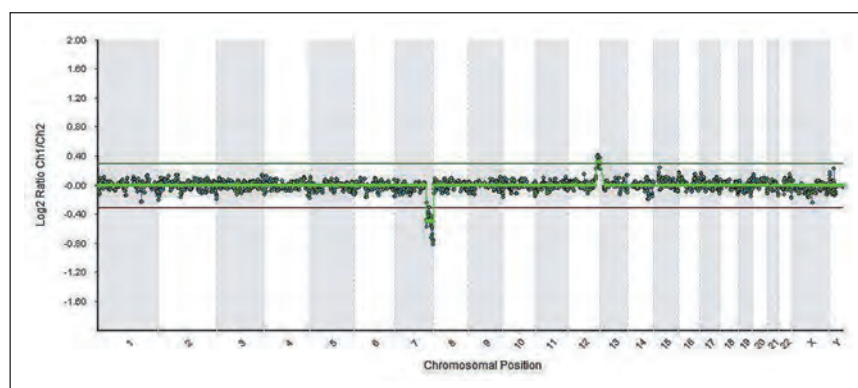


Figure 7. Array CGH profile of an unbalanced rearrangement of chromosomes 7 and 12 after PGD of a trophectoderm sample (TE1).

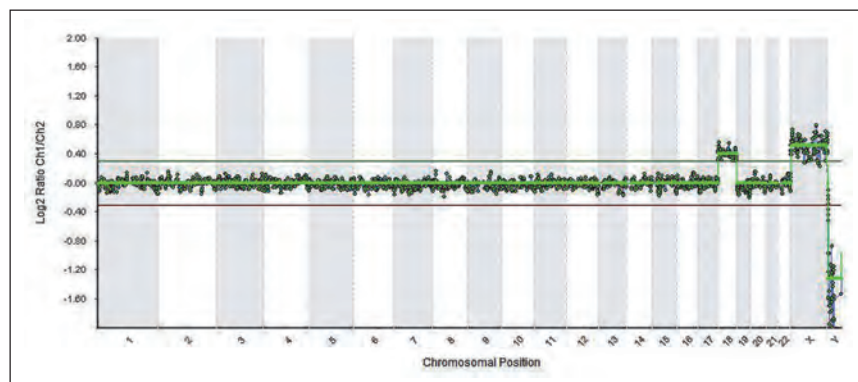


Figure 8. Array CGH profile of an aneuploid (trisomy 18) of another trophectoderm sample (TE2) of the same cycle.

chromosome (BAC) arrays have been modified in such way as to enable the analysis within the challenging time frame of 12–24 hours. BlueGnome Ltd. developed the 24sure technology which is comprised of a whole genome amplification system (WGA, SurePlex), DNA fluorescent labelling kits, BAC arrays and evaluation software (BlueFuse Multi). After amplification of the sample DNA, it is fluorescently labelled and

hybridised together with a differentially labelled reference DNA on a 24sure array, which consists of several thousand BAC clones of human DNA. This array format is optimized for PGD in such way that it only reflects the very robust regions of the human genome; genomic variants of unknown significance are absent from the array, thus avoiding questionable interpretations of the results. Figure 7 illustrates the unbalanced

array CGH result of a trophectoderm sample (TE1); the male partner is carrier of a balanced reciprocal translocation (karyotype 46,XY,t(7;12)(q32;q24.1)); Figure 8 illustrates the array CGH result of another trophectoderm sample (TE2) of the same cycle presenting balanced for the translocation, but aneuploid for chromosome 18 (trisomy 18); Figure 9 illustrates the array CGH result of a balanced and euploid trophectoderm sample (TE3). Array CGH is a highly reliable procedure that detects chromosomal imbalances in as few as 12 hours, thus avoiding cryopreservation (vitrification) of the embryos.

Treff et al. applied a different type of array based upon single nucleotide polymorphism (SNP) probes (262K SNP array, Affimatrix) [23]. These arrays also accomplish the diagnosis in a very short period of time. Handyside et al. [28] combined closely spaced informative SNP loci to detect not only copy number variations but also single gene mutations by haplotyping the genome of the embryo in one single approach (Karyomapping).

■ Aneuploidy Testing

Compared to the targeted investigation of embryos from translocation or inversion carriers, the term preimplantation genetic screening (PGS) describes the testing of chromosomal aneuploidies oocytes or embryos of women with recurrent miscarriages, repeated IVF failure or advanced maternal age. Data for aneuploidy testing mainly revealed through FISH testing of only a limited number of chromosomes showed no or uncertain benefit [29, 30], whereas array CGH revealed a striking improvement in pregnancy rates. Yang et. al. [31] published results from a pilot study, which revealed a pregnancy rate of 69,1% after transfer of a single embryo employing array CGH of day 5 blastocysts compared to a rate of 41,7% in a control group. However, more randomized controlled trials have to prove the benefit of the aneuploidy testing before it can be offered couples without a familial risk in the context of an assisted reproductive technology. Aneuploidy testing of blastocysts may then become a powerful tool to select a single euploid embryo for transfer, which may lead to an improved pregnancy rate.

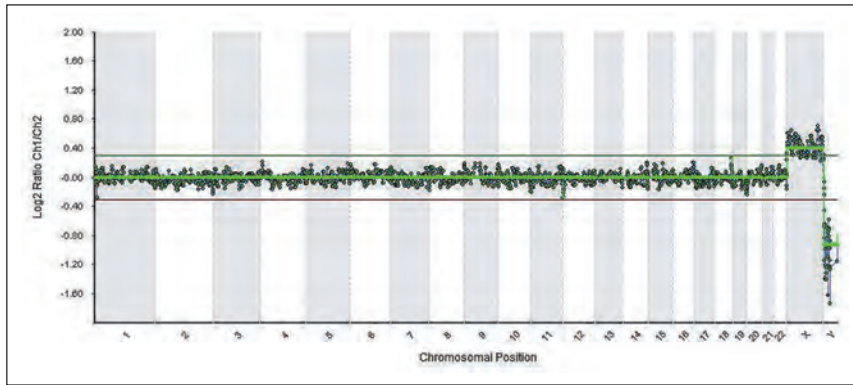


Figure 9. Array CGH profile of a balanced, euploid trophectoderm sample (TE3) of the same cycle.

Conclusion

Since the first preimplantation genetic diagnosis in the late 1980s and early 1990s, PGD has evolved into a well-established technique for the analysis of both single gene mutations, chromosome rearrangements and aneuploidies [32, 33]. Array CGH has replaced FISH and its limited informative value [34]. It can be expected that the pregnancy rate after comprehensive chromosome screening will improve remarkably and that a transfer of a single embryo is aspired to avoid twin pregnancies [31, 35]. Scott et al. [36] were the first to report the delivery of a chromosomally normal child from an oocyte with reciprocal aneuploid polar bodies but an euploid blastocyst, which calls polar body diagnosis into question. Improved PGD protocols including PCR based detection of chromosomal imbalances [37], karyomapping [28, 38] and the approach of Brezina et al. [39] who employed a SNP-based array CGH for the simultaneous detection of aneuploidies and single gene disorders are very promising and document the high standard of genetic analyses. However, further randomized clinical trials have to be evaluated in order to strengthen support for the benefits of PGD [40]. Best practice guidelines for preimplantation genetic diagnosis are supported though the ESHRE PGD consortium [41–44]. Despite the undoubted positive value of PGD, it is worth mentioning that PGD may lead to a higher amount of ART cycles without transferable embryos, because no unaffected or wild type embryos is left after PGD. The legal regulation varies greatly in countries offering PGD - a fact that is unlikely to change in the near future. For instance, social sexing or the selection of a so-called savior sibling (HLA matching)

is routinely performed within a PGD setting in some countries. Hence, legal and ethical issues have to be considered very closely [45]. As of May 2012 the legal situation in Germany is still ambiguous; a rapid regulation of the law is required in order to serve couples with these very promising techniques.

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Conflict of Interest

No potential conflict of interest to this article was reported.

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