Preimplantation genetic diagnosis

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History of PGD


**Sexing of Live Rabbit Blastocysts**
R. G. EDWARDS & R. L. GARDNER

Physiological Laboratory, University of Cambridge
History of PGD

Human IVF in 1978
R. Edwards: Nobel prize for medicine in 2010

PCR in 1983
K. Mullis: Nobel prize for chemistry in 1993
History of PGD

- 1990: Handyside et al.: first PGD for X-linked disease
- 1992: Handyside et al.: baby after PGD for Cystic Fibrosis

Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification

A. H. Handyside, E. H. Kontogianni, K. Hardy & R. M. L. Winston
Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK

OVER 200 recessive X chromosome-linked diseases, typically affecting only hemizygous males, have been identified. In many of these, prenatal diagnosis is possible by chorion villus sampling (CVS) or amniocentesis, followed by cytogenetic, biochemical or molecular analysis of the cells recovered from the conceptus. In others, the only alternative is to determine the sex of the fetus. If the fetus is affected by the defect or is male, abortion can be offered. Diagnosis of genetic defects in preimplantation embryos would allow those unaffected to be identified and transferred to the uterus. Here we report the first established pregnancies using this procedure, in two couples known to be at risk of transmitting adrenoleukodystrophy and X-linked mental retardation. Two female embryos were transferred after in vitro fertilization (IVF), biopsy of a single cell at the six- to eight-cell stage, and sexing by DNA amplification of a Y chromosome-specific repeat sequence. Both women are confirmed as carrying normal female twins.
Preimplantation Genetic Diagnosis

- an alternative to prenatal diagnosis and TOP

- involves genetic testing of cells biopsied from *in vitro* obtained oocytes and/or *in vitro* fertilised embryos and selective transfer of unaffected embryos

- for couples at *high* risk of transmitting a genetic condition to their children
Preimplantation Genetic Screening

- PGS or aneuploidy screening involves selection of euploid embryos to improve IVF results and reduce miscarriage rates

- for specific IVF patients groups at low risk (advanced maternal age, recurrent IVF failure or repeated miscarriages)

- RCTs: no benefit for PGS with FISH at cleavage stage (Checa et al., 2009) => biological and technical reasons => current multicentre RCT with PB biopsy and aCGH
PGD workflow in daily practice

- multidisciplinary team
  - collaboration between IVF and diagnostic genetics unit:
    - in-house and/or transport PGD cycles

DIAGRAM:

- **D** diagnostic unit
- **IVF** IVF unit
- **different location**
- **same location**

**PGD consortium**
PGD workflow in daily practice

- Intake and evaluation of PGD request
  - is PGD acceptable, is PGD possible?
- Consultation at the IVF/genetics unit
  - counselling and informed consent
- Pre-PGD workup in the genetics lab
  - development of single-cell test
- PGD clinical cycle
- Follow-up
  - of cycles, pregnancies and children
PGD workflow in daily practice

- Intake and evaluation of PGD request
  - Is PGD acceptable, is PGD possible?

- Consultation IVF and genetics unit - counselling and informed consent

- Pre-PGD workup in the genetics lab - development and validation of single-cell test

- PGD clinical cycle

- Follow-up
PGD clinical cycle

Day 0

Oocyte collection after hormonal stimulation (day 0)

Intracytoplasmic sperm injection (day 0)
PGD clinical cycle

- *in vitro* culture
- incubator
PGD clinical cycle: biopsy

1 or 2 polar bodies from oocytes
day 0/1

1 or 2 blastomeres from early cleavage stage
day 3

5-10 trophectoderm cells from blastocysts
day 5/6
PGD clinical cycle: biopsy

polar body (1 and/or 2) from oocytes (day 0/1):
- extraembryonic cells
- only for maternally inherited disorders
- no gender determination possible

TE cells from blastocysts embryos (day 5/6):
- sexing + maternally and paternally inherited disorders
- extraembryonic cells
- several cells => higher accuracy
- good blastocyst culture systems required
- limited analysis time (cryopreservation)
- TE representative? interference? mosaicism rate?
PGD clinical cycle: biopsy

Chromosomal mosaicism in preimplantation embryos is a biological phenomenon.

blastomere(s) from cleavage stage embryos (day 3):
- for maternally and paternally inherited disorders
- gender determination possible
- inherent high chromosomal mosaicism rate
- impact of 1 or 2 embryonic cell removal on embryonic development/implantation?
PGD clinical cycle: biopsy: 1 vs 2 cells?

- 2 cells: Lowest risk for misdiagnosis
  - efficient and accurate diagnosis

- 1 cell: Highest chance for a pregnancy
  - implantation potential unaffected by biopsy
PGD clinical cycle: biopsy: 1 vs 2 cells?

RCT:  8- cell Embryos on day 3
> single Embryo transfer on day 5  
Ref: De Vos et al., 2009

Live birth delivery rates:  
37.4 % (1 cell)  
22.4 % (2 cells)  
35.0 % (control)

clinical outcome of 1-cell biopsy was  
significantly better than that of 2-cell biopsy
PGD clinical cycle: standard genetic testing

Embryo biopsy with laser (day 3)

FISH

Embryo transfer (day 5)

Interphase FISH: for sex determination (X-linked disorders) and chromosomal aberrations (numerical and structural (translocations)) was also used for PGS.
PGD clinical cycle: standard genetic testing

embryo biopsy with laser (day 3)

amplification

multiplex PCR of linked STR markers w/wo mutation(s): for monogenic disorders and HLA typing

embryo transfer (day 5)
PGD for monogenic diseases: indications

- any single gene disorder with known genomic location
- hereditary cancer syndromes and other late-onset diseases
- PGD for mtDNA mutations
  - reducing instead of eliminating risk
  - criteria for PGD on mtDNA diseases
- Human Leucocyte Antigens (HLA) typing
  aim: to select an embryo that is HLA identical with a sick sibling, so that at birth cord blood can be collected and used for transplantation and cure of the sick child
  - HLA-typing alone for acquired disease or in combination with mutations underlying immunodeficiencies or hemoglobinopathies

Ref Bredenoord et al., 2008
Single cell PCR amplification

- **Regular PCR**: 100-500 ng purified genomic DNA

- **Single-cell PCR**: single cell with 6 pg or 2 copies in lysis buffer

- more cycles, problems of specificity, allele drop out and contamination => extensive optimisation and validation of PCR conditions
Single cell PCR amplification

- **specificity**: use hot start PCR, < 45 cycles
- **contamination**: from carry-over or extraneous DNA
- **allele drop out**: random amplification failure of one of two alleles in a single heterozygous cell – may affect up to 5% of single-cell PCRs
- apply measures to prevent and detect contamination and ADO

[Diagram showing accurate amplification, ADO affecting normal allele, and ADO affecting mutant allele]
**Single cell fluorescent multiplex-PCR**

- simultaneous amplification of multiple loci per cell (flanking STR markers closely linked (< 1 Mb) +/- mutation locus)
- more accurate: allows diagnosis AND reveals contamination & ADO
- fluorescent: allows detection on automated sequencers

*capillary electrophoresis*
*5 fluorochromes*
Single cell fluorescent multiplex-PCR

- **Direct diagnosis**: direct mutation testing with linked STR markers (<1 Mb range)
- **Indirect diagnosis**: use of at least 2 closely linked (within 1 Mb) STR markers that flank the region/gene of interest, at least two STRs at either side improve robustness of test

![Graph showing short tandem repeat (CA)n, (CAG)n, (GATA)n repeats](image_url)
PGD indirect diagnosis

- When mutation analysis is not possible (unknown, region too large or refractory for single cell PCR, pseudogene interference, ...)

- For disorders with many private mutations
  Advantage: same PCR useful for several couples

- Limiting factor: informative couples with family history and requirement for samples from affected family members
PGD direct diagnosis

- mutations => differences in fragment length
- pointmutations => *post-PCR* reactions for further allele discrimination
  → restriction enzyme analysis, ARMS, minisequencing….
PGD workflow in daily practice

- Intake and evaluation of PGD request
  - is PGD acceptable, is PGD possible?
- Consultation at the IVF/genetics unit
  - Counselling and informed consent
- Pre-PGD workup in the genetics lab
  - Informativity and segregation analysis, confirmation mutation
  - Development and validation of single-cell test
- PGD clinical cycle
- Follow-up
  - Of cycles, pregnancies and children
Pre-PGD workup

PGD request of couple, both carriers of the c.1521_1523del, p.F508del mutation in the CFTR gene. The couple has an affected child, homozygous for this mutation.

→ confirmation mutation, eventually
→ informativity and segregation analysis to check whether an existing single cell test can be used for the couple
→ if not, or in case of a request for a new gene, develop new single cell test
Pre-PGD workup: informativity testing

STR genotyping to select STRs which are informative, allowing to distinguish alleles from each other.
Pre-PGD workup: segregation analysis

Affected child

♂
- 7 repeats
- p.F508del
- 11 repeats

♀
- 9 repeats
- wild type
- 11 repeats

♂
- 10 repeats
- wild type
- 9 repeats

♀
- 14 repeats
- p.F508del
- 16 repeats

♂
- 14 repeats
- p.F508del
- 16 repeats

♀
- 7 repeats
- p.F508del
- 11 repeats
Pre-PGD workup

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<th>Date</th>
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### Genotypes expected in the embryos

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<th>Linked markers</th>
<th>CFTF/Del</th>
<th>IVS1/IVS2</th>
<th>IVS3/IVS4</th>
<th>D7S833</th>
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<th>EFRS1/G612C</th>
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<td>274-278</td>
<td>237-237</td>
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</table>
Single cell fluorescent multiplex-PCR

Marker 1       Mutation       Marker 2

♂

♀

Contamination

Recombination
Pre-PGD workup

→ if an existing single cell test cannot be used, or in case of a request for a new gene, develop new single cell test
Pre-PGD workup: development

- primer design for flanking STR markers (+ mutation)
- informativity analysis to select useful STR markers (check mutation)
- establish multiplex single cell PCR conditions
  - multiplex of STR markers (+ mutation) (10 ng genomic DNA)
  - multiplex of STR markers (+ mutation) (100 pg genomic DNA)
  - multiplex of STR markers (+ mutation) (single cell)
- validation: several sets of single cells (lymphoblasts) and controls
  - determine amplification efficiency, ADO and contamination rate
PGD: standard genetic tests

request for mutation/gene/locus 1  =>  develop single cell PCR 1

request for mutation/gene/locus n  =>  develop single cell PCR n

customised protocols: optimisation and validation at the single cell level has to be repeated each time  =>  pre-PGD workup is labour-intensive and time-consuming and yields high costs
PGD: emerging genetic tests

- single-cell WGA and SNP arrays
  - mutation analysis by haplotyping
  - full chromosomal constitution
  - aneuploidy, deletions, duplications, uniparental disomy, parental origin

- single-cell WGA and NGS
  - reveal also point mutations, balanced chrom. rearrangements
  - high cost, still under validation
PGD: emerging array tests

Emerging array platforms are genome-wide and allow standardisation and automation.

- **universal single cell WGA**
  - several µg of DNA
  - multiple downstream analyses

Optimisation and validation of single cell **whole genome amplification (WGA)**: only 1 time!

=> Pre-PGD workup labour, time and costs are reduced.
Molecular Displacement Amplification, (MDA) isothermal amplification (30°C) => DNA fragments up to 70 kb, low error rates

Dean et al., 2002
SNP bead array preparation
MDA based

1. Whole genome amplification and fragmentation
2. Denaturation and hybridisation on BeadChip
3. Single base extension and staining
4. Array scanning and genotype scoring

SNP bead array: workflow
SNP array: principle

LaFramboise T, 2009
SNP array: principle

- Target: GTT ...
- Probe: CAGA/C TC ...
- Denaturation and hybridisation on beadChip
- Single base extension

LaFramboise T, 2009
SNP array: principle

A = A/T base
B = G/C base

target removal and staining
SNP bead array

A = A/T base
B = G/C base
NC = no call
SNP array: interpretation

1) identify informative SNPs in region of interest

2) phase SNPs in embryo vs reference
### SNP array: interpretation

2) phase SNPs in embryo vs reference

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<th>father</th>
<th>mother</th>
<th>reference</th>
<th>embryo</th>
<th>phase</th>
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<tr>
<td>AB</td>
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Examples:

- Informative allele in blue
SNP array: interpretation

genotype + copy number information

Can et al., 2011
## SNP array: interpretation

<table>
<thead>
<tr>
<th>CNV type</th>
<th>Possible SNP genotypes</th>
<th>Expected A+B signal</th>
<th>Expected BAF</th>
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</table>

#B in AA = 0/2 = 0
#B in AB = 1/2 = 0.5
#B in BB = 2/2 = 1
PGD: quality assurance

Implementation to provide standardisation and assure quality

● accreditation process (ISO15189)
  → Ref. Accreditation of the PGD laboratory, Harper et al., Hum. Reprod. 2010

● best practice PGD guidelines
  → for amplification based PGD , for FISH-based PGD, for organisation of a PGD centre for PGD/PGS, Ref. Harton et al., Hum. Reprod. 2011
  → guidelines for array-based PGD/PGS are under development

● external quality assessment (EQA)
  → molecular EQA and pilot EQA arrayCGH (run by UK National External Quality Assessment Service (http://www.uknegas-molgen.org.uk))
  → FISH-based EQA (run by Cytogenetics European Quality Assessment (http://www.ceqa-cyto.eu))
PGD: accuracy and safety

- constant technical progress > high efficiency and accuracy

- general misdiagnosis rate < 1%  (Ref. Wilton et al., Hum Reprod 2009)
  possible causes: human & technical errors, intrinsic factors

- > 5000 PGD children: follow-up results are reassuring

  Neonatal follow-up of 995 consecutively born children after embryo biopsy for PGD

  main conclusion: embryo biopsy does not introduce extra risks
PGD team of UA Brussel

Centre for Reproductive Medicine
- IVF team
- Biopsy team
- Fertility specialists
- Embryologists
- Prof. Tournaye

Centre for Medical Genetics
- DNA and cytogenetics lab
- Clinical geneticists
- PGD coordinator: P. De Becker
- Prof. M. Bonduelle

PGD lab team:
- Technicians
- C. Staessen
- A. Gheldof
- V. Berckmoes
- P. Verdyck
- M. De Rycke