Genetherapy:
Viral applications in Research and Gene therapy

M.H. Modarressi  MD, PhD.
Medical Genetics Dept.
Tehran University of Medical Sciences
http://www.tums.ac.ir/faculties/modaresi
modaresi@tums.ac.ir

Exam: In English – from slides and whatever said in the class
Definition:
-Gene therapy is a technique
-Treating genetic disease using the normal (unmutated) version of the affected gene
There are two strategies that can be used to correct a disease by gene therapy

1. **Somatic cell transgenesis** - delivering normal gene to patients with defective gene, can't be passed on to progeny

2. **Germline transgenesis** - correcting the defective gene in the gamete, fertilised egg or early embryo (before germline has split from the cells that make up the rest of the body). This is currently prohibited for ethical reasons in some countries
Technical methods molecular therapy:

1- Gene replacement therapy (expression)
2- Antisense therapy (stop expression)
3- Stem cell therapy
4-…….
Two methods for introducing genes

*Ex vivo*- cells are removed from the organism and manipulated to introduce the gene before being put back. Suitable for bone marrow stem cells.

*In vivo*- Organism is treated. Suitable for diseases such as cystic fibrosis and Duchennes muscular dystrophy where the affected cells are no longer dividing.
Delivery of Genes

EX VIVO

Human Cells

Vectors

IN VIVO

Therapeutic Gene

Altered Cells

Vector

Figure 19.11 Diagram of augmentation gene therapy approach
**Target of gene therapy:**

1- Single gene disorders

Cystic Fibrosis, Adenosine Deaminase Deficiency (ADA), Severe Combined Immunodeficiency (SCID), Thalassemia and etc.

2- Non-heritable disorders

Cancers, Parkinson's disease, Huntington's, viral infections (HIV, CMV and …. ) and etc.

The most promising use of gene therapy is for the treatment of non-heritable disorders such as cancer therapies. There are several possible applications which are already in clinical trial.
<table>
<thead>
<tr>
<th>Gene product</th>
<th>Disease and symptoms</th>
<th>Frequency</th>
<th>Current therapy</th>
<th>Prognosis</th>
</tr>
</thead>
</table>
| Adenosine deaminase| Severe combined immunodeficiency  
Loss of T and B cells                                                                   | 1:1,000,000 | Bone marrow transplant;  
adenosine deaminase replacement | Without therapy: fatal by 2 years of age  
With therapy: clinical improvement                  |
| LDL receptor      | Familial hypercholesterolemia  
Elevated blood serum cholesterol level,  
coronary artery disease                                                               | 1:500 (heterozygotes) | Diet, drugs, liver transplant                               | Some clinical improvement                             |
| Glucocerebrosidase| Gaucher disease  
Accumulation of glucocerebrosid in macrophages causing liver, spleen, and bone damage | 1:2,500 (Jewish populations); rare in non-Jewish populations | Symptomatic treatment: removal of spleen, antibiotics,  
repairing bone damage, bone marrow transplant, enzyme replacement | Some clinical improvement                             |
| Blood clotting factor VIII | Hemophilia A  
Altered plasma protein that causes defective blood clotting,  
chronic internal bleeding into joints, excessive bleeding after wounding | 1:10,000 (males) | Concentrates of factor VIII by transfusion                  | Extended life expectancy, requires continual treatment, risk of viral infection from transfusions |
| Phenylalanine hydroxylase | Phenylketonuria  
Excess phenylalanine in the bloodstream of newborns causes mental retardation | 1:10,000 | Restriction of dietary phenylalanine                        | Good in many cases, if treatment is started early and maintained |
| α1-Antitrypsin     | Emphysema  
Deficiency of serum protein protease inhibitor, damage to the lungs, cirrhosis of the liver | 1:3,500 | Replacement therapy, lowering environmental risks          | Progression of the disease is slowed but not stopped  |
<table>
<thead>
<tr>
<th>Gene product</th>
<th>Disease and symptoms</th>
<th>Frequency</th>
<th>Current therapy</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic fibrosis transmembrane regulator</td>
<td>Cystic fibrosis, Multisystem disease, pancreatic insufficiency in some cases, intestinal blockage, blocked airways of the lungs</td>
<td>1 : 2,500 (Caucasians)</td>
<td>Antibiotics, physical clearing of the lungs; upgrading diet</td>
<td>Fatal by late 20s</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>Hyperammonemia, Urea cycle defect, ammonia accumulation, arginine deficiency Early form: within 72 h of birth, lethargy, vomiting, coma, death, and, if survival, irreversible brain damage Late form: vomiting, lethargy, seizures</td>
<td>1 : 40,000</td>
<td>Restricted protein diet, arginine-supplemented diet, drugs, liver transplant</td>
<td>Late form: good Early (severe) form: symptoms diminished</td>
</tr>
<tr>
<td>Dystrophin</td>
<td>Duchenne muscular dystrophy, Progressive muscle wasting</td>
<td>1 : 7,500 (males)</td>
<td>Only supportive treatments: good nutrition, aid in respiratory function, confinement to a wheelchair</td>
<td>Fatal by early 20s</td>
</tr>
<tr>
<td>β-Globin</td>
<td>Sickle-cell disease, Chronic anemia, multisystem disease, damage to spleen, heart, kidneys, liver, and brain; heterozygotes have a mild form of the disease</td>
<td>1 : 500 (heterozygotes in populations of black African origin; less frequent in other populations)</td>
<td>Blood transfusions, drugs, analgesics, bone marrow transplant</td>
<td>Symptoms diminished; treatments suboptimal</td>
</tr>
<tr>
<td>Gene therapy</td>
<td>Condition(s)</td>
<td>Target cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------------------------------------</td>
<td>---------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>Adenosine deaminase deficiency</td>
<td>Lymphocytes, bone marrow cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Melanoma</td>
<td>Tumor-infiltrating lymphocytes, autologous tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>Melanoma, glioblastoma, renal cell cancer</td>
<td>Autologous tumor cells, tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor IX</td>
<td>Hemophilia B</td>
<td>Autologous skin fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL receptor</td>
<td>Hypercholesterolemia</td>
<td>Autologous liver cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histocompatibility locus antigen class 1-B7 plus $\beta_2$-microglobulin</td>
<td>Melanoma, colorectal cancer, renal cell cancer</td>
<td>Tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus thymidine kinase</td>
<td>Glioblastoma, AIDS, ovarian cancer</td>
<td>Tumor cells, T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis transmembrane conductance regulator</td>
<td>Cystic fibrosis</td>
<td>Nasal and airway epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multidrug resistance 1</td>
<td>Breast cancer</td>
<td>Blood CD34* cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor</td>
<td>Melanoma</td>
<td>Tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-1 receptor antagonist</td>
<td>Arthritis</td>
<td>Autologous fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CNTF</td>
<td>Amyotrophic lateral sclerosis</td>
<td>Encapsulated transduced xenogeneic cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Head and neck squamous carcinoma</td>
<td>Tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fanconi anemia C</td>
<td>Fanconi anemia</td>
<td>Bone marrow cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For example gene therapy of Cancer using **p53 Tumors Suppressor Protein:**

p53 acts in late G1 phase. It prevents the cell progressing to the S phase i.e. is antiproliferative.

p53 function almost always disrupted in high grade tumors cells.
What factors have kept gene therapy from becoming an effective treatment for genetic disease?

1- Short-lived nature of gene therapy

2- Immune response

3- Problems with viral vectors

4- Multigene disorders

5- Control of the expression
Why should we use virus for gene therapy?

More than 120000 publications in gene therapy

What is the most important issue in gene therapy?

Gene Delivery
Methods of Gene delivery

Bare DNA-

Physical methods-

*Electroporation*, sonication & Gun, *Microinjection* or direct DNA injection, Biolistic Particle Delivery, Ca phosphate and etc.

Non-viral vectors-

*Lipofection:*

liposomes, emulsion, peptides, cationic polymers etc.

Lipid based system, Solid-lipid nanoparticle, nanoparticle Polymers

Viral vectors-

*Viruses:* adenoviruses, retrovirus,....
Gene Delivery Mechanisms

• Biological
  – viruses
Transfected HEK293 cells with plasmids containing GFP using Lipofection system

Infected HEK293 cells with adenovirus containing GFP after 24h
Viruses are classified on the basis of morphology, chemical composition, and mode of replication.

Common viruses for gene delivery:

- Retrovirus
- Adenovirus
- Adenoassociated virus
- Lentinivirus
- Phages*
- Herpes virus
- Reovirus

\[\text{Vehicle}\]

\[\text{Lytic}\]
Real treatments performed with retroviral system

- SCID means “severe combined immuno deficiency disease”
- “bubble boy disease”
- Recessive genetic disorder
- Mutation in gene for blood enzyme adenosine deaminase (ADA)
- No Precursor cells so no immune system function
What precisely has been done to the patient?
Ashanti de Silva successfully treated for ADA deficiency - 1990

Ryes Evans successfully treated for SCID - 2001

**SCID**

- No therapy to treat this disease except gene therapy
- Normal ADA gene is transplanted into patient
- Restarts immune system
- Best to attack disease at the source
- Put wild gene in stem cells of bone marrow that produce immune system using avirulent retrovirus
- A transgene integrates with body cells
Effect of AAV-mediated Hepatic Gene Therapy in Hemophilia B Dogs

**Normal:** blood clots in about 8 to 10 minutes

**Diseased:** blood clots in about 50 to 60 minutes
The first gene therapy clinical trial began in 1990.

Two halts by FDA:

1- In 1999, death of 18-year-old Jesse Gelsinger. Jesse was participating in a gene therapy trial for ornithine transcarboxylase deficiency (OTCD). He died from multiple organ failures 4 days after starting the treatment. His death is believed to have been triggered by a severe immune response to the adenovirus carrier.

2- In January 2003, when the FDA placed a temporary halt on all gene therapy trials using retroviral vectors in blood stem cells. Gene therapy trial had developed a leukemia-like condition in a child who had been successfully treated by gene therapy for X-linked severe combined immunodeficiency disease (X-SCID), also known as "bubble baby syndrome."
Risks

• Other risks with gene therapy:
  - Cells injected may cause an immune response (specially for adenovirus and HSV)
  – Random insertion of retrovirus into host chromosome- may be likely in non-coding DNA, but what if it interrupts the coding DNA?
Adenovirus type 2 and 5
An icosahedral protein shell (252 structural capsomeres) surrounding a protein core (70 to 100 nm in diameter)
A linear, double-stranded DNA genome.

The genome is divided into early functions (E1A, E1B, E2A, E2B, E3, and E4 regions), which are expressed first during viral replication, and late functions (L1 to L5 regions), which are usually expressed after the early functions and after the beginning of viral DNA replication. The late genes encode the viral structural proteins. In the case of Ad2, DNA replication begins 6 to 8 hours after infection of cultured human cells.
# Comparison of viral vectors

## Table 1 | The main groups of viral vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Genetic material</th>
<th>Packaging capacity</th>
<th>Tropism</th>
<th>Inflammatory potential</th>
<th>Vector genome forms</th>
<th>Main limitations</th>
<th>Main advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enveloped</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Dividing cells only</td>
<td>Low</td>
<td>Integrated</td>
<td>Only transduces dividing cells; integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in dividing cells</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Broad</td>
<td>Low</td>
<td>Integrated</td>
<td>Integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in most tissues</td>
</tr>
<tr>
<td>HSV-1</td>
<td>dsDNA</td>
<td>40 kb* 150 kb†</td>
<td>Strong for neurons</td>
<td>High</td>
<td>Episomal</td>
<td>Inflammatory; transient transgene expression in cells other than neurons</td>
<td>Large packaging capacity; strong tropism for neurons</td>
</tr>
<tr>
<td><strong>Non-enveloped</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV</td>
<td>ssDNA</td>
<td>&lt;5 kb</td>
<td>With the possible exception of haematopoietic cells</td>
<td>Low</td>
<td>Episomal (&gt;90%) Integrated (&lt;10%)</td>
<td>Small packaging capacity</td>
<td>Non-inflammatory; non-pathogenic</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>8 kb* 30 kb†</td>
<td>Broad</td>
<td>High</td>
<td>Episomal</td>
<td>Capsid mediates a potent inflammatory response</td>
<td>Extremely efficient transduction of most tissues</td>
</tr>
</tbody>
</table>

*Replication defective. †Amplicon. ‡Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.
Adenovirus Delivery Systems

W. C. Russell
Packaging of the virus in HEK293 cells

2 days

5 days

7 days

10 days

infected HEK 293 cells after 24h

CPE is starting after about 36h

CPE after 48H
Perspectives and future directions

Target specific gene therapy

Promoters
Inducible promoters (for example: Tet-on & off)
Tissue specific promoters
Is it possible to have a manufactured adenovirus which can be a tool for manipulating a cell? When? Too soon

آدنوویروس برای اتصال به سلول‌های سرطانی fiber & Knob ساخت لیگاند اختصاصی در محل
Manipulation of viral receptors to make tissue or cell specific viruses

High titer in blood stream ➔ Attack metastases
PND & PGD

Genetic screening
Genetic Diagnosis

• Genetic diagnosis - Determining whether or not a particular gene defect is present in an individual.– adults – e.g. Huntington’s disease, BRCA and ....

• Preimplantation Genetic Diagnosis (PGD) or Embryonic diagnosis –in vitro fertilization; implant disease free embryos

• Prenatal Diagnosis (PND) – done before baby is born

• Genetic Screening
Prenatal Diagnosis (PND)

What are you looking for?
1- Family History

Have you examined?
2- Clinical exam, Ultrasound scan and Paraclinics

Why are you going to check chromosome and genes?
3- cytogenetic and genetic analysis
Prenatal Diagnosis

Healthy fetus

Carrier (Heterozygote) Fetus Affected (Homozygote)

Fetus is a patient

Is it accepted by parents and did they know that?
Is the mother ready for a physical and emotional stress?
What about legal and ethics?
Is it the right time for abortion?

..........
Methods of screening in pregnancy

Chorionic villus sampling via the vagina

Chorionic villus sampling through the abdomen

Amniocentesis  Ultrasound

Figure 14.9 Amniocentesis procedure
PND will be done using:

1- **TROPHOBLAST / PLACENTA (CVS)**  
   CARYOTYPE, DNA, BIOCHEMISTRY

2- **AMNIOTIC FLUID**  
   CELLS: CARYOTYPE, ADN, BIOCHEMISTRY  
   FLUID: BIOCHEMISTRY

3- **FETAL BLOOD** (cord blood)  
   CARYOTYPE, DNA, SEROLOGY, HEMATOLOGY, BIOCHEMISTRY

4- **FETUS**  
   TISSUES (biopsy of BLOOD, SKIN, MUSCLE, LIVER)  
   "PHENOTYPE" "WELL-BEING" "BEHAVIOUR"

5- **MATERNAL BLOOD**  
   FETAL CELLS, FETAL DNA
Time points:

GESTATIONAL AGE

13 WKS : CVS

15 WKS : AMNIOCENTESIS

20 WKS : FETAL BLOOD SAMPLING

Figure 14.11 Procedure for transcervical chorionic villus sampling

Figure 14.12 Chorionic villus material
Some of Chemical Screening tests for Prenatal Diagnosis

In advance for PND using genetical tests
Screening tests:
- Cheap and specific not NOT specific
Results may lead to the identification of the disease

NEURAL TUBE DEFECTS
Between 15 and 20 weeks of pregnancy, Maternal serum (MS) measurement of
- a-fetoprotein (AFP)
- human chorionic gonadotropin (beta hCG &h-hCG)
- unconjugated estriol (UnE3)
- Inhibin -A
- blood test between 10 and 14 weeks for PAPP-A
…..
It individualizes 70% of the Trisomia 21 (Down Syndrome) but also Edwards Syndrome and Spina Bifida.

+ HISTORY OF NTD
Box 14.2 Some causes of increased maternal serum α-fetoprotein concentration

- Underestimated gestational age
- Threatened abortion
- Multiple pregnancy
- Fetal abnormality
  - Anencephaly
  - Open neural tube defect
  - Anterior abdominal wall defect
  - Turner syndrome
  - Bowel atresia
  - Skin defects
- Maternal hereditary persistence of α-fetoprotein
- Placental haemangioma
DETERMINATION OF FETAL KARYOTYPE:

1- ADVANCED MATERNAL AGE (>35 YRS)
2- HIGH RISK OF ANEUPLOIDY ON SCREENING
3- HISTORY OF CHROMOSOMAL ANOMALY
4- PARENTS CARRIERS OF A BALANCED TRANSLOCATION
5- X-LINKED DISEASES
6- ULTRASOUND-DETECTED FETAL ANOMALIES
7- MATERNAL ANXIETY

Box 14.1 General criteria for prenatal diagnosis

- High genetic risk
- Severe disorder
- Treatment not available
- Reliable prenatal test available
- Acceptable to parents
## COMPLICATIONS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorioamnionitis</td>
<td>&lt; 1/1000</td>
</tr>
<tr>
<td>Fetal lesions</td>
<td>-</td>
</tr>
<tr>
<td>Amniotic fluid leakage</td>
<td>&lt; 1/100</td>
</tr>
<tr>
<td>Fetal death, miscarriage</td>
<td>0.5 - 1/100</td>
</tr>
<tr>
<td>Rh sensitization</td>
<td>~5%</td>
</tr>
</tbody>
</table>
Methods for PND:

**Molecular methods:**

PCR*, PCR-RFLP, Nested PCR, RT-PCR, PCR based methods

Reverse dot blotting

Sequencing

**Cytogenetics:**

Karyotyping

FISH and CGH
Critical points: Do not forget it

1- You have just a few amount of fetal sample. So be careful: do not lose it and label it.

2- Almost the sample is contaminated with maternal cells. So you should have controls with (STRs).

3- You must know what you are looking for (mut., del., etc.). So You must check parents in advance before pregnancy.

4- Do not forget that result can be a permit for abortion. So confirm the result with another method.
Definition of **PGD**: Preimplantation genetic diagnosis (PGD) is a genetic-testing tool that allows for the detection of abnormalities in 3-day-old embryos via molecular or cytogenetic methods.
Preimplantation Genetic Diagnosis (PGD)

*Helps patients at risk of transmitting an inherited disease to their children

*Main option is natural conception and prenatal diagnosis

*In PGD embryo generated by IVF

*Test the preimplantation embryo

*Pregnancy started knowing fetus is unaffected
Why should we do PGD: PGD Applications

MEDICAL USES
PGD for single-cell disorders
PGD for aneuploidy detection
PGD for late-onset disorders (Huntington's disease, Cancer predisposition, …)
PGD for HLA typing/preimplantation genetic typing

Single Gene Disorders:
For Known specific mutations, AR, AD, X-linked & Mitochondrial Disorders and Linkage analysis

Heterogenic X-linked Disorders
Retinitis Pigmentosa (RP), FXS, DMD, BMD, Hemophilia

Chromosomal abnormalities
Reciprocal or Robertsonian translocations & Inversions,…

NON-MEDICAL USES OF PGD
PGD for gender selection
Patients requesting PGD:

- Repeated termination or Recurrent Spontaneous abortion (RSA) > 3

- Moral or religious objections to termination

- Repeated miscarriages due to chromosomal abnormality

- Infertile couple carrying genetic or chromosomal abnormality such as cystic fibrosis, Y chromosome deletion, Deafness due to mutation in Canexine26 or… and etc.

- To find the best HLA matched sibling for BM transplantation

Box 14.3 Potential applications of preimplantation genetic diagnosis

- Fetal sexing for X linked disorders, for example
  - Duchenne muscular dystrophy
  - Haemophilia
  - Hunter syndrome
  - Menke syndrome
  - Lowe oculocerebrorenal syndrome

- Chromosomal analysis:
  - Autosomal trisomies (21, 18 and 13)
  - Familial chromosomal rearrangements

- Direct mutation analysis:
  - Cystic fibrosis
  - Childhood onset spinal muscular atrophy
  - Huntington disease
  - Myotonic dystrophy
  - β thalassaemia
  - Sickle cell disease
Stages of PGD:

1. IVF
2. Fertilization
3. 6-8 cell embryo
4. Blastomere biopsy
5a. FISH
5b. PCR
6. Embryo transfer
Viable and Desirable?

“This information is helping parents choose which embryos they want--and which to reject as unhealthy, or merely undesirable.” (Zitner 2002)
Ovarian stimulation & oocyte retrieval

• The success rate of PGD is associated with the number of retrieved oocyte (>9)

ICSI

- Similar to in vitro fertilization
- Eggs are removed from woman
- A single sperm is injected a single egg using a needle.
- High success rate
How long do you have time for PGD?

Less than a day
Stages of PGD:

1- Embryo Biopsy
   A- polar body*
   B- Cleavage Stage
   C- Blastocyst

A- Polymerase Chain Reaction (PCR)
   - Analyse specific mutation
   - single gene defect
   - Sexing for X-link disease
   - Triplet repeat disorders
   - Linkage analysis for a locus

B- Fluorescent In Situ Hybridization (FISH)
   - Analyse chromosome
   - Sexing for X-link disease
   - Chromosome abnormalities
   - Aneuploidy

2- Single cell diagnosis
Possibility for contamination during PGD
Successfully-fertilized egg (two pronuclei and second polar body)
DNA Markers: 13 CODIS Core STR Loci with Chromosomal Positions

For contamination detection in PGD and PND
--Diseases which PGD has detected include:
• Tay-Sachs disease
• sickle cell anemia
• spinal muscular atrophy (type I)
• Gaucher disease
• Factor V Leiden
• Fanconi’s anemia
• congenital adrenal hyperplasia

--Autosomal dominant monogenic diseases that have been detected with PGD include:
• myotonic dystrophy
• Charcot-Marie-Tooth disease IA
• Marfan’s syndrome
• osteogenesis imperfecta

--Single-gene X-linked conditions detected using PGD include:
• Duchenne/Becker muscular dystrophy
• hemophilia
• Fragile X syndrome
• mental retardation
• agammaglobulinemia
• Wiskott-Aldrich syndrome
• Lesch-Nyhan syndrome
Important points in PGD: Be careful

1- You are working with single cell. So, I would say take two cells out of 8 cells. If you lost one you have another.

2- You are trying to amplify just two double strand DNA from a cell. If you brake the DNA in the target gene, You get nothing. It makes error. It is Allele Drop Out (ADO). ADO is serious for AD disorders.

3- To confirm your PGD do PND
Ethics & informed consent in research

From a signature... for legal coverage

To accurate and adequate information

for free decision-making
Good Luck