Molecular therapies

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Functional Genomics</td>
</tr>
<tr>
<td>1.1</td>
<td>Definitions</td>
</tr>
<tr>
<td>2</td>
<td>About diseases</td>
</tr>
<tr>
<td>3</td>
<td>Approaches to understanding disease mechanisms</td>
</tr>
<tr>
<td>3.1</td>
<td>Gene expression is regulated in several basic ways</td>
</tr>
<tr>
<td>3.2</td>
<td>Microarrays: functional genomics in cancer research</td>
</tr>
<tr>
<td>3.3</td>
<td>A Variety of Genetic Alterations Underlie Developmental Abnormalities and Disease</td>
</tr>
<tr>
<td>3.4</td>
<td>Genomic microarrays</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Array based comparative genome hybridization (aCGH)</td>
</tr>
<tr>
<td>2</td>
<td>Recombinant proteins</td>
</tr>
<tr>
<td>2.1</td>
<td>Overview: Protein pharmaceuticals</td>
</tr>
<tr>
<td>2.2</td>
<td>Cell-free systems: In vitro transcription and translation</td>
</tr>
<tr>
<td>3</td>
<td>Recombinant protein expression in isolated cells (cell culture)</td>
</tr>
<tr>
<td>4</td>
<td>Non-prokaryotic expression systems</td>
</tr>
<tr>
<td>4.1</td>
<td>Cloning in Pichia pastoris</td>
</tr>
<tr>
<td>4.2</td>
<td>Baculovirus mediated protein expression in insect cells</td>
</tr>
<tr>
<td>4.3</td>
<td>Mammalian expression systems</td>
</tr>
<tr>
<td>5</td>
<td>Purification of recombinant proteins</td>
</tr>
<tr>
<td>3.3</td>
<td>Gene Therapy: Vectors and Strategies</td>
</tr>
<tr>
<td>3.1</td>
<td>Vectors for Gene Therapy</td>
</tr>
<tr>
<td>3.2</td>
<td>Types of gene transfer, vectors for gene therapy</td>
</tr>
<tr>
<td>3.3</td>
<td>General gene therapy strategies</td>
</tr>
<tr>
<td>3.4</td>
<td>Human gene therapy</td>
</tr>
<tr>
<td>4</td>
<td>Protein replacement therapies</td>
</tr>
<tr>
<td>5</td>
<td>Recombinant antibodies and the phage display technology</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>5.1.1</td>
<td>The structure of antibodies and their production in the body</td>
</tr>
<tr>
<td>5.1.2</td>
<td>Antigen-antibody binding</td>
</tr>
<tr>
<td>2.5</td>
<td>The production of therapeutic antibodies</td>
</tr>
<tr>
<td>2.5.1</td>
<td>The production of antibodies in hybridoma cells</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Humanized antibodies</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Production of human antibodies</td>
</tr>
<tr>
<td>3.5</td>
<td>Generation of antibodies by phage display</td>
</tr>
<tr>
<td>3.5.1</td>
<td>The phage display technology</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Generation of phage libraries</td>
</tr>
<tr>
<td>4.5</td>
<td>Administration of therapeutic antibodies</td>
</tr>
<tr>
<td>6</td>
<td>Anti-cytokine Therapy (sepsis)</td>
</tr>
<tr>
<td>6.1</td>
<td>The consequences of developing inflammation</td>
</tr>
<tr>
<td>6.2</td>
<td>Development of Inflammatory Response: Synthesis of Lipid Mediators</td>
</tr>
<tr>
<td>6.3</td>
<td>Role of the Liver in Maintenance of Homeostasis: Acute Phase Response</td>
</tr>
<tr>
<td>6.4</td>
<td>Time-Course of the Inflammatory Response During Sepsis</td>
</tr>
<tr>
<td>7</td>
<td>Animal models and transgenesis in biotechnology</td>
</tr>
<tr>
<td>1</td>
<td>Animal models and transgenesis in biotechnology</td>
</tr>
<tr>
<td>8</td>
<td>Embryonic and adult stem cells in regenerative medicine I</td>
</tr>
<tr>
<td>8.1</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>8.2</td>
<td>Somatic cell reprogramming into pluripotent stem cells</td>
</tr>
<tr>
<td>8.3</td>
<td>Adult stem cells</td>
</tr>
<tr>
<td>9</td>
<td>Embryonic and adult stem cells in regenerative medicine II</td>
</tr>
<tr>
<td>9.1</td>
<td>Pluripotent stem cells for regenerative medicine</td>
</tr>
<tr>
<td>9.2</td>
<td>Clinical application of stem cells</td>
</tr>
<tr>
<td>9.3</td>
<td>Stem cell therapy to cure various diseases</td>
</tr>
<tr>
<td>10</td>
<td>Cell Cycle and Cancer Therapy, p53 I</td>
</tr>
<tr>
<td>10.1</td>
<td>Cell Cycle</td>
</tr>
</tbody>
</table>
2. 10.2. Mitogenic Signaling in Eukaryotic Cell Controls the Rate of Cell Division ........ 85
3. 10.3. Biochemical Events of Cell-cycle – in M Phase ........................................ 88
4. 10.4. Protooncogenes ...................................................................................... 88
5. 10.5. ErbB/HER Receptors ............................................................................. 90
6. 10.6. Therapeutic targets ................................................................................ 90
11. 11. Cell Cycle and Cancer Therapy, p53 II. ....................................................... 96
   1. 11.1. Tumor Suppressor Genes and p53 ......................................................... 96
   2. 11.2. Biochemical Pathways of Apoptosis and its Therapeutic Utilization .......... 100
12. 12. Gene Silencing Technologies. ..................................................................... 104
   1. 12.1 Introduction ............................................................................................. 104
   2. 12.2 Action of antisense oligonucleotides .................................................... 104
   3. 12.3 Chemical modifications of gene silencing oligonucleotides; general considerations 106
   4. 12.4 Inhibition of transcription by triple helix forming oligonucleotides ............. 110
   5. 12.5 Gene silencing by ribozymes ................................................................. 112
   6. 12.6 Gene silencing with short RNA fragments ............................................ 115
   7. 12.7 Important final note ............................................................................... 120
# List of Figures

1.1. Figure 1.1. Global analysis of gene expression ................................................................. 6
1.2. Figure 1.2. Mapping of genetic aberration ........................................................................ 7
1.3. Figure 1.3. Different arrays for different purposes ............................................................. 7
1.4. Figure 1.4. Array CGH maps DNA copy number, alteration to position in the genome ...... 8
2.1. Figure 2.1. Linearization of template .................................................................................. 10
2.2. Figure 2.2. Baculovirus/insect expression system ............................................................. 11
2.3. Figure 2.3. Methotrexate (MTX) selection ..................................................................... 11
2.4. Figure 2.4. Conventional purification strategy ................................................................. 12
3.1. Figure 3.1. In vivo gene therapy ....................................................................................... 13
3.2. Figure 3.2. Ex vivo gene therapy ..................................................................................... 13
3.3. Figure 3.3. Liposomes ..................................................................................................... 14
3.4. Figure 3.4. Naked DNA .................................................................................................. 14
3.5. Figure 3.5. Gene particle bombardment ......................................................................... 15
3.6. Figure 3.6. Retrovirus ..................................................................................................... 16
3.7. Figure 3.7. Life cycle of retroviruses .............................................................................. 16
3.8. Figure 3.8. Moloney Murine Leukemia Virus Based Retroviral Vector I. ..................... 16
3.9. Figure 3.9. Moloney Murine Leukemia Virus Based Retroviral Vector II. ..................... 17
3.10. Figure 3.10. Retroviral gene therapy ............................................................................. 18
3.11. Figure 3.11. Lentiviral vector ....................................................................................... 19
3.12. Figure 3.12. Adenoviruses ......................................................................................... 20
3.13. Figure 3.13. Evolution and adenoviral vectors .............................................................. 20
3.14. Figure 3.14. Adenoviral gene therapy ......................................................................... 21
3.15. Figure 3.15. Adeno-associated viruses ....................................................................... 21
3.16. Figure 3.16. Gene therapy strategies I. ....................................................................... 22
3.17. Figure 3.17. Gene therapy strategie II. ..................................................................... 23
3.18. Figure 3.18. Gene therapy strategie III. ..................................................................... 23
3.19. Figure 3.19. Severe combined immunodeficiency (SCID); lack of adenosine deaminase (ADA) 24
3.20. Figure 3.20. Gene therapy for severe immunodeficiency synfrom I. ............................. 25
3.21. Figure 3.21. Gene therapy for severe immunodeficiency synfrom I. ............................. 25
3.22. Figure 3.22. Ornithine transcarbamolase (OTC) deficiency .......................................... 26
3.23. Figure 3.23. Transports of lipids with plasma lipoproteins ........................................... 27
3.24. Figure 3.24. Regulation of the mevalonate pathway ....................................................... 27
3.25. Figure 3.25. Levels of the genetic deficiencies of LDL receptor .................................. 28
3.26. Figure 3.26. Correlation between the LDL cholesterol level of blood and the number of LDL receptors in liver ................................................................. 28
3.27. Figure 3.27. General treatments of the high plasma cholesterol level ......................... 29
4.1. Figure 4.1. Structure of insulin. .................................................................................... 32
4.2. Figure 4.2. Cohn fractionation. ..................................................................................... 33
4.3. Figure 4.3. Activation of Factor XI. ........................................................................... 34
4.4. Figure 4.4. Structure and activation of Factor VIII. ...................................................... 34
4.5. Figure 4.5. Steps in a human Factor VIII gene transfer protocol. ................................. 35
5.1. Figure 5.1. The structure of antibodies. ........................................................................ 37
5.2. Figure 5.2. The structure of antibody heavy chain. ....................................................... 38
5.3. Figure 5.3. The structure of antibody light chain. .......................................................... 38
5.4. Figure 5.4. Production of antibodies by B cells. ............................................................ 38
5.5. Figure 5.5. The clonal selection and clonal expansion. ................................................... 39
5.6. Figure 5.6. Polyclonal antibodies. .................................................................................. 39
5.7. Figure 5.7. Monoclonal antibodies. .............................................................................. 40
5.8. Figure 5.8. Production of antisides in hybridoma cells. ................................................ 41
5.9. Figure 5.9. Humanized antibodies. .............................................................................. 41
5.10. Figure 5.10. Production of human antibodies in genetically modified mice. ................ 42
5.11. Figure 5.11. The structure of M13 phage. ................................................................. 43
5.12. Figure 5.12. Specific elution of immobilized phage particles. ....................................... 43
5.13. Figure 5.13. Enzyme phage display. ........................................................................... 44
5.14. Figure 5.14. Substrate phage display I. ..................................................................... 44
5.15. Figure 5.15. Substrate phage display II. ..................................................................... 44
Index

LIST OF FIGURES

• Figure 1.1. Global analysis of gene expression
• Figure 1.2. Mapping of genetic aberration
• Figure 1.3. Different arrays for different purposes
• Figure 1.4. Array CGH maps DNA copy number, alteration to position in the genome
• Figure 2.1. Linearization of template
• Figure 2.2. Baculovirus/insect expression system
• Figure 2.3. Methotrexate (MTX) selection
• Figure 2.4. Conventional purification strategy
• Figure 3.1. In vivo gene therapy
• Figure 3.2. Ex vivo gene therapy
• Figure 3.3. Liposomes
• Figure 3.4. Naked DNA
• Figure 3.5. Gene particle bombardment
• Figure 3.6. Retrovirus
• Figure 3.7. Life cycle of retroviruses
• Figure 3.8. Moloney Murine Leukemia Virus Based Retroviral Vector I.
• Figure 3.9. Moloney Murine Leukemia Virus Based Retroviral Vector II.
• Figure 3.10. Retroviral gene therapy
• Figure 3.11. Lentiviral vector
• Figure 3.12. Adenoviruses
• Figure 3.13. Evolution and adenoviral vectors
• Figure 3.14. Adenoviral gene therapy
• Figure 3.15. Adeno-associated viruses
• Figure 3.16. Gene therapy strategies I.
• Figure 3.16. Gene therapy strategies II.
• Figure 3.16. Gene therapy strategies III.
• Figure 3.17. Severe combined immunodeficiency (SCID); lack of adenosine deaminase (ADA)
• Figure 3.18. Gene therapy for severe immunodeficiency synfrom I.
• Figure 3.19. Gene therapy for severe immunodeficiency synfrom I.
• Figure 3.20. Ornithine transcarbamoylase (OTC) deficiency
• Figure 3.21. Transports of lipids with plasma lipoproteins
• Figure 3.22. Regulation of the mevalonate pathway
• Figure 3.23. Levels of the genetic deficiencies of LDL receptor
• Figure 3.24. Correlation between the LDL cholesterol level of blood and the number of LDL receptors in liver
• Figure 3.25. General treatments of the high plasma cholesterol level
• Figure 4.1. Structure of insulin.
• Figure 4.2. Cohn fractionation.
• Figure 4.3. Activation of Factor XI.
• Figure 4.4. Structure and activation of Factor VIII.
• Figure 4.5. Steps in a human Factor VIII gene transfer protocol.
• Figure 5.1. The structure of antibodies.
• Figure 5.2. The structure of antibody heavy chain.
• Figure 5.3. The structure of antibody light chain.
• Figure 5.4. Production of antibodies by B cells.
• Figure 5.5. The clonal selection and clonal expansion.
• Figure 5.6. Polyclonal antibodies.
• Figure 5.7. Monoclonal antibodies.
• Figure 5.8. Production of antibodies in hybridoma cells.
• Figure 5.9. Humanized antibodies.
• Figure 5.10. Production of human antibodies in genetically modified mice.
• Figure 5.11. The structure of M13 phage.
• Figure 5.12. Specific elution of immobilized phage particles.
- Figure 5.13. Enzyme phage display.
- Figure 5.14. Substrate phage display I.
- Figure 5.15. Substrate phage display II.
- Figure 5.16. Enzyme-substrate phage display I.
- Figure 5.17. Enzyme-substrate phage display II.
- Figure 5.18. Generation of phage libraries.
- Figure 5.19. Generation of protease substrate phage library.
- Figure 5.20. Substrate phage display –engineering of protease substrate sequences.
- Figure 5.21. In vivo phage display – mapping vascular endothelial cells.
- Figure 5.22. Generation of antibody libraries from whole blood.
- Figure 5.23. The mechanism of antibody dependent cell mediated cytotoxicity (ADCC).
- Figure 5.24. Administration therapeutic antibodies with immunosupressant activity.
- Figure 5.25. Forms of therapeutic antibodies.
- Figure 5.26. Forms of small-sized therapeutic antibodies.
- Figure 6.1. Migration of neutrofil from vascular space to tissues
- Figure 6.2. Stream of cytokines in sepsis
- Figure 6.3. Inflammatory cytokines and anti-cytokines
- Figure 6.4. Development of inflammatory response, synthesis of lipid mediators
- Figure 6.5. Development of inflammatory response I.
- Figure 6.6. Indication of cytokine synthesis of inflammation
- Figure 6.7 Synthesis of NO controlled by cytokine
- Figure 6.8. Acute phase response
- Figure 6.9. Inflammatory mediators that modulate hepatic APR synthesis
- Figure 6.10. Time-course of the inflammatory response during sepsis
- Figure 6.11. Endothelial activation, coagulation and fibrin clot formation I.
- Figure 6.12. Coagulation response
- Figure 6.13. Endothelial activation, coagulation and fibrin clot formation II.
- Figure 6.14. Coagulation, fibrin clot formation and inhibition of fibrinolysis
- Figure 6.15. Endogenous activated Protein C has multiple Mechanisms of activation
- Figure 6.16. Contribution of high-mobility group box1 (HMGB1) to sepsis
- Figure 6.17. Collapse of homeostasis
- Figure 6.18. Development of septic shock and organ failure
- Figure 8.1. Stem cells I.
- Figure 8.2. Stem cells II.
- Figure 8.3. The most important characteristics of embryonic stem (ES) cells
- Figure 8.4. Generation of human ES cells
- Figure 8.5. Generation of transgenic and knock out mice (blastocyst injection)
- Figure 8.6. Nucleic reprogramming of somatic cells into pluripotent cells
- Figure 8.7. Animal cloning is based on somatic cells nuclear transfer (SCNT)
- Figure 8.8. IN human only therapeutic cloning
- Figure 8.9. Induced pluripotent stem (iPS) cell generation
- Figure 8.10. Lineage specific reprogramming (trans-differentiation) by transcription factors
- Figure 8.11. The promise of ES/iPS cell research
- Figure 8.12. Adult stem cell example: hematopoetic stem cells (HSC)
- Figure 9.1. ES (iPS) based transplantation medicine
- Figure 9.2. iPS cells based gene correction
- Figure 9.3. Transgene delivery strategies
- Figure 9.4. Curative potential 34CD+ hematopoetic stem cells (HSC) to treat disorders of the blood system
• Figure 9.5. Are there any spontaneous lineage conversions (trans-differentiation) in vivo?

• Figure 10.1. The functional cell cycle

• Figure 10.2. Cyclin-CDK regulators of cell cycle

• Figure 10.3. Constitutive and inducible cell cycle kinase inhibitors

• Figure 10.4. Mitogen-Activated Protein (MAP) kinase cascade

• Figure 10.5. Transcriptional events in G1 phase of cell-cycle

• Figure 10.6. Mechanisms of gene-suppression by the Retinoblastoma Protein

• Figure 10.7. Biochemical events of cell-cycle in M-phase

• Figure 10.8. Cancer causing genes in mitotic signal pathway

• Figure 10.9. Proto-oncogenes and oncogenes

• Figure 10.10. Different families of Receptor Tirosi Kinase recognize a diverse set of different ligands

• Figure 10.11. Ligand binding activates RTKs by dimerization

• Figure 10.12. Proto-oncogenes are normal genes that can become oncogenes

• Figure 10.13. Neu, EGFR targeting methods

• Figure 10.14. Expression of HER2 receptor on the surface of normal and tumor cells

• Figure 10.15. Therapeutic targets

• Figure 10.16. Blocking of oncoproteins of EGFR and Mitogen-Activated Protein Kinase signalization via monoclonal abs and specific inhibitors

• Figure 11.1. Transcriptional events in G1 phase of cell-cycle

• Figure 11.2. Tumor suppressor genes: retinoblastoma and P53

• Figure 11.3. Regulation transcription factor of P53 I.

• Figure 11.4. Regulation transcription factor of P53 II.

• Figure 11.5. Primary structure of transcription factor p53

• Figure 11.6. Restoration of p53 function in tumor cells I.

• Figure 11.7. Restoration of p53 function in tumor cells II.

• Figure 11.8. Loss of p53 is observed in 50% of all human cancers

• Figure 11.9. Morphology of apoptosis

• Figure 11.10. Biochemical pathways of caspase activation dependent cell death

• Figure 11.11. Killing tumors by induction of apoptosis

• Figure 11.12. Cell death prevention after Stroke

• Figure 12.1. The basic concept of antisense activation

• Figure 12.2. Potential mechanisms of action of antisense oligonucleotides

• Figure 12.3. Stability, cellular uptake of antisense oligonucleotides, accessibility to the target

• Figure 12.4. Chemical modification of gene silencing oligonucleotides I.

• Figure 12.5. Chemical modification of gene silencing oligonucleotides II.

• Figure 12.6. Chemical modification of gene silencing oligonucleotides III.

• Figure 12.7. Chemical modification of gene silencing oligonucleotides IV.

• Figure 12.8. Chemical modification of gene silencing oligonucleotides V.

• Figure 12.9. Half lives of natural DNA, phosphorothioate (PS) and end-blocked oligonucleotides with 2'-OCH3 or locked nucleotides (LNA in human serum

• Figure 12.10. Gene silencing in the laboratory for experimental purposes

• Figure 12.11. Schematic representation of triple helix

• Figure 12.12. A comparison of the anti-gene and anti-sense strategy

• Figure 12.13. Structure of parallel triplets

• Figure 12.14. Structure of antiparallel triplets

• Figure 12.15. General structures of ribozymes often utilized for gene silencing
• Figure 12.16. Mechanism of celf-splicing of the rRNA precursor of Tetrahymena

• Figure 12.17. Construction of hammerhead ribozymes against M1 RNA

• Figure 12.18. Group I intron ribozyme designed to bind and repress mutant p53

• Figure 12.19. Possible mechanisms of action A: siRNA, B: miRNA

• Figure 12.20. miRNA and siRNA pathways

• Figure 12.21. miRNA and siRNA biosynthetic pathway

• Figure 12.22. miRNA and siRNA pathways, and various methods to induce RNA interference

• Figure 12.23. Functionally active siRNA

• Figure 12.24. Roles of Ago proteins in gene silencing induced by siRNA and miRNA

• Figure 12.25. Plasmids expressing functional shRNA

• Figure 12.26. Action of shRNA-plasmid gene silencer

• Figure 12.27. Gene silencing with 21 nt long dsRNA oligos

• Figure 12.28. Lentiviral delivery of shRNA and its mode of action
Chapter 1.1. Functional Genomics

1.1.1 Definitions

Genomics means the study of genomes (the DNA comprising an organism) using the tools of bioinformatics. The prerequisite of genomics is the accessibility of genome sequences in well annotated databases. This is static data: the genome sequence is not supposed to change (or changes very slowly) over time. Bioinformatics is the study of protein, genes, and genomes using computer algorithms and databases.

Functional genomics is investigating the correlations between genome and phenotype in:

- Normal and pathological conditions of an organism
- When the organism is responding to changes in the environment
- different organisms

The prerequisite of functional genomics was the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics. It is characterized by high throughput or large-scale experimental methodologies combined with statistical or computational analysis of the results (Hieter and Boguski 1997).

Functional genomics as a means of assessing phenotype differs from more classical approaches primarily with respect to the scale and automation of biological investigations. A classical investigation of gene expression might examine how the expression of a single gene varies with the development of an organism in vivo. Modern functional genomics approaches, however, would examine how 1,000 to 10,000 genes are expressed as a function of development (UCDavis Genome Center)

2.1.2 About diseases

Genetic variation is responsible for the adaptive changes that underlie evolution. Some of these changes improve the fitness of a species, while other changes are maladaptive. For the individual in a species, these maladaptive changes may represent disease, or elevated risk to disease development. From the molecular perspective we may talk about mutation and variation (characteristic to the individual, static), whereas from the medical perspective we distinguish healthy and pathological condition, which may change during the lifetime of the individual.

Previously, a large distinction was made between monogenic (single gene) and polygenic (complex) disorders. They are now seen to be more on a continuum. We may define a single-gene disorder as a disorder that is caused primarily by mutation(s) in a single gene. However, all monogenic disorders involve altered functions of many genes. 90% of monogenic diseases appear by puberty, only 1% have onset after age 50. Diseases of complex origin tend to appear later; if the onset is early, the burden is greater. Examples are anomalies of development, early onset asthma, high blood pressure, cancer, diabetes, autism, obesity, osteoporosis. For complex disorders there is a gradient of phenotype in the affected population. Multiple genes are involved in the development of complex diseases, and the combination of specific sequence variants (sometimes including mutations as well) in those genes define the development and the severity of the disease. Complex diseases are non-Mendelian: they show familial aggregation, but not segregation. This means that they are heritable, but it is not easy to identify the responsible genes in pedigrees (e.g. by linkage analysis). Cancer is a special type of complex disease characterized by genetic instability. Thus, genetically cancer is characterized by multiple genetic (chromosomal) aberrations, including deletions, duplications, or rearrangements of chromosomal DNA. Multiple genes are affected, and the unique combination of loss and gain of function aberrations leads to the manifestation of different types of cancers. Apart from familial cancer syndromes, genetic aberrations of tumor cells are not present in other cells of the body, and they are usually not heritable.

3.1.3 Approaches to understanding disease mechanisms
The usual approach to decipher monogenic diseases is using classical genetics and genomics, including linkage analysis, genome-wide association studies (GWAS), identification of chromosomal abnormalities and genomic DNA sequencing. The study of complex, multigenic diseases on the other hand requires utilization of functional genomics, genomics, genetics, molecular biology etc. Data from global analyses may identify targets for molecular therapy, which may be quite variable:

- Genes that cause disease (cardiovascular, diabetes, Alzheimer’s)
- Interactions between genes and the environment that lead to chronic disease
- Various aspects of cancer: response to treatment, prognosis, recurrence
- Basic biological questions involving regulation of genes

### 3.1. 1.3.1 Gene expression is regulated in several basic ways

One of the most used functional genomics approaches is global gene expression analysis, since there is a good correlation between RNA expression patterns and expression levels and phenotype (disease). Gene expression microarrays are widely used for this purpose.

**Figure 1.1. Global analysis of gene expression**

![Gene expression and microarrays](image)

### 3.2. 1.3.2 Microarrays: functional genomics in cancer research

Global gene expression analysis may contribute to personalized cancer medicine in several ways:

- It may help identify who is at risk (Prognosis)
- It may help identify who will and won’t respond to each agent
- It may help identify alternatives for patients with chemo-resistant disease
- It may lead to better utilization of existing and new drugs, or development of strategies for unique combinations of drugs (lecture 2, slide 5).

### 3.3. 1.3.3 A Variety of Genetic Alterations Underlie Developmental Abnormalities and Disease
In addition to gene expression analyses, global analysis of DNA sequence variations, mutations and larger chromosomal aberrations are equally important. In contrast to RNA, DNA is relatively robust and can be assayed specimens that have been treated in multiple ways, including archival tissue from hospital laboratories. Global genomic analyses can be performed e.g. by genomic microarrays.

**Figure 1.2. Figure 1.2. Mapping of genetic aberration**

![Mapping of genetic aberration](image)

**Figure 1.3. Figure 1.3. Different arrays for different purposes**

<table>
<thead>
<tr>
<th>Array type</th>
<th>Probes</th>
<th>Target</th>
<th>Sample Biological Question</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression arrays</td>
<td>cDNA, long/short oligos</td>
<td>mRNA</td>
<td>Transcriptome profiling; differential expression, eQTL</td>
</tr>
<tr>
<td>Array CGH</td>
<td>(DNA) BAC clones (~150kb)</td>
<td>DNA</td>
<td>Genomic changes in cancer</td>
</tr>
<tr>
<td>SNP arrays</td>
<td>(DNA) long/short oligos</td>
<td>DNA</td>
<td>Genotyping</td>
</tr>
<tr>
<td>Methylation arrays</td>
<td>Methylation-specific oligos</td>
<td>DNA</td>
<td>Methylation status in genes</td>
</tr>
<tr>
<td></td>
<td>(DNA) promoter sequences (~1kb)</td>
<td>DNA</td>
<td>Methylation status in genes</td>
</tr>
<tr>
<td></td>
<td>Splice-specific oligos</td>
<td>mRNA</td>
<td>Splice isoform-specific gene expression</td>
</tr>
<tr>
<td>Tiling arrays</td>
<td>(DNA) high-density oligos</td>
<td>DNA/mRNA</td>
<td>Sequencing, SNP genotyping, gene expression, transcription factor binding</td>
</tr>
</tbody>
</table>

**3.4. 1.3.4 Genomic microarrays**

Genomic microarray is a microarray technology that detects chromosomal abnormalities and/or sequence variations. In the clinical lab it is complementary to fluorescence in situ hybridization (FISH), although genomic microarrays provide information at a genome wide scale. In the research lab it helps the discovery of the genetic basis of diseases. Its significance lies in the fact that many disorders are likely to be caused by microdeletions and other chromosomal abnormalities that cannot be detected by FISH. SNP arrays may offer even more resolution, and additional information (both genotype and copy number).
3.4.1. 1.3.4.1 Array based comparative genome hybridization (aCGH)

Figure 1.4. Array CGH maps DNA copy number, alteration to position in the genome

This technique measures the amount (copy number) of DNA, not RNA. It compares two samples: the ‘Test’ sample and the ‘Reference’ sample. For instance, tumor copy number profiles are a reflection of two processes:

- Selection for alterations in gene expression that favor tumor development. Selective advantage to maintain set of aberrations.
- Mechanisms of genetic instability promoting changes in the genome. (Initiating oncogenetic event in murine models and methotrexate resistance in MMR deficient and proficient cell lines. aCGH aids cancer research in several ways:
  - Based on the results better tests can be performed that measure the DNA copy number of oncogenes and TSGs.
  - Monitor cancer progression and distinguish between mild and metastatic cancerous lesions using FISH (Fluorescence in situ hybridization) probes on regions of recurrent copy number aberrations in several tumor types.
  - It can be used to reveal more regional copy number markers that can be used for cancer prediction.
  - Identifying and understanding the genes that are involved in cancer will help to design therapeutic drugs that target the dysfunction genes and/or avoid therapies that cause tumor resistance.
Chapter 2. 2. Recombinant proteins

1. 2.1 Overview: Protein pharmaceuticals

Biotechnology have been used to make bioactive products for medical or research purposes for a long time. The biotechnology industry have been hugely successful in producing simpler, smaller molecules, such as antibiotics, but the mass production of human proteins (or even smaller peptides) proved to be significantly more complicated. Many hormones are in fact small peptides (calcitonin, oxytocin, vasopressin) and can be synthesized using the method developed by Bruce Merrifield in the sixties. Chemical peptide synthesis is very efficient (over 99% per bond), but that means that for even a short 50-amino acid peptide the overall yield will be 60.5%. Traditionally, human or animal proteins intended for medical purposes are isolated from natural sources, using complicated protein purification techniques. Human and animal tissues, including blood can be sued as natural source for several important proteins. 8-9% of blood consists of 10000 different proteins, although the bulk of this is really made up from about 20 major blood proteins. Blood clotting factors for the treatment of coagulation diseases were for a long time isolated from human blood using Cohn fractionation developed in 1946. A great advantage of proteins isolated from human blood is the species-identity, which prevents the mounting of immune response against the protein product, and the human proteins also carry all posttranslational modifications necessary for their biological activity. At the same time, there never is a sufficient supply of human blood, and any protein pharmaceuticals isolated from human or animal tissues may contain traces of infectious agents (known or unknown), and hazardous contaminants incompletely removed during purification. Recombinant DNA technology provides the means to produce human protein pharmaceuticals in simple organisms, utilizing well-established fermentation technologies. There are four general approaches to produce recombinant proteins:

1. Expression in cell-free systems (in vitro)
2. Expression in isolated cells (cell cultures)
3. Expression in transgenic plants/animals
4. Gene therapy in humans

During this lecture we will discuss the first two systems, comparing the advantages and disadvantages of the in vitro and cell culture systems, and their applications.

2. 2.2 Cell-free systems: In vitro transcription and translation

Cell-free systems are exceptionally useful for research purposes, but the low yields excludes their utilization for the mass production of proteins. There advantages over in vivo gene expression are:

- When the protein is: toxic to the host cell, insoluble or forms inclusion bodies, degraded rapidly by intracellular proteases
- Speed and directness of all procedures
- Absence of constraints from a living cell
- Pure product

Disadvantages over in vivo gene expression are:

- Lack of cellular membranes
- Lack of post translational modifications

Eukaryotic transcription in vivo is based on the specific interaction of several proteins, the DNA template and the newly produced RNA. The majority of the proteins are involved in regulation and posttranscriptional processing. Since the majority of these functions are not needed in an in vitro system, we can utilized the much
simpler transcriptional system of the bacteriophages. For this we need the phage RNA polymerase, nucleotides and the appropriate buffer.

On the other hand, eukaryotic translation requires ribosomes, tRNAs, amino acids, template RNA and a host of other proteins, not all well characterized. Therefore, in vitro translation systems are always provided as crude extract of cells.

Figure 2.1. Linearization of template

3. 2.3 Recombinant protein expression in isolated cells (cell culture)

The first step of recombinant protein expression is identification and cloning of its gene. This process is the subject of recombinant DNA technology, and will not be discussed in this lecture. A critical decision point is determining which expression system to use: in vitro or in vivo, and if in vivo, which species to choose. Culturing and protein purification conditions are based on standard approaches, but always require empirical optimization. Initially prokaryotes were used to produce recombinant proteins - however, for larger proteins, or proteins with extensive posttranslational modifications euakaryotic sytems are better suited.

4. 2.4 Non-prokaryotic expression systems

4.1. 2.4.1 Cloning in Pichia pastoris

This system uses a special plasmid that works both in E. coli and yeast. Once the gene of interest is inserted into this plasmid, it must be linearized, then yeast cells are transfected with linear plasmid. Following transfection double cross-over recombination event occurs to cause the gene of interest to insert directly into P. pastoris chromosome where the old AOX gene used to be. From now on the gene of interest is under control of the powerful AOX promoter.

4.2. 2.4.2 Baculovirus mediated protein expression in insect cells

This system uses the Autographica californica multiple nuclear polyhedrosis virus (Baculovirus), which commonly infects insects cells of the alfalfa looper (small beetle) or armyworms (and their larvae). The system uses super-strong promoter from the polyhedrin coat protein to enhance expression of proteins while virus resides inside the insect cell - protein is not required for infection or viral life cycle. Secreted proteins better expressed by stably transfected insect cell lines, from the ie-1 promoter (infection interferes with secretory pathways).
2. Recombinant proteins

4.3. 2.4.3 Mammalian expression systems

In these systems the gene is initially cloned into plasmid, and propagated in bacterial cells, then the mammalian cells are transformed by electroporation (with linear plasmid) and the gene integrates (1 or more times) into random locations within different chromosomes. The cells are typically derived from the Chinese Hamster Ovary (CHO) cell line. Often, multiple rounds of growth and selection using methotrexate to select for those cells with highest expression and integration of DHFR and the gene of interest.

5. 2.5 Purification of recombinant proteins
Protein purification protocols produce proteins with variable purity - as always, therapeutical proteins have to be extremely pure. Steps of the purification strategies are based on different physicochemical properties of the proteins, such as size, charge, side chains, hydrophobicity. This requires several purification steps, lengthy optimization for each protein, and sensitive and specific detection of the protein at each step. For recombinant proteins on the other hand, it is possible to attach a short protein sequence, a so called tag to the N or C terminal of the protein using recombinant DNA technology. The tags are designed to bind smaller molecules or metal ions with high affinity and specificity, and can greatly shorten and standardize the purification of a wide variety of proteins.

**Figure 2.4. Conventional purification strategy**

- Use different properties of protein in purification scheme
- Multiple intermediate steps may be required
- Need to detect low amounts

Gene therapy is one of the key technologies of the twenty-first century. Hopes are high to find new treatments for cancer, AIDS, heart attack, stroke, and other common ailments.

1. 3.1 Vectors for Gene Therapy

The transfer of therapeutic genes into patient cells depends critically on the further development of in vivo gene transfer systems. Such vectors should have certain properties in order to deliver the gene efficiently to the target cell. They must be easy to produce and high titer preparations of vector particles should be reproducible. From a safety aspect, they should be non-toxic and should not elicit undesired effects such as immune reactions in the host. The therapeutic gene should be expressed persistently at a high level. Viral vectors should target defined types of cells or tissue and infect stationary as well as dividing cells, because most cells in an adult patient are in a postmitotic state. The insertion to the host genome must be specific in order to avoid insertional mutagenesis. Specific insertion would render the repair of gene defects possible.

Figure 3.1. Figure 3.1. In vivo gene therapy

![In vivo gene therapy](image1)

Figure 3.2. Figure 3.2. Ex vivo gene therapy

![Ex vivo gene therapy](image2)
2. 3.2. Types of gene transfer, vectors for gene therapy

Over the past years, a wide range of vector systems of viral and nonviral origin have been developed. While methods such as the direct injection of naked plasmid DNA, gene transfer through a gene gun, or as liposome vesicle showed low transfection efficiency, experiments using viral vectors looked more promising.

Figure 3.3. Liposomes

Advantages: non-pathogenic, no immunity problems, no gene size limit
Disadvantages: low transfection efficiency, low rate of stable integration

Figure 3.4. Naked DNA

Genomes of retroviruses, adenoviruses and adeno-associated viruses (AAV) make up the bulk of the most frequently used viral vectors. Other, less commonly used viral vectors are derived from the Herpes simplex virus I (HSV-1), the baculovirus, and others. Viruses have evolved and adopted many properties of cells in the process, which enables them to identify target cells efficiently and penetrate them. They migrate from the cytoplasm to the nucleus to express their genes in the host cell. This viral life cycle enables infectious virions to transfer genetic information with great success.

Retroviral Vectors

Retroviruses are a large versatile group of viruses with a genome consisting of single or double-stranded RNA. They have a diameter of about 100 nm and they are covered by an envelope. The envelope contains a viral glycoprotein which binds to cellular receptors, thus defining the specificity of the host and cell type that is infected. The envelope protein furthers fusion with a cellular surface membrane or with endosomal compartments inside the cell. Depending on the arrangement of their genome, retroviruses are divided into two categories, the simple and the complex retroviruses. All retroviruses contain three essential genes, gag, pol, and env. Gag codes for structural proteins that constitute the matrix, the capsid, and the nucleoprotein complex. Pol codes for reverse transcriptase and integrase, while env codes for the proteins of the envelope. There is also a psi packaging signal and two LTRs (long terminal repeats) with regulatory functions within the virus. The prototype for a simple retrovirus carrying only a small set of information is the Moloney murine leukemia virus (MoMLV). Complex retroviruses such as lentiviruses (e.g., the human immunodeficiency virus, HIV) contain additional regulatory and accessory genes. Initially, vectors for gene therapy were developed from simple retroviruses, very often MoMLV. In order to develop retroviral vectors, knowledge of the viral life cycle became fundamental. After the infection of the host cell, the viral RNA is reversely transcribed into linear double-stranded DNA by reverse transcriptase. This process takes place in the cytoplasm, and the viral DNA is then introduced into the nucleus followed by a stable insertion into the host genome.
Figure 3.6. Retrovirus

Figure 3.7. Life cycle of retroviruses

Figure 3.8. Moloney Murine Leukemia Virus Based Retroviral Vector I.
The mechanism by which retroviruses are introduced into the nucleus of the host cell differs between simple and complex retroviruses. Whereas simple retroviruses can only enter the nucleus where the nuclear membrane is being dissolved during the mitotic process, lentiviruses have a pre-integration complex that relies on an active cellular transport mechanism through the nuclear pore without destroying the nuclear membrane. Unlike MoMLV, lentiviruses are therefore able to transduce stationary host cells. Once the virus has entered the nucleus, the viral enzyme integrase initiates the integration of the viral DNA into the host genome. The integrated viral DNA is called a provirus. It limits expression of cellular genes and uses the host cell for viral gene expression. The transcriptional activity of the host is controlled by cis-acting proviral LTR regions.

Complex retroviruses have additional trans-acting factors that activate RNA transcription (e.g., HIV-1 tat). After the translation of the viral genes, the resulting protein products and the viral RNA form viral particles that are released from the cell via the cell membrane by budding.

Most retroviral vectors currently used in gene therapy studies are based on MoMLV, one of the first gene vehicles used in human gene therapy experiments. In order to produce viruses with a deficient replication mechanism that only replicate in the packaging cell and not in the host cell, the viral genes have been removed and replaced by a therapeutic gene. Gag, pol, and env are expressed in trans in the packaging cell. When the modified viral genome containing the therapeutic gene is transfected into the packaging cell, all required components are brought together to form a recombinant virus. This virus can transfect target cells, but is unable to form infectious particles because genes code for viral proteins are missing from its genome. This is a safety device often used in viral vectors. The viral genes responsible for the replication of the virus are separated from the rest of the genome, thus reducing the risk of a recombination of infectious particles.

**Figure 3.9.** Moloney Murine Leukemia Virus Based Retroviral Vector II.
Lentiviruses are a subfamily of retroviruses with all the advantages of retroviral constructs plus the ability to transduce also postmitotic cells and tissue, including neurons, retinal, muscle, and hematopoietic cells. Recently developed lentiviral vectors are largely based on the HIV genome. In order to avoid a recombination of infectious HIV particles, as many endogenous HIV proteins as possible have been deleted without reducing the transduction and expression rate. Furthermore, more recently developed vectors carry regulatory elements that have been added later. The cPPT (central polypurine tract) sequence facilitates the synthesis of the second strand and the transport of the pre-integration complex into the nucleus, while the WPRF (woodchuck hepatitis virus posttranscriptional regulatory element) sequence enhances the expression of the transgene via a higher efficiency of the transduction and translation processes. An additional mutation of the 3'–LTRs results in self-inactivation (SIN), thus reducing the risk of a recombination of infectious HIV particles. In order to improve safety further, efficient vectors have been developed from lentiviruses that are not pathogenic to humans, but also have the ability to transduce stationary cells. The basic structure comes from, e.g., the monkey-specific simian immunodeficiency virus (SIV), the cat-specific feline immunodeficiency virus (FIV), or the horse-specific equine infectious anemia virus (EIAV). So far, most lentiviral vectors have been produced through transient transfection of packaging and vector plasmids. The cells used are the easily transfectable 293T cells, which yield titers between 1 x 10^9 and 1 x 10^10 infectious units per mL. The virus particles are further concentrated through ultracentrifugation. However, it is not easy to standardize virus production in transient infections. It would be an advantage to be able to develop stable production cells, especially in view of the use of HIV-based vectors in clinical trials, but the toxicity of the VSV-G envelope protein and of other lentiviral proteins such as Gag, and Tat is a major obstacle in the production of lentiviral packaging cell lines. It would be useful if these toxic proteins could be expressed in tetracycline-regulated systems, a strategy currently under investigation. The potential risk for the application of lentiviral vectors lies in the possibility of insertion mutagenesis and a strong tendency in retroviruses to recombine with infectious foreign retroviruses either within the transfected or the target cells. It is also possible that new viruses emerge through recombination with endogenous sequences. Thus, new infectious viruses with hitherto unknown properties could be spread by the use of retroviral vectors. These could not only affect other organs, but also the germ cells.

Figure 3.10. Retroviral gene therapy
Adenoviral Vectors

Until recently, adenoviral viruses were very popular because they can be easily produced on an industrial scale, the virus titers are high and they can transfect stationary as well as dividing cells. The linear double-stranded DNA of adenoviruses codes for 11 proteins. The genome is packed into an icosahedral protein capsule, which is not surrounded by an envelope, but contains fiber envelope proteins. The fiber proteins combine with the surface receptors of the host cell to form a high-affinity complex. The endosomes are lysed by the adenoviral enzymes, but the genome is not inserted into the cell DNA and remains episomal. This results in a serial dilution of the adenoviral genome over several cell divisions. Unlike retroviruses, adenoviruses cannot be passed on via the germ line. Their high expression rate on a short-term basis makes them suitable for tumor treatment. Due to their wide host tropism adenoviral viruses are not restricted to one compartment but spread into surrounding tissue. This leads to toxic side effects particularly on the liver. Furthermore most patients have already been exposed to adenoviruses during their lifetime and thus developed antibodies, which makes therapeutically relevant target tissues such as the epithelium of the respiratory tract as well as various tumors refractory to an adenoviral infection. This could reduce the efficacy of adenoviral gene therapy. What is more, conventional...
adenoviral vectors could elicit a strong immune reaction in the host mainly caused by the adenoviral E2 protein. While such an inflammatory reaction might well have an antitumor effect, there is also a high safety risk as the death of a patient has demonstrated. The replication defect in first generation adenoviral vectors was the result of a deletion of the E1A and E1B genes. In some of these vectors, the E3 gene was also deleted in order to improve their uptake capacity. However, they retain the other early and late viral genes that are expressed in small quantities after infection. In second generation adenoviral viruses where the E2 and E4 regions have also been deleted, and only the late genes are retained. Viral gene products induce an immune response against the transduced cells, resulting in a reduced expression of the transgene. New strategies aim to completely avoid the immune response and to achieve a higher uptake capacity for foreign DNA in adenoviral viruses. This led to the development of adenoviral vectors in which all viral reading frames have been deleted. These are known as gutless vectors and contain only those viral DNA sequences that are active in cis and are essential for the replication and packaging of viral DNA, such as inverse terminal repeats (ITRs), which contain the polymerase binding sequence for the start of DNA replication and the DNA packaging signal psi. The original adenoviral gene region between the two ITRs has been replaced by foreign non-coding DNA. In recombinant vectors derived from gutless vectors, this space is partially taken by the transgene. Gutless vectors can only be produced with the assistance of a helper virus, which provides the proteins required for viral replication and packaging.

Figure 3.12. Adenoviruses

Figure 3.13. Evolution and adenoviral vectors

Figure 3.14. Adenoviral gene therapy

Adeno-Associated Virus (AAV)

The adeno-associated virus (AAV), member of the parvovirus family, is a new promising candidate for the transfer of genes. AAV has an icosahedral structure and contains a single-stranded DNA genome of only 4.7 kb. It can only be replicated with the assistance of helper viruses such as adenoviruses or herpes viruses. Although a large proportion of the population is AAV-seropositive, so far no pathogenicity has been observed. In contrast to adenoviruses, AAV are only weakly immunogenic. They can infect dividing as well as stationary cells and integrate into the host genome, which is advantageous for long term expression. Wild type AAV contains no more than two genes, rep for replication and cap for encapsulation. The coding sequences are flanked by ITRs, which are needed for packaging DNA into capsids. In AAV vectors, the genes rep and cap have been replaced by a therapeutic gene. In order to produce a recombinant virus, the AAV genes and adenoviral helper genes are expressed in a packaging cell in trans. The major advantage of AAV-derived vectors is the ability to stably integrate into the target cell genome at a defined location in the chromosome. Location-specific insertion is mediated by a usually inactive 100 bp long region in the REP protein. However, since AAV vectors no longer contain the rep gene, targeted integration could only be detected using wild type AAV. Furthermore, since AAV is widespread among the human population, the question arises if the AAV-specific insertion location has been occupied by other genetic material. This would have to be removed first before an AAV vector could be used efficiently. It is also unclear what would happen if the insertion location of an AAV-derived vector were not available - whether sequence-independent insertion or even chromosomal relocation would take place. Another interesting property in AAV vectors derives from their specific chromosomal insertion: the capacity of homologous recombination. It was also possible to correct point mutations and deletions using an AAV vector, albeit at a very low frequency. This approach might also hold promising therapeutic possibilities.

Figure 3.15. Adeno-associated viruses
At this stage, the production of AAV vectors is still a major problem, being very difficult and time-consuming. The rep gene and some of the adenoviral helper genes are cytotoxic to packaging cells, and there are no cell lines available for the large-scale production of pure recombinant viruses. Despite many limitations, AAV vectors are quite useful gene transfer systems, since they achieve excellent expression in muscle, brain, hematopoietic precursor cells, neurons, photoreceptor cells, and hepatocytes.

### 3. 3.3. General gene therapy strategies

Gene augmentation therapy (GAT). For diseases caused by loss of function of a gene, introducing extra copies of the normal gene may increase the amount of normal gene product to a level where the normal phenotype is restored. As a result GAT is targeted at clinical disorders where the pathogenesis is reversible. It also helps to have no precise requirement for expression levels of the introduced gene and a clinical response at low expression levels. GAT has been particularly applied to autosomal recessive disorders where even modest expression levels of an introduced gene may make a substantial difference. Dominantly inherited disorders are much less amenable to treatment: gain-of-function mutations are not treatable by this approach and, even if there is a loss-of-function mutation, high expression efficiency of the introduced gene is required: individuals with 50% of normal gene product are normally affected, and so the challenge is to increase the amount of gene product towards normal levels.

**Figure 3.16.** Gene therapy strategies I.

Figure 3.17. Figure 3.17. Gene therapy strategy II.

Figure 3.18. Figure 3.18. Gene therapy strategy III.

Targeted killing of specific cells. This general approach is popular in cancer gene therapies. Genes are directed to the target cells and then expressed so as to cause cell killing. Direct cell killing is possible if the inserted genes are expressed to produce a lethal toxin (suicide genes), or a gene encoding a prodrug is inserted, conferring susceptibility to killing by a subsequently administered drug. Alternatively, selectively lytic viruses can be used. Indirect cell killing uses immune-stimulatory genes to provoke or enhance an immune response against the target cell.

Targeted inhibition of gene expression. If disease cells display a novel gene product or inappropriate expression of a gene (as in the case of many cancers, infectious diseases, etc.), a variety of different systems can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels. Allele-specific inhibition of expression may be possible in some cases, permitting therapies for some disorders resulting from dominant negative effects. (The example shows correction of a mutation in a mutant gene by homologous recombination, but mutation correction may also be possible at the RNA level. ODN, oligodeoxynucleotide; TFO, triplex-forming oligonucleotide.)

Targeted mutation correction. If an inherited mutation produces a dominant-negative effect, gene augmentation is unlikely to help. Instead the resident mutation must be corrected. Because of practical difficulties, this approach has yet to be applied but, in principle, it can be done at different levels: at the gene level (e.g. by gene targeting methods based on homologous recombination); or at the RNA transcript level (e.g. by using particular types of therapeutic ribozymes or therapeutic RNA editing).

4. 3.4. Human gene therapy

Human gene therapy is a complex, multiphase process involving the identification of genes causing or related to disease, development, in vitro testing and manufacturing of the gene transfer vectors, preclinical testing and toxicology studies in animal models, and clinical development of gene therapy products through the three phases of clinical trials. Many metabolic diseases are potential candidates for treatment with gene therapy. Candidate diseases include those caused by a single gene defect such as severe combined immunodeficiency (SCID) and familial hypercholesterolemia (FH), more complex multifactorial diseases, including diabetes mellitus and atherosclerosis, and acquired diseases like AIDS.

Figure 3.19. Figure 3.19. Severe combined immunodeficiency (SCID); lack of adenosine deaminase (ADA)
Figure 3.20. Gene therapy for severe immunodeficiency synfrom I.

Figure 3.21. Gene therapy for severe immunodeficiency synfrom I.
The first phase 1 gene therapy clinical trial, which aimed to provide detailed information on the safety and feasibility of the gene therapy procedure, began in 1990 when a 4-year-old girl with SCID was injected with autologous T cells transduced ex vivo with a retroviral vector containing the human adenosine deaminase (ADA) cDNA. A 12-year follow-up study reported that ten years after the last ex vivo transduced cell infusion, approximately 20% of the patient's lymphocytes still carry and express the retroviral gene; however, the expression was not considered sufficient to allow withdrawal of the supplementary PEG-ADA treatment. Two other patients, treated with the same transduction protocol, developed precipitating antibodies against fetal bovine serum, present in the infused cell suspension. Antibodies to MMLV p30 core protein were also detected from the patients following the cell infusions. Subsequently, successful gene therapy after retroviral ex vivo gene transfer to the autologous hematopoietic stem cells of nine out of eleven infants with sustained (up to over four years) correction of SCID phenotype has been reported. Unfortunately, almost three years after completing therapy, two children developed T cell leukemia, possibly owing to insertional mutagenesis associated with retrovirally mediated gene transfer. Another adverse event, systemic inflammatory response syndrome, leading to the death of an 18-year-old male who participated in a safety study of E1, E4 deleted Adenovirus-mediated gene transfer of human ornithine transcarbamoylase (OTC) into the right hepatic artery at high dose, has been reported in 2003.

**Figure 3.22. Ornithine transcarbamoylase (OTC) deficiency**

- most common disorder of urea cycle
- X-linked recessive disorder
- Low-protein diet and administration of medications scavenging nitrogen
Genetic defects in the LDL receptor

Four classes of mutations that disrupt the structure and function of the LDL receptor and cause FH. Each class of mutation affects a different region in the gene and thus interferes with a different step in the process by which the receptor is synthesized, processed in the Golgi complex, and transported to coated pits. Class I Mutations: no receptors synthesized; Class II Mutations: receptor synthesized, but transported slowly from ER to Golgi; Class III Mutations: receptors processed and reach cell surface, but fail to bind LDL normally; Class IV Mutations: receptors reach cell surface and bind LDL, but fail to cluster in coated pits.

**Figure 3.23. Transports of lipids with plasma lipoproteins**

![Image of lipoprotein transport](image1)

**Figure 3.24. Regulation of the mevalonate pathway**

![Image of mevalonate pathway](image2)

The therapeutic implications of the LDL receptor studies center on strategies for increasing the production of LDL receptors in the liver, thereby lowering plasma LDL-cholesterol levels. In FH heterozygotes this goal can be attained by stimulating the normal gene to produce more than its usual number of LDL receptors, thus...
compensating for the defective allele. Inasmuch as the liver is the major site of expression of LDL receptors, the therapeutic problem is reduced to the development of methods to increase hepatic demands for cholesterol. This can be achieved by two techniques: 1) inhibition of the intestinal reabsorption of bile acids; and 2) inhibition of cholesterol synthesis. These techniques can be used alone or in combination. The liver requires cholesterol for conversion into bile acids, which constitute the major route by which cholesterol is excreted from the body. However, only a fraction of the bile acids secreted by the liver actually leaves the body. The vast bulk of bile acids are reabsorbed in the terminal ileum and returned to the liver for reutilization. As a result, the liver converts only a minimal amount of cholesterol into bile acids. The liver’s demand for cholesterol can be enhanced by the ingestion of resins that bind bile acids in the intestine and prevent their reabsorption. Since the liver can no longer re-use old bile acids, it must continually make new bile acids and the liver’s demand for cholesterol increases. In order to obtain this cholesterol, the liver makes a dual response: 1) it synthesizes increased amounts of cholesterol through an increase in the activity of HMG-CoA reductase; and 2) it attempts to take up additional plasma cholesterol by increasing the production of LDL receptors. The increased LDL receptor activity causes plasma LDL levels to fall.

**Figure 3.25. Levels of the genetic deficiencies of LDL receptor**

![Image of LDL receptor deficiencies](image)

**Figure 3.26. Correlation between the LDL cholesterol level of blood and the number of LDL receptors in liver**

![Image of cholesterol and LDL receptors](image)
The second method for increasing LDL receptor production, namely, inhibition of hepatic cholesterol synthesis, is much more powerful than bile acid depletion. The principles applied to treatment of FH heterozygotes cannot, unfortunately, be applied to homozygotes, especially those who have totally defective LDL receptor genes. These individuals do not respond to the above-mentioned drugs because they cannot synthesize LDL receptors. The first gene therapy technique developed for FH utilized the ex vivo approach. In it recombinant amphotropic retroviruses carrying the LDL receptor gene were used to transduce hepatocytes in rabbits, and eventually in the first human FH gene therapy clinical trial in 1994-1995. In the procedure, the patients underwent hepatic resection and placement of a portal vein catheter. Primary hepatocyte cultures were prepared from the resected liver and transduced with the retrovirus. The autologous hepatocytes were subsequently transplanted back into the donor liver via the portal circulation.

**Figure 3.27.** General treatments of the high plasma cholesterol level

Cystic fibrosis (CF)

CF, the most common recessive disease seen in Caucasians (frequency 1:3000), is caused by a mutation in the CFTR gene. Patients homozygous for the CF mutation suffer problems associated with the production of thick and sticky mucus. Their lung function is compromised, they require pancreatic enzymes to digest their food and many are infertile. Current therapies include physiotherapy, heart—lung transplantation, DNase treatment and antibiotics. Despite available treatments CF is a lethal disease with patients rarely living beyond 40 years.
The CFTR gene product is a membrane protein that acts as a channel. The normal gene has been cloned and expressed in CFTR negative cells, restoring function. Animal models are available for the disease. Gene therapy for CF focuses on treatment of the lungs, in spite of the fact that other organs may be affected, as it is the lung pathology that is difficult to control and will ultimately kill the patient. The lungs are easily accessible by use of aerosols such as those used in asthma treatment.

A recent review of CF gene therapy has stated that although clinical studies have demonstrated proof-of-principle for correction of the defect in CF patients using viral vectors, this has not yet resulted in gene therapy for patients. Problems associated with inefficient gene transfer and host immune responses caused by viral vectors have effectively halted trials. Non-viral delivery is being more thoroughly investigated using cationic liposome - plasmid DNA complexes and DNA nanoparticles to deliver the CFTR gene. These have shown some promise in phase I clinical trials but the levels of CFTR expression achieved in the respiratory epithelium were too low and only of limited duration.

Summary

Despite these drawbacks, human gene therapy clinical trials are ongoing and success has been achieved. More than 900 gene therapy phase I to III clinical trials have been approved worldwide during the last 15 years. The majority (66%) of these trials were directed towards the fight against cancer, about 10% to the treatment of monogenic diseases and 8% to the treatment of vascular diseases (http://www.wiley.co.uk/genmed/clinical/).
Chapter 4. 4. Protein replacement therapies.

1.

The development and use of protein therapeutics have increased substantially in recent years, mostly as a consequence of improvements of recombinant technologies. Protein therapeutics have several advantages over small-molecular-weight drugs: they are typically highly specific and provide complex functions. Protein therapeutics used nowadays can be classified in various ways. One of their most important groups involves proteins utilized to replace proteins that are not produced or abnormal. Many protein therapeutics are used to modify signal transduction pathways (e.g. interferons), supplement growth hormones (e.g. platelet-derived growth hormone, PDGF) or influences haemostasis (e.g. tissue-type plasminogen activator, tPA). Other protein therapeutic enzymes are utilized to degrade molecules (like Asparaginase used in therapy of acute lymphocytic leukaemia). Therapeutic proteins can also be utilized in vaccination (e.g. against human papilloma virus HPV).

There are several current practical examples for the use of therapeutic proteins to supply missing proteins or to replace dysfunctional ones. Diabetes is one of the most common human diseases, and the use of recombinant insulin products is critical for its treatment. Albumin was first produced in mass quantities after the II. World War, from outdated pooled human donor plasma, due to its military importance. Lactose intolerance is actually the lack of a gain of function mutations, nevertheless, taking into account the widespread dietary use of milk and milk products, the use of lactase is very beneficial for the persons suffering from this condition. The classical examples of hemophilia are the deficiencies of blood coagulation factors VIII and IX. These factors were originally produced from pooled donor plasma for the use in therapy, than later were replaced by proteins made by recombinant technologies. The therapeutic concentrate used in protein C deficiency was also produced from outdated human donor plasma. In the patients suffering from Gaucher’s disease, the beta-glucocerebrosidase enzyme is missing. As a consequence, lipids accumulate in cells and in certain organs.

Examples of commercially available therapeutic protein products: Humulin and Novolin are recombinant insulin-containing products. Flexbumin 25% is a 25% solution of albumin prepared by alcoholic precipitation from pooled outdated human plasma. Lactaid is a lactase formulated into pills. ReFacto and Benefix are recombinant Factor VIII and Factor IX products, respectively. The active component of Ceprotin concentrate is activated protein C.

The first example of therapeutic protein utilization is the “protein vaccine” of cowpox used to prevent smallpox by Edward Jenner (1796). The first example of a protein utilized as a drug was the use of insulin by Banting and Best to treat a diabetic patient (1922). Currently more than 200 peptide or protein have been approved in USA for use in therapy.

As mentioned above, therapeutic proteins may come from different sources. Originally insulin was purified from pig and bovine pancreas, while nowadays only recombinant protein products are utilized. The source for therapeutic albumin production is still outdated human plasma. Factor VIII and IX were originally also purified from human plasma, but these products have been replaced by recombinant ones. The source of calcitonine was originally salmon. Anti-venoms originated from horse or donkey blood. Beta glucocerebrosidase was originally purified from human placenta.

Although therapeutic proteins isolated from natural sources are still in use, most of the protein therapeutics utilized are produced by recombinant technologies. The use of recombinant technologies provides several advantages over the purification of proteins from natural sources. The natural source of a given protein is usually sparse and expensive, the isolation of the protein is hard, and due to the limited source it is typically difficult to satisfy demands. For example, to isolate beta-glucocerebrosidase to treat a patient for a year required 50000 human placentas as source material. Furthermore, use of animal proteins could trigger immune response, as observed originally with insulin preparations of animal origin. Proteins isolated from natural sources could contain viral and pathogen contaminations. For example, a substantial number of HIV-infected individuals in the 1980’s in USA were hemophilic patients infected by contaminated blood products.

The first protein replacement therapy was performed in January 1922 by Banting and Best who first used insulin isolated from animal pancreas to treat a 14-years-old patient named Leonard Thomson. Leonard became more ill...
as the consequence of the injection (due to immune reaction), but his blood glucose level decreased, therefore the improvement of the preparation technique was decided. Six weeks later a better extract was able to decrease the blood glucose level from 520 mg/dL to 120 mg/dL within 24 hours. Leonard lived for additional 13 years; he died of pneumonia at the age 27.

The relatively simple structure of the small-sized insulin (Figure 4.1.), made it possible that insulin became the first commercially available recombinant protein therapeutics. Sanger was awarded by a Nobel Prize in Chemistry for the determination of insulin structure.

**Figure 4.1. Figure 4.1. Structure of insulin.**

![Insulin structure diagram](image)

Insulin has two polypeptide chains. The A-chain is 21 amino acid residues-long, while the B-chain is 30 amino acid residues-long. The two chains are held together by a disulfide bridge. Later the insulin gene, located at chromosome 11 was cloned. This cloning made it possible to use insulin as the first human recombinant therapeutic protein.

In spite of the rapid developments of recombinant technologies, the human blood still remains an important protein source for therapeutic purposes. The human body contains approx. 6 liters of blood of which 60-70% is plasma, 8-9% proteins. Human plasma contains about 10,000 different proteins and about 20 proteins make up the 99% of the total protein content of plasma. As annually more than 2 million liters of outdated transfusion plasma is generated, it is an excellent and relatively abundant protein source.

Blood coagulation factors VIII and IX were traditionally produced from blood, however, these products have been replaced by recombinant ones. Albumin, as well as intravenous immunoglobulin solution that generally can be utilized in infections, are still produced from human plasma. Some minor products, like Antithrombin III and Alpha I protease inhibitor that can be used in protein replacement therapy in coagulation disorder and emphysema, respectively, are also produced from plasma, the recombinant technology of their production is being developed.

The traditional method still used in production of blood proteins is Cohn fractionation, originally developed in 1946. In Cohn fractionation plasma proteins are selectively precipitated by using ethanol, salt, temperature change, then separation of the fractions is achieved by centrifugation.
The dramatic evolution of molecular biological techniques in the 1970’s and 80’s made it possible to develop the recombinant protein expression technologies. One of the milestones of these advances was the discovery of restriction enzymes by Paul Berg (1973). The cloning of human insulin gene in E. coli by Herbert Boyer (1978, Genentech) was a critical step in the production of recombinant insulin as the first recombinant protein therapeutics. The current recombinant technologies utilize two fundamental approaches. The expression in individual cells is the most common method, while the expression in transgenic plants or animals provides several advantages in the future.

Treatment of bleeding disorders developed as a consequence of coagulation factor deficiencies requires the supplementation of the given factor.

The coagulation cascade leads to crosslinked fibrin formation required to cover the blood vessel wall injuries. Traditionally, the coagulation cascade is divided into two pathways: the intrinsic and the extrinsic pathway. The in vivo activation of blood coagulation is mediated exclusively through the extrinsic pathway and is initiated by the binding of the enzyme factor VIIa to tissue factor (TF). TF (an integral membrane protein), becomes exposed whenever the endothelial cell lining is damaged. TF acts as a cofactor for FVIIa in the activation of FX, a reaction that occurs on the negatively charged phospholipid surface contributed by the damaged tissue. The FXa will generate small amounts of thrombin, which in turn activates FV into FVa and FVII to FVIIa, the cofactors for further prothrombin activation. The main role of thrombin in the advanced stage of coagulation is to generate crosslinked fibrin polymers establishing a stable thrombus. Thrombin is also capable to activate FXI into FXIa, which in turn activates FIX. FIXa, then assembles on negatively charged phospholipids with its cofactor FVIII(a). The importance of the formation of the complex of FIXa and FVIIIa on negatively charged phospholipids (known as the intrinsic tenase complex) is manifested by the classical hemophilias, the lack of properly functioning FVII or FIX.

Hemophilias A and B are caused by deficiencies in factors VIII or IX, respectively. These genetic disorders affect ~1 in 5,000 or 30,000 males in Hemophilia A and B, respectively, and are inherited as a recessive X-linked trait (mother would be an unaffected carrier). Hemophilia is also called as a "royal disease": Queen Victoria was a carrier for this mutation. The most famous related story is the manifestation of the hemophilia in...
The disease can be treated by administration of factor VIII or factor IX concentrates, as well as by recombinant factor VIII or XI. Recently several gene therapy trials are ongoing to provide a better cure.

Factor IX, missing of which causes Hemophilia B is a zymogenic form of a serine protease, synthesized by the liver in a pre-pro-form, and secreted to the bloodstream. The removal of the signal peptide occurs in the secretory pathway, while in the advanced stage of blood coagulation either XIa or the VIIIa-TF having an altered substrate specificity removes an activation peptide from the precursor and hence activate it.

**Figure 4.3. Activation of Factor XI.**

Factor VIII shares many structural and functional features with FV. It is a large (300 kD) glycoprotein, which is synthesized in the liver and secreted by it to the bloodstream. It is circulating in the blood in a complex with von Willebrand Factor. It binds to the phospholipid surface of activated platelets together with FIXa and FX to form the “intrinsic X-ase” (tenase) complex and exerts a double effect. It enhances the activity of FIXa but by binding to FX it also enhances its proteolysis. Thrombin activates FVIII yielding FVIIIa, which three fragments are held together by Ca2+, and has a much higher accelerating effect.

**Figure 4.4. Structure and activation of Factor VIII.**
The evolution of the hemophilia A treatment with protein replacement therapy provides a good example how the technology is advancing. The original use of blood protein preparations was replaced by recombinant proteins, and nowadays the gene transfer technologies are being developed. The hemophilia A is a good candidate for gene therapy developments. Severe symptoms (spontaneous bleeding into joints, vital organs) develop only if the FVIII level in circulation is lower than 1% of the normal level, therefore even low levels of proteins is beneficial. Tight control of Factor VIII expression is not required. The broad therapeutic index of FVIII minimizes the risk of overdose, and delivery to the bloodstream does not require expression in the liver. Interestingly, domain B is not required for the haemostatic functions of FVIII and in its absence the eukaryotic protein expression levels are higher. A gene transfer can be achieved in many ways. A procedure already introduced to clinical trials used fibroblast cells obtained by skin biopsy. The cells were transfected with a plasmid harboring the FVIII gene. After the selection and cloning of the properly transfected cells, the cell cultures were expanded and implanted into the omentum of the patients. The procedure provided sufficient amount of FVIII in the bloodstream for up to 10 months.

**Figure 4.5.** Steps in a human Factor VIII gene transfer protocol.
Skin biopsy to isolate fibroblasts

Transfection with factor VIII plasmid

Selection and cloning of cells expressing factor VIII

Expansion and characterization of clone

Implantation of cells in omentum
Chapter 5. 5. Recombinant antibodies and the phage display technology

1. 5.1. Introduction

Recombinant antibodies are antibodies containing sequences originating from different species (usually human and mice) and are able to specifically recognize antigens. The production of recombinant antibodies constitutes a specific part of recombinant protein technology as far as during the production/isolation process it is extremely important to preserve the antigen binding capacity of the antibody. In this chapter the structure of antibodies, their production in the body and the possibilities for recombinant antibody production will be discussed.

1.1. 5.1.1. The structure of antibodies and their production in the body

Antibodies are molecules made up of heavy and light chains and produced in specialized B cells and plasma cells. The combinatorial diversity makes possible the generation of a huge variety of antibody molecules, while the junctional diversity and affinity maturation increase even more the number of possible antibody variants. The immune system is able to recognise 10^7 antigents and to produce 10^9 different antibodies. On the 3' end of the V (variable) genes, on the 5' end of the J (junction) genes and on both sides of the D (diversity) genes, there are recombination signals recognized by a V(D)J recombinase enzyme. In the course of recombination, the randomly selected V, D and J genes combine, giving rise to the variable region to which a randomly selected C gene attaches, generating the gene encoding for a specific antibody. In the case of light chain, the process is similar but the light chain genes lack the D genes. In the course of recombination, the deletions or insertions at the junction sites can increase the variability by a process called junctional diversity. In the B cells the mutation rate is much higher than in the other cells of the body. The B cell receptors can be further modified by these somatic hypermutations and can give rise to variants which can bind the epitope better or for a longer time.

The clonal selection and clonal expansion make possible the appearance of a huge number of specific antibody-producing B cells in a very short period of time. The antibodies produced by the B cells are transported to the cell surface, giving rise to B cell receptors (BCR). The cells having B cell receptor that can bind to proteins in the bone marrow will enable the recombination to happen again. Such cells will be reprogrammed by receptor editing as the bone marrow is a relatively sterile environment and the protein recognized is most probably a self-protein. Another possibility to remove the self-recognizing B cells is cell death by apoptosis. Those cells which do not recognize self-proteins will be targeted to the periphery and as soon as they are activated by binding to pathogens in the spleen, they will become antigen producing plasma cells or memory cells.

Figure 5.1. Figure 5.1. The structure of antibodies.
5. Recombinant antibodies and the phage display technology

Figure 5.2. Figure 5.2. The structure of antibody heavy chain.

Figure 5.3. Figure 5.3. The structure of antibody light chain.

Figure 5.4. Figure 5.4. Production of antibodies by B cells.
1.2. 5.1.2. Antigen-antibody binding

The antibody recognizes the antigen and specifically binds to the epitope or antigen determinant, which is the region of the antigen specifically recognized by the antibody. The polyclonal antibody is a combination of immunoglobulins originated from different B cells recognizing different epitopes of the antigen while the monoclonal antibody contains immunoglobulins originated from a single type of B cells recognizing one epitope.

Figure 5.6. Figure 5.6. Polyclonal antibodies.
2. 5.2. The production of therapeutic antibodies

At the beginning of the 20th century Paul Ehrlich devised his magic bullet theory. According to this theory, the magic bullet is a substance selectively binding the pathogen and, with the help of the attached toxin, specifically kills it. The first monoclonal antibody was produced in 1973 by Jerrold Schwaber and George Pieczenik in human-mouse hybrid cells. The technique was later refined by Georges J.F. Kohler and César Milstein who were awarded the Medical Nobel prize for their work in 1984 together with Niels K. Jerne. The humanization of antibodies was started in 1988 by Georg Winter.

The production of antibodies is done by different techniques:

• Mice – generation of hybridoma cells – mouse antibody production
• Cell culture – production of recombinant human antibodies
• Genetically modified mice – human/humanized antibody production
5. Recombinant antibodies and the phage display technology

- Phage display – modified human antibody production.

2.1. 5.2.1. The production of antibodies in hybridoma cells.

Hybridoma cells are immortalized cells capable of the production of antibodies. The hybridoma cells are generated by the fusion of myeloma cells (providing infinite cell division) and immunized mouse spleen cells (providing antibody production). For the generation of hybridoma cells, the mice are immunized with an antigen and after several weeks the spleen cells are isolated and fused with myeloma cells. The myeloma cells lack the hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) enzyme thus only the fused cells will be able to survive in the special medium. The non-fused spleen cells have HGPRT enzyme as well but they cannot survive for a longer time as they are not immortalized. The generated hybridoma cells are further propagated and the antibodies are isolated from the cell culture medium. Monoclonal antibodies can be generated with this technique. This is a widely used method for antibody production especially for diagnostic and other purposes. For generation of therapeutic antibodies other methods are used because of the appearance of human anti mouse antibody (HAMA) against the mouse antibodies.

Figure 5.8. Figure 5.8. Production of antibodies in hybridoma cells.

2.2. 5.2.2. Humanized antibodies

Humanized antibodies are recombinant proteins containing both mouse and human sequences. In the course of the humanization some parts of the mouse antibody are changed to human ones in order to prevent HAMA production. The humanized antibodies are usually produced in mammalian cell cultures.

Figure 5.9. Figure 5.9. Humanized antibodies.
2.3. 5.2.3. Production of human antibodies

The major advantage of human antibodies is that the production of anti non-human antibodies is inhibited. The antibody generation can be done in vitro by two widely used techniques:

- Phage display
- In genetically modified mice producing human antibodies.

Figure 5.10. Production of human antibodies in genetically modified mice.

3. 5.3. Generation of antibodies by phage display

3.1. 5.3.1. The phage display technology

Phage display is an in vitro molecular biology technique, which uses the advantage of displaying different proteins/peptides on the surface of phages fused with the phage surface proteins. The technique can be used for antibody production as the number of possible variations achieved by the administration of phage libraries is equal to (or even higher than) the number of variations produced by organism. This is an easily designed system, which provides with the production of antibodies with different features and binding characteristics.
Most often, the M13 phage is used for this purpose. M13 is a lytic virus not killing the cell but generating growth retardation. The phage is able to infect E. coli cells bearing F-pilus as the phage p3 protein can specifically bind the F-pilus. The genes of small proteins or peptides can be attached to the genes encoding phage surface proteins forming the recombinant gene encoding for the recombinant phage surface protein. For this purpose the most widely used protein is the p3 (gpIII) protein.

The major advantage of the phage display technique is that the selection step can be precisely designed; only those phages remain bound to the matrix, which can harbour the protein specifically binding the immobilized proteins. The technique can be applied for antigen-antibody, enzyme-substrate, protein-ligand and protein-protein interaction studies.

During enzyme phage display, the enzyme is on the phage surface and can bind to its immobilized substrate.

The substrate phage display can be done in two ways: either the phage containing the product of the enzyme substrate reaction can bind to the matrix or only the phages having the substrate of the enzyme bound to the matrix can be removed from the surface.

During enzyme-substrate phage display both the enzyme and the substrate are displayed on the surface of the phage but only the product can bind to the matrix. In this case, only those phages where the enzyme interacts with the substrate and the enzyme substrate reaction happens can be immobilized:

- The enzyme cleaves the substrate and the phages are released from the matrix
- Only those phages bind to the matrix, which have an interaction between the substrate and its interaction partner.

Figure 5.11. Figure 5.11. The structure of M13 phage.

Figure 5.12. Figure 5.12. Specific elution of immobilized phage particles.
5. Recombinant antibodies and the phage display technology

Figure 5.13. Enzyme phage display.

Figure 5.14. Substrate phage display I.

Figure 5.15. Substrate phage display II.

Figure 5.16. Enzyme-substrate phage display I.
3.2. 5.3.2. Generation of phage libraries

In the course of library generation, the sequence series aimed to be expressed on the phage surface are fused to the p3 gene of the M13 phage giving rise to phagemids. The series of sequences to be inserted can originate from a genomic or cDNA library, error prone PCR etc. The generated phagemid is a plasmid which bears both features of plasmids and phages as well: it contains replication origin, bacterial selection marker (antibiotic resistance gene), the phage genes and packing signals. Each vector from a phagemid library will contain a different sequence, thereby making possible the generation of phage particles with different proteins/peptides expressed on their surface.

The phage libraries were successfully used in vitro for protease or other enzyme substrate design but there are in vivo administrations known as well for example in studies mapping the surface proteins of vascular endothelial cells. The phage libraries generated from cDNA libraries isolated from whole blood cells can lead to a library producing as much as 108 different antibody variants.

Figure 5.18. Figure 5.18. Generation of phage libraries.
5. Recombinant antibodies and the phage display technology

Figure 5.19. Generation of protease substrate phage library.

Figure 5.20. Substrate phage display – engineering of protease substrate sequences.
Figure 5.21. Figure 5.21. In vivo phage display – mapping vascular endothelial cells.

Figure 5.22. Figure 5.22. Generation of antibody libraries from whole blood.
4. 5.4. Administration of therapeutic antibodies

The recombinant antibodies are used as therapeutics, during diagnosis and in research as well. The therapeutic antibodies are antibodies administrated in different human therapies. In their case the high purity (>99%) is extremely important and can be achieved with the administration of different recombinant protein purification techniques. The therapeutic antibodies are used in many cases to harbour substances, which being enriched on the surface of the cells, will kill the target cells. In the course of antibody dependent cell mediated cytotoxicity (ADCC), the constant region of the antibody will attract the killer cells of the immune system and through their Fc receptors the killer cells can bind tumour cells and kill them. The therapeutic antibodies can be used in the treatment of psoriasis, rheumatoid arthritis, Crohn disease, spondilitis etc. In these cases anti TNF alpha antibodies are administrated and injected into the blood stream. To inhibit the rejection of transplanted organs, anti IL2 antibodies are used.

The administrated antibodies can have various structures. In several cases, the whole antibody or the Fab region is used, in other cases the variable region or the combination of these regions is administrated.

Figure 5.23. The mechanism of antibody dependent cell mediated cytotoxicity (ADCC).
Figure 5.24. Administration therapeutic antibodies with immunosuppressant activity.

Figure 5.25. Forms of therapeutic antibodies.
Figure 5.26. Forms of small-sized therapeutic antibodies.
Chapter 6.6. Anti-cytokine Therapy (sepsis).

1. 6.1. The consequences of developing inflammation

Anti-cytokine based therapy for systemic and local inflammatory or immunologically-based diseases has some unique features. They tend to be specific to blocking of specific cytokine, most of them based on naturally occurring molecules, such as soluble molecules. They are non-toxic, and if they have some side effects, they tend to be host-defense related and non toxic as in usual sense of a small organic molecules. The most important thing is, however, that they are naturally based and we are using molecules to enhance what takes place in various diseases between agonism and antagonism.

The most important ones are IL-1, tumor necrosis factor (TNF), chemokine family, and particularly IL8.

Effects of IL1 and TNF-α on Endothelium

The basic mechanism of action for IL1 and TNF-α in inflammatory disease are their actions on endothelium. IL1 and TNF-α activate endothelium. There is a capillary leak that is observed in the presence of IL1 and TNF-α. There are up-regulation of platelet activating factor (PAF), prostaglandins (PG), nitric oxide (NO) and endothelial-leukocyte adhesion molecule (ELAM). In addition to these productions of cytokines, there is a high level expression of IL-8, which is important for neutrophil trafficking. The end results of this are vessel dilatation, capillary leak, emigration and degranulation of neutrophils in tissues, which cause injury.

In many of these animal models we can block neutrophil emigration, adhesion molecules, the synthesis of NO and PG. The rationale of utilization in anti-cytokine therapy constitutes block excitatory cytokines like IL-1 and TNF-α upstream that prevent the diversification of cascade. Most importantly, we prevent the usual production of these small molecules, for example the (cyclooxygenase) COX II for PG2, and inducible endothelial nitric oxide synthase (iNOS) for NO. Blocking IL-1 and TNF-α tend to block both activities of COX II and nitric oxide synthase inducible form, but not the endogenous ones. During this process we do not influence the level of endogenous regulatory molecules, however we only block the inducible cytokines, such as the main inducible inflammatory cytokines. That is the reason of attacking IL-1 and TNF-α.

Septic shock is a very tragic shock clinically. It is induced by infection or trauma and the first thing that was noticed in animal experiment was inflammatory cytokine storm. This is a storm, as we suddenly see from no level of cytokine to huge level of cytokines. The second wave is the small mediator molecules, such as PG and NO. All of which decrease the vascular resistance leading to hypotension, acidosis, and a decreased tissue oxygenation, and then myocardial suppression, refractory shock, organ ischemia, which can cause multiple organ failure, sometimes intravascular coagulation and death for multiple organ failure.

In experimental animal model, the cytokine cascade is the following: there is an early activation of C5a, then a pick of TNF-α, then pick of IL-1 and IL-6. The clinical and physiological consequences of these cytokines expression are hypotension, tissue injury, organ failure and death. Human data support animal data.

Figure 6.1. Migration of neutrophil from vascular space to tissues
6. Anti-cytokine Therapy (sepsis).

Figure 6.2. Stream of cytokines in sepsis

Figure 6.3. Inflammatory cytokines and anti-cytokines
2. 6.2. Development of Inflammatory Response: Synthesis of Lipid Mediators

In mammalian cells, two major pathways of arachidonic acid, a 20:4 fatty acid derived from linoleate, metabolism produce important mediators of cellular and bodily functions: the cyclooxygenase and the lipoxygenase pathways. The substrate for both pathways is free arachidonic acid. The cyclooxygenase pathway leads to a series of compounds including prostaglandins and thromboxanes. The lipoxygenase pathways lead to the synthesis of leukotriens. A series of prostaglandins is synthesized by reductases and isomerases. The major classes are designated PGA through PGI. Prostaglandins with two double bonds, such as PGE2, are derived from arachidonate. Prostacyclin and thromboxanes are related compounds that arise from a nascent prostaglandin. They are generated by prostacyclin synthase and thromboxane synthase respectively. Alternatively, arachidonate can be converted into leukotrienes by the action of lipoxygenase. These compounds, first found in leukocytes, contain three conjugated double bonds—hence, the name. Prostaglandins, prostacyclin, thromboxanes, and leukotrienes are called eicosanoids because they contain 20 carbon atoms. Prostaglandins and other eicosanoids are local hormones because they are short-lived. They alter the activities both of the cells in which they are synthesized and of the adjoining cells by binding to seven-transmembrane domain receptors. The nature of these effects may vary from one type of cell to another. Prostaglandins stimulate inflammation, regulate blood flow to particular organs, control ion transport across membranes, modulate synaptic transmission, and induce sleep.

Figure 6.4. Figure 6.4. Development of inflammatory response, synthesis of lipid mediators
Signaling Through Toll-Like Receptors

Several pathogen-associated molecular patterns (PAMPs) can stimulate TLR4. LPS is an important structural component of the outer membrane of Gram-negative bacteria. LPS consists of three parts: lipid A, a core oligosaccharide, and an O side chain. Lipid A is the main PAMP of LPS. LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4. LBP is a soluble shuttle protein which directly binds to LPS and facilitates the association between LPS and CD14. CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition. MD-2 is a soluble protein that non-covalently associates with TLR4. Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains. TIR domains contain three highly conserved regions, which mediate protein–protein interactions between the TLRs and signal transduction adaptor proteins such as MyD88. MyD88 with the help of other adaptor proteins (IRAK, IL-1 receptor-associated kinase-4 and TRAF6, TNF receptor-associated factor 6) activates TAK1 (transforming growth factor-β-activated kinase 1). TAK1 then activates downstream IKK (IκB kinase) which consists of IKKα, IKKβ and IKKγ proteins. IKKα, IKKβ and IKKγ form a complex and phosphorylate IκB (inhibitor of κ light chain gene enhancer in B cells) proteins. This phosphorylation leads to the degradation of IκB proteins and the subsequent translocation of the transcription factor NF-kB into nucleus, which controls the expression of proinflammatory cytokines, in addition to other immune related genes.

Figure 6.5. Figure 6.5. Development of inflammatory response I.
Synthesis of nitric oxide (NO)

NO is synthesized from L-arginine by the enzyme NO synthase (NOS), which exists in three isoforms, of which two are constitutive and the third is an inducible form (iNOS). Constitutive NOS, responsible for the continuous basal release of NO under physiological conditions. Inducible NOS, slowly expressed in response to cytokines and lipopolysaccharides, can generate large amounts of NO even in the absence of Ca²⁺. NO diffuses out of the endothelial cell and into adjacent smooth muscle cells where it binds to and activates soluble guanylate cyclase,
which makes cGMP. cGMP activates protein kinase G, a cGMP-dependent kinase that phosphorylates a variety of channels, including L-type calcium channels, and receptors, all leading to inhibition of calcium influx into the smooth muscle cell. This leads to decreased calmodulin stimulation of muscle light chain kinase (MLCK) and decreased phosphorylation of myosin light chain (MLC), causing diminished development of smooth muscle tension and, thus, vasodilation.

**Figure 6.7. Synthesis of NO controlled by cytokine**

3. 6.3. Role of the Liver in Maintenance of Homeostasis: Acute Phase Response

The acute phase of the inflammatory response refers to the wide ranging physiological changes that are initiated immediately after an infection or physical trauma has occurred. The mammalian acute phase response is characterized by fever, changes in vascular permeability, along with changes in the biosynthetic, metabolic and catabolic profiles of many organs. The response is initiated and coordinated by a large number of diverse inflammatory mediators, which include cytokines anaphylatoxins and glucocorticoids. Some of these are released initially at the site of inflammation by activated mononuclear phagocytes, lymphocytes or other differentiated cell types and have potent local and systemic effects. The ensuing cascade of mediators induces activation, proliferation, altered behaviour and changes in the biosynthetic capacities of a variety of target cells and tissues. This enhances host-survival by neutralizing the inflammatory agent and promoting repair process, thus initiating a return to normal function. Normally, the acute phase response lasts only a few days; however, in cases of chronic or recurring inflammation, an aberrant continuation of some aspect of the acute phase response may contribute to the underlying tissue damage that accompanies the disease, and may also lead to further complication, for example cardiovascular disease.

An important aspect of acute phase response is the radically altered biosynthetic profile of the liver. Under normal circumstances, the liver synthesized a characteristic range of plasma proteins at steady state concentrations. Many of these proteins have important functions and higher plasma levels of these “acute phase reactants” (APRs) are required during the acute phase response following an inflammatory stimulus. Although most APRs are synthesized by hepatocytes, some are synthesized by other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes. So-called “negative APRs” are decreased in plasma concentration during the acute phase response to allow an increase in the capacity of the liver to synthesize the induced APRs.

Of the many soluble factors that initiate and maintain an inflammatory response, several hormones specifically regulate the transcription of human APRs. These include interleukin 1 (IL-1), IL-6, TNF-α, transforming growth factor β (TGF-β) interferon-α (INF-α) and glucocorticoids. An important features of the acute phase
response is that IL-1 and TNF-α stimulate via the CNS, the synthesis of glucocorticoids by the adrenal glands, which results in cooperative enhancement of the IL-1 and TNF-α-mediated induction of APR synthesis in the liver. This effects is coincident with the glucocorticoid-mediated downregulation of IL-1 synthesis by macrophages, thereby creating a negative-feedback loop between the immune and CNS systems to reduced de novo cytokine synthesis.

APRs have a wide range of activities that contribute to host defence. They can directly neutralize inflammatory agents, help to minimize the extent of local tissue damage, as well as participate in tissue repair and regeneration. There is a rapid increase in the plasma concentration of many complement cascade components the activation of which ultimately results in the local accumulation of neutrophils, macrophages and plasma proteins. These participate the killing of infectious agents, the clearance of foreign and host cellular debris, and the repair of damaged tissue. Coagulation components, such as fibrinogen, play an essential role in promoting wound healing.

Proteinase inhibitors neutralize the lysosomal hydrolases released following the infiltration of activated macrophages and neutrophils, thus controlling the activity of the pro-inflammatory enzyme cascades mentioned above. The increased plasma levels of some metal-binding proteins help prevent iron loss during infection and injury, also minimizing the levels of heme iron available for uptake by bacteria and acting as scavengers for potentially damaging oxygen free radicals.

Most APRS are induced between 50% and several-fold over normal level. In contrast, the so-called major APRs’ can increase to 1000-fold over normal levels. This group includes serum amyloid A (SSA), C-reactive protein (CRP) and serum amyloid P component (SAP).

**Figure 6.8.** Acute phase response

**Figure 6.9.** Inflammatory mediators that modulate hepatic APR synthesis
4. 6.4. Time-Course of the Inflammatory Response During Sepsis

Infectious process can generalize very severe inflammatory response called sepsis, which is manifested by organ dysfunction (i.e., hypoperfusion, tissue hypoxia, lung injury, etc.). The hyper-inflammatory responses are mediated by excessive production of cytokines (TNF-α), involving both hyperactive cellular and humoral defense mechanism. Both lead to tissue injury and organ dysfunction. Inflammation is a normal protective response of the body in which cytokine production and leukocytes activation are properly regulated to eliminate harmful stimuli, such as pathogens. In sepsis dysregulated hyper-inflammatory responses can occur by the abnormal innate immune response to infection. Pro-inflammatory cytokines, chemokines and nitric oxide are synthesized by activated macrophages/monocytes through their toll-like receptors (TLRs) which leads to the activation of nuclear factor κB (NF-κB). TNF-α plays an important role in the pathogenesis of an early shock state (i.e. hypotension, fever) and at least part of organ dysfunction related to septic shock. TNF-α elicits neutrophil-mediated tissue injury by acting on endothelial cells and neutrophils. Endothelial cells exposed to TNF-α become activated, and commence the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) as well as chemokines. TNF-α also activates neutrophils by up-regulating integrin adhesiveness and promoting extravasation to the organs. Extravasated neutrophils damage tissues by releasing oxygen free radicals and proteases. In addition, TNF-α amplifies inflammatory cascades in an autocrine and paracrine manner by activating macrophages/monocytes to secrete other pro-inflammatory cytokines.

At the onset of sepsis, the inflammatory system becomes hyperactive, involving both cellular and humoral defense mechanisms. It is important to point out that during at onset of sepsis, the early appearance of cytokines and chemokines in the serum is well-established in rodents, whereas such patterns are less evident in humans. Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes, produce powerful pro-inflammatory mediators (TNF-α, IL-6, IL-1 and IL-8). Simultaneously, robust production of acute-phase proteins, such as C-reactive protein, occurs and humoral defense mechanisms such as the complement system are activated, resulting in production of pro-inflammatory mediators, including C5a, the complement split product. C5a ultimately enhances cytokine and chemokine production. Furthermore, the coagulation system becomes activated through various mechanisms, often resulting in disseminated intravascular coagulopathy. The described mediators are produced early at the onset of sepsis and reflect the overactive status of the inflammatory response. Phagocytic cells (neutrophils and macrophages) respond to many of these mediators by releasing granular enzymes and producing reactive oxygen species (ROS) such as H2O2, which is a crucial product for the killing of bacteria. H2O2 is also capable of causing tissue damage, which ultimately leads to increased vascular permeability and organ injury. In later stages of sepsis, anti-inflammatory mediators are produced (such as IL-10, transforming growth factor-β and IL-13), leading to abatement in the production of many of the pro-inflammatory mediators. In this phase, various innate functions are suppressed, especially the functions of neutrophils, leading to a hyporeactive host defense system and immunoparalysis.

Figure 6.10. Time-course of the inflammatory response during sepsis
Crosstalk Between Inflammation and Coagulation

Complex interactions between inflammation and coagulation are involved in the pathogenesis of sepsis. Patients with sepsis exhibit platelet activation, up-regulation of pro-coagulation pathways and down-regulation of anti-coagulation pathways. These abnormal coagulation pathways lead to the formation of micro-vascular thromboses that compromise tissue perfusion and give rise to organ dysfunction. Inflammation in sepsis skews the balance of haemostasis to the pro-coagulation states. In endothelial cells, neutrophils and monocytes, TNF-α and IL-1 induce the expression of tissue factor, which is typically exposed by sub-endothelial cells upon vascular injury. The aberrant expression of tissue factor by TNF-α initiates blood coagulation cascades. Tissue factor then binds to factor VIIa (FVIIa) in circulating blood, thereby forming a tissue factor-FVIIa complex that activates FX to FXa. The latter subsequently converts prothrombin to thrombin. Thrombin, a serine protease, converts fibrinogen to fibrin, which is further stabilized by inter-molecular cross-linking via a transglutaminase FXIII similarly activated by thrombin. In fact, thrombin activates other coagulation factors such as FXI, FV, FVIII and FXIII. In addition, thrombin activates platelets, monocytes and endothelial cells through thrombin receptors. Thus, thrombin acts to form a positive feedback loop that can amplify coagulation as well as other inflammatory cascades.

Figure 6.11. Endothelial activation, coagulation and fibrin clot formation I.
6. Anti-cytokine Therapy (sepsis).

Figure 6.12. Coagulation response

Figure 6.13. Endothelial activation, coagulation and fibrin clot formation II.
6. Anti-cytokine Therapy (sepsis).

Figure 6.14. Figure 6.14. Coagulation, fibrin clot formation and inhibition of fibrinolysis

The thrombin pathway serves as a negative feedback loop that dampens coagulation. Protein C, a plasma protein synthesized in the liver and that circulates in the blood as an inactive zymogen, plays a critical role in this negative feedback process. Protein C is converted by thrombin to its active form, activated protein C (APC) and is deposited onto the surface of endothelial cells to form the thrombin-thrombomodulin-endothelial protein C complex.
receptor complex. APC with its co-factor protein S inactivates FVa and FIIIa, thereby negatively regulating blood coagulation. Defects in the negative feedback by APC can lead to hyper-coagulation states.

In addition to acting as an antithrombotic factor, APC possesses anti-inflammatory properties. It inhibits the production of inflammatory cytokines and the transduction of NF-κB signalling in monocytes stimulated by LPS and thrombin. APC also suppresses endothelial permeability as well as the transendothelial migration of neutrophils. Therefore, APC plays a pivotal role in the negative regulation of coagulation and inflammation. In patients with sepsis, the following observations have been made: serum concentration of protein C is decreased by inflammation, the conversion to APC is reduced and decreased protein C level is associated with poorer outcomes.

**Figure 6.15.** Figure 6.15. Endogenous activated Protein C has multiple Mechanisms of activation

Identification of a Late Mediator HMGB1

Kinetic analysis of cytokine production in sepsis has revealed that plasma levels of TNF-α and IL-1 peak at the early stage and decrease to undetectable levels in the late stage. Despite symptoms of shock during the early stage (e.g. hypotension, fever), most instances of mortality in experimental sepsis, as well as in sepsis patients, occur in the late stage. This partially explains why late neutralization of TNF-α, an early mediator of sepsis, failed to halt its progression in clinical trials. Searching for mediators that played a critical role in the late phase of sepsis, the cytokine HMGB1 (high-mobility group box 1) has been identified. HMGB1 acts as a late mediator in LPS-induced toxicity. Being produced by macrophages, it appears in plasma between 8 and 32 h after LPS infusion into mice.

Before its re-discovery as a cytokine that mediates lethality in the late phase of sepsis, HMGB1 was originally identified as a chromatin-binding protein in the nucleus that associated with chromosomal DNA. HMGB1 exists ubiquitously in the nucleus of all eukaryotic cells, where by binding to DNA and several transcription factors it plays a critical role in stabilizing nucleosome formation and in regulating transcription. HMGB1 is released from the nucleus to the extracellular space when cells undergo necrotic cell death. As the membrane integrity is disrupted during necrosis, HMGB1 is passively leaked into the extracellular space where it elicits inflammation. Of note, however, is the fact that apoptotic cells do not release HMGB1 extracellularly, since HMGB1 is sequestrated within the nucleus and prevented from release. The differing ability of necrotic and apoptotic cells
to release HMGB1 supports the idea that necrotic, but not apoptotic, cells evoke inflammation. In addition to passive release, HMGB1 is actively secreted from macrophages and monocytes upon activation with TNF.

The HMGB1 released into the extracellular space, either from necrotic cells in damaged tissue or from activated macrophages at sites of infection, generates signals that activate immune cells and induce inflammation. Extracellular HMGB1 signals via cell surface receptors such as Receptor for Advanced Glycation End-products (RAGE) and TLR2 and TLR4. Extracellular HMGB1 activates macrophages through RAGE and TLR2/4 to secrete TNF-α and other pro-inflammatory cytokines, thereby amplifying inflammatory cascades. By binding to RAGE on endothelial cells, HMGB1 up-regulates adhesion molecules ICAM-1, VCAM-1, TNF-α, chemokines, PAI-1 and tissue plasminogen activator (tPA) expression, and has been implicated in the enhanced accumulation of leukocytes. Thus, in addition to sustaining inflammation by acting on monocytes and macrophages, the induction of endothelial and epithelial damage could constitute a mechanism of HMGB1 released during sepsis to evoke organ dysfunction.

**Figure 6.16.** Contribution of high-mobility group box1 (HMGB1) to sepsis

**Figure 6.17.** Collapse of homeostasis
Apoptosis of Lymphocytes to Cause Immunosuppression

Sepsis is characterized by a surge of the pro-inflammatory cytokines at the early stage. However, as the disease progresses, the hyper-inflammatory state during the early stage coverts to the anti-inflammatory state, marked by decreased levels of TNF and increased levels of IL-10. IL-10 inhibits TNF-α production by macrophages and suppresses expression of costimulatory molecules in macrophages and T-cells. The increased production of IL-10 in the late phase of sepsis is believed to contribute to ‘immunosuppression’. In addition to increased levels of IL-10, depletion of immune cells by apoptosis has emerged as a potential pathological mechanism for immunosuppression in sepsis. Apoptosis of lymphocytes has been observed both in animal models and in autopsies of patients who have died from sepsis. The extent of apoptosis correlates with the severity of the disease. Depletion of lymphocytes is believed to compromise the immune system’s ability to control infection, thereby contributing to increased morbidity in sepsis.

Figure 6.18. Development of septic shock and organ failure

Summery
We have sufficient background to say that cytokine blockade in chronic autoimmune or inflammatory disorders is a valid strategy today. Although some risks are associated with the anti-cytokine therapeutics, the protocols are getting safe and efficient. The benefits, they provide to most of the patients, are not questionable.
Chapter 7.7. Animal models and transgenesis in biotechnology

1.

The physiology of animals is surprisingly similar to ours. For this reason, animals have long been used as experimental models. With the development of genetically modified animals, now we can generate “design” experimental animals that have a predefined set of genetic modifications and therefore can be used to investigate the role of genes in disease models in which putative causative genes have already been identified. This led to a revolution in biomedical sciences and significantly broadened our understanding of animal and human pathophysiology. Concomitant to the biomedical application of genetically modified animals, it became apparent that genetically modified animals could also be successfully used outside of the scope of biomedical research. The overall goal of this lecture is to present an intriguing and rapidly developing field of generating cloned or transgenic animals. In the lecture, we will discuss both cloning and genetic manipulation, which are very different from practical and principal standpoints. We will discuss the practical advantages and the accompanying ethical considerations pertaining to genetic modification.

There are several reasons why we turn to cloning and genetic modifications. One area where the above manipulations play very important roles is biomedical research. But genetic manipulations have a role to play in fields outside of basic science or medical research. One area where cloning/genetic manipulations are predicted to become more and more important is the production of small molecules or recombinant macromolecules in transgenic animals or plants. In human medicine, countless proteins are used to remedy diseases. Traditionally, some of these proteins (such as human growth hormone or insulin) were originally isolated from animal sources. However, neither the quantity nor the purity of these proteins isolated from animal tissues meet the requirements of human medicine. Later, some of the above proteins (such as insulin) were produced in bacteria. That was already a major step in biotechnology, since the production could be easily upscaled. However, bacterial production of human proteins still has drawbacks. One of the common problems of production of human proteins in bacteria derives from the fact that proteins often undergo posttranslational modifications, such as glycosylation and limited proteolysis. Not all of the eukaryotic posttranslational modifications can be recapitulated in bacterial cells. With clever genetic manipulation, it is possible to generate farm animals that produce human proteins in their milk. This means that a “renewable” and relatively safe source of human proteins will be available for use in medicine.

In biomedical research, animals are used with the basic assumption that the knowledge learned about a disease when using animals as model organisms is applicable to human medicine. With this principle in mind, there is a great need for animals that suffer from medical conditions affecting humans. However, a large number of medical conditions/diseases that cause great health concern to humans have no clear animal counterparts. Therefore, in order to study these diseases in animals, we need to create genetically modified animals that are susceptible to these diseases. The generation of genetically modified animals that show susceptibility to diseases reminiscent of specific human conditions often involves precise modifications of the genome. Gene targeting, the main method for precise modification of genomes is relatively simple to accomplish in the mouse. For this reason, the mouse is the most typical model organism whose genetically modified strains are used to create and study mouse diseases that are considered to be the equivalents of human diseases. Such mouse models were developed for the study of a wide range of diseases, including Alzheimer disease, cancer, obesity, diabetes or Crohn’s disease.

Once genetically modified animals are created, they can be used in a number of different applications. Such an application in which genetically modified animals play a major part is basic biomedical research. The genetic background of human diseases is often poorly understood. There are instances in which candidate genes are suspected to play an important part in the pathobiology of disease. Often, it is very difficult to prove the suspected link between the suspected gene and the disease in simple experimental systems. In these cases the generation of genetically modified animals (most often mice) in which the candidate gene is specifically inactivated/mutated can provide evidence of the suspected role of the gene in diseases.

Another major field of application of genetically modified animal models is drug/product testing and screening. For preclinical drug development, animal models are essential to the understanding of disease etiology and to ascertain that drugs are efficacious and have minimal side effects. Candidate bioactive molecules, drugs must
have no toxic effect in vivo. Screening for drug toxicity is impossible without using some form of whole animal pharmacology study. Another important field in which transgenic animals are expected to play a role is generating organs for xenotransplantations. There is a chronic shortage of transplantable organs. Some of the animals have organs that could potentially be used for human transplantations (such as hearts of pigs). The overall goal of genetic manipulation in these cases would be to create “humanized” animals, whose tissues would not be recognized as foreign by the human immune system. Naturally, cloning for organ replacement is the field that sees the most ethical concerns about the use of cloning and genetic manipulation.

There are 3 main sources of animal models used in biomedical research. Some animal models show up spontaneously. In these cases a serendipitous finding of a spontaneous mutation will result in an animal (most often mouse) strain that show characteristics that make it useful for biomedical research, and these characteristics are passed on to the next generation. Two important advances in basic research have been made possible due to such models. The ob/ob mouse, which suffers from early onset diabetes and is heavily overweight was not derived from a genetic manipulation but was created by spontaneous mutation of a gene. After years of hard work, scientists found that the gene that suffered mutation in this mouse is the gene for leptin. Leptin, an important metabolic hormone produced in the fat tissue and reports satiety to the brain was identified as a result of the work done on the spontaneous animal model. In another spontaneous model, mice were born that displayed little sensitivity to bacterial endotoxins (namely to LPS). These mice were found to have a mutation in a cell surface receptor. With the help of this strain, it was found that this receptor, TLR4, is the main receptor on our cells to detect LPS (and therefore the presence of bacteria in our body). Animals can also be cloned, and a large number of genetically identical animals can be created. Genetic manipulation of animals, on the other hand, generates animals whose genome is modified in a precise manner.

As already mentioned, we can generate a large number of genetically identical animals by cloning.克隆 animals can be used not only in biomedical research but also in agriculture, as it often happens. There are several current technologies by which cloning can be performed. Embryo splitting was the first method developed. In embryo splitting, early stage embryos are mechanically split. This allows the generation of identical twins from a single embryo. The other main technology used to create cloned animals is SCNT. Today, SCNT is the predominant technology. The first successful cloning of a mammal by SCNT was the generation of Dolly, the sheep. Since then, other mammals, including primates have been cloned by SCNT. This suggests that in theory human cloning would also be possible by SCNT. Naturally, this has caused huge ethical concerns.

Problems involved in animal cloning:

In the case of cloning Dolly, 200-300 attempts had been made before the experiment was successful (think about the time-, money- and ethical consequences). This raises practical and principal questions. As for the practical ramifications, the poor success rate means that unless major advances are being made in the technology of animal cloning, few farm animals can be propagated by cloning for commercial purposes with a reasonable outlook of financial gain. Additionally, Dolly died (much) earlier than sheep usually die (6 vs 12 years). It is uncertain to what extent this is due to the fact that Dolly was a cloned animal. (i.e., is cloning “bad for your health?”). Cloned animals are often in poor health and age faster than normal (respiratory or circulatory problems are common). It is not only farm animals that can be cloned: in 2001, scientists cloned a gaur, an endangered species for bioconservation purposes. Also, in 2004 the first sale of a cloned kitten took place (the kitten was not produced by SCNT, but a later technique that involved the transfer of the chromosomes (and not nucleus) to the egg.

With cloning, we can generate exact copies of animals of preexisting genetic makeup. A principally different way of generating animals for biomedical (or biotechnological purposes) is transgenesis. With transgenesis, we create animals whose genome is modified in a way that the animals can pass their modified genome to the next generation. With transgenesis, we create animals whose genome is unique. Such genetically modified animals can be created by two competing technologies.

By classical transgenesis, the cells of the germline are modified. A typical example for this is the manipulation of the fertilized mouse egg by a direct injection of foreign DNA into the male pronucleus. This is a relatively fast method to generate genetically modified animals. However, we can only insert a foreign DNA (typically a promoter and a gene, therefore an expression unit) into the host chromosome at a random location. Therefore, with classical transgenesis, we cannot modify the genome in a precise manner. The other technology, gene targeting, is carried out in mouse ES cells. This technology allows an extremely precise modification of the genome.
In different animal species, the different technologies mentioned earlier are used to various degrees. Several factors determine which technologies are preferred in a certain species. The availability of embryonic stem cells, the success rate of in vitro fertilization, the length of gestation period, the success rate of the genetic manipulation etc. all taken into account when choosing a technology to genetically modify animals. Although nowadays animals are either cloned (by SCNT) or their genome is modified by an available technology (such as classical transgenesis or gene targeting), it can be predicted that in a lot of applications cloning and genetic manipulation will converge. This means that cloning will be carried out on animals whose genome will have been successfully modified. This will enable scientists to create large numbers of identical animals whose genome would be modified in the exact same way.

Slide 20 summarizes the different technologies that can be applied in cloning or in genetic manipulation of animals. Principally, the different technologies can be divided into two major groups. Manipulation of ES cells has two advantages. One advantage is that genetic manipulation of ES cells allows (but not necessarily involves) a precise modification of genomes when foreign DNA is introduced by transfection (in gene targeting). Foreign DNA can also be introduced into ES cells by an infection in which the retroviruses that are used to infect ES cells carry an exogenous gene (recombinant retroviruses). The retroviral infection of ES cells will result in a random integration of the recombinant retroviral genetic material into the host genome. This has unpredictable consequences on the expression of the foreign gene. Random integration can also disrupt important host genes. Additionally, the expression of retrovirally introduced genes is often lost in cells, presumably due to various defense mechanisms of the host cell. As a result of the above disadvantages of retroviral transfection, the genome of ES cells are typically modified by transfection of foreign DNA followed by homologous recombination.

Cells of the germline (such as eggs, or early embryos) can also be genetically manipulated. This allows a faster but less precise manipulation of the genome.

Summary

In this lecture we have demonstrated the importance of animal models in biomedical research. We wanted to illustrate that today we no longer rely only a handful of “serendipitous” animal models, but we are able to generate genetically modified animals that are “tailor-made” to serve as experimental models for specific diseases. The lecture also showed that such genetically modified animals could be used in a number of seemingly unrelated fields, such as agriculture, biotechnology and pharmaceutical production. It is more than probable than we are to see a revolution in the utilization of genetically modified animals and therefore we have to be prepared to address the ethical questions that are raised by the usage of such organisms.
Chapter 8. 8. Embryonic and adult stem cells in regenerative medicine I.

The aim of this lecture is to define and characterize the embryonic and adult stem cells. This lecture also covers the necessary basic concepts of cell reprogramming and lineage conversion.

1. 8.1 Embryonic stem cells

Definition of stem cells: Cells which are characterized by the ability to renew themselves (self-renewal) through cell division and they have the potential to develop into many different cell types.

**Figure 8.1.** Figure 8.1. Stem cells I.

Classification of stem cells:

- **Totipotent cells:** Only zygotes and early morulas retain the ability to give rise to all embryonic and extra-embryonic cells and tissues. Fertilized egg is considered as a totipotent cell.

- **Pluripotent cells:** These cells can form all somatic cells (ectoderm, endoderm and mesoderm) and germline cells of the developing embryo, but they are unable to form extra-embryonic tissues. Embryonic stem (ES) cells are pluripotent cells. Multipotent cells: These cells, can only give rise to cell types within their lineage. Most adult stem cells are multipotent cells.

**Figure 8.2.** Figure 8.2. Stem cells II.
Embryonic stem cells:

The most important characteristics of embryonic stem (ES) cells: Unlimited proliferation capacity without differentiation (self-renewal). They are able to develop into all types of somatic cells that make up the body (pluripotency). ES specific markers: transcription factors (Oct4, Nanog, Sox2), Alkaline phosphatase, SSEA1 (mouse ES cells), SSEA3/4 (human ES cells) and telomerase activity.

**Figure 8.3. The most important characteristics of embryonic stem (ES) cells**

**The most important characteristics of embryonic stem (ES) cells:**

- **Self-renewal:** Unlimited proliferation capacity without differentiation (immortality).
- **Pluripotency:** They are able to develop into all types of somatic cells that make up the body.

**ES specific markers:**

- Transcription factors: Oct4, Nanog, Sox2
- Alkaline phosphatase
- SSEA1 (mouse ES cells)
- SSEA3/4 (human ES cells)
- Telomerase activity

**Factors for maintaining the pluripotent state:**

- LIF, BMP4 (mouse ES cells)
- bFGF, activin (human ES cells)

Factors for maintaining the pluripotent state: LIF, BMP4 (mouse ES cells), bFGF, activin (human ES cells).

Generation of human ES cells:
Human ES cells are established from preimplantation stage embryos. ES cells are usually derived from blastocysts that developed from eggs that had been fertilized in vitro in an IVF clinic and then donated for research purposes. Establishment of ES cell lines: The inner cell mass of the blastocyst contains pluripotent cells. These cells are isolated and propagated in tissue culture. ES cells can proliferate indefinitely and maintain their pluripotent state. ES cells can be also derived from other mammalian species (e.g., mouse, rat) using similar techniques.

**Figure 8.4. Generation of human ES cells**

ES cells can be injected into blastocysts and these cells will contribute to the embryo proper and form a chimeric animal. In addition, the injected cells may form germ cells (germ line transmission) and later these cells will produce oocytes or sperms.

**Figure 8.5. Generation of transgenic and knock out mice (blastocyst injection)**

2. 8.2 Somatic cell reprogramming into pluripotent stem cells
There are 3 ways to reprogram somatic cells into pluripotent cells:

a. Somatic cell nuclear transfer (SCNT)
b. Cell fusion
c. Transcription factor mediated reprogramming

Figure 8.6. Nucleic reprogramming of somatic cells into pluripotent cells

Animal cloning is based on somatic cell nuclear transfer.

Figure 8.7. Animal cloning is based on somatic cells nuclear transfer (SCNT)

In human only therapeutic cloning!

The overwhelming consensus of the world’s scientific and medical communities is that human reproductive cloning should be banned.
Figure 8.8. IN human only therapeutic cloning

- retrovirus
- lentivirus
- transposon
- adenovirus
- plasmids

Future strategy: Reprogramming genes will be replaced with pharmacological agents.

Figure 8.9. Induced pluripotent stem (iPS) cell generation

Future strategy: Reprogramming genes will be replaced with pharmacological agents.
ES versus iPS cells advantage:

Oocytes or embryos do not need for iPS cell generation.

There are no ethical issues (disruption of human embryos).

iPS cells can be obtained from various somatic cells.

iPS cells can be generated from patients.

disadvantage:

iPS cells contain transgenes (reprogramming factors), these ectopic genes might interfere cell differentiation and over-activation of these genes (e.g. c-myc) can promote tumorgenesis.

iPS cells are very similar to ES cells but several recent reports suggested that iPS cells have an impaired developmental potential. For example, getting tetraploid complementation with adult tissue derive iPS cells has not been successful.

iPS cells are often partially reprogrammed.

**Lineage specific reprogramming (trans-differentiation) by transcription factors:**

a. Skeletal muscle differentiation from fibroblast by MyoD

b. Neuron formation from fibroblast by Ascl1, Brn2 and Myt11

c. Macrophage differentiation from B cells by c/EBPa

d. Cardiomyocyte development from fibroblast by Gata4, Mef2c andTbx5

**Figure 8.10.** Lineage specific reprogramming (trans-differentiation) by transcription factors

The promise of ES/iPS cell research:

Study of cell differentiation.

Cell, tissue and organ transplantation.

In vitro drug test and development of disease models.

**Figure 8.11.** The promise of ES/iPS cell research
3. 8.3 Adult stem cells

Adult (tissue specific) stem cells:

Adult stem cells are multipotent (or unipotent) cells, found throughout the body after embryonic development. These cells have the capacity to self-renew and to regenerate tissues in long-term, in addition, they only differentiate to cell types within their lineage.

Examples:

Hematopoietic stem cells (blood stem cells)

Mesenchymal stem cells

Muscle stem cells (satellite cells)

Intestinal stem cells

Neural stem cells

Adult stem cell example:

Hematopoietic stem cells (HSCs) are multipotent stem cells that give rise to all the blood cell types including myeloid, erythroid, and lymphoid lineages.

Figure 8.12. Adult stem cell example: hematopoietic stem cells (HSC)
Summary

Stem cells have the capacity to self-renew and to sustain cell turnover in vivo. There are two basic stem cell types: embryonic stem (ES) cells and adult (tissue specific) stem cells. Both cell types have an important role in regenerative medicine. Adult blood stem cells (hematopoietic stem cells) have been used in the clinical practice for almost 40 years to treat patients with leukemia and hereditary blood cell diseases. Recently bone marrow derived adult stem cells have been applied to treat other ailments using their inherent plasticity. ES cells have demonstrated their potential to develop into all types of somatic cells that makes up the body, therefore these cells have an enormous potential for cell therapy in the future. Researchers think ES cells may hold cures for conditions such as Alzheimer’s disease, spinal cord injuries, heart failure or diabetes. In addition, ES cells are important research tools to study cell differentiation and embryonic development. Moreover, patient derived stem cells can be used to establish disease models and serve as in vitro assays for screening and testing potential drugs.
Chapter 9. 9. Embryonic and adult stem cells in regenerative medicine II.

The aim of this lecture to discuss the potential application of the embryonic and adult stem cells for cell therapy. This lecture provides general information on stem cell based regenerative medicine.

1. 9.1 Pluripotent stem cells for regenerative medicine

Potential applications of stem cells in regenerative medicine: It is hoped that stem cells will play an important role in the treatment of a number of incurable diseases (Alzheimer’s disease, spinal cord injuries, heart failure or diabetes) via transplantation therapy.

Stem cells can replace the dysfunctional and degenerated cells and the replacement of these cells could offer a lifelong treatment for these disorders. Stem cells can be used to make in vitro a complete organ for autologous transplantation.

Human stem cell sources:

Donation of excess oocytes (blastocysts) for research purposes from IVF clinics. Intentional in vitro production of early embryos (blastocysts) for ES generation. Generation of stem cells by somatic cell nuclear transfer (SCNT).

Somatic cells reprogramming into iPS cells.

Cell harvesting from aborted embryos (fetal stem cells).

Umbilical cord blood cells (mainly hematopoietic stem cells).

Adult stem cell isolation from tissues, organs obtained from surgical procedures.

ES cells for regenerative medicine

Advantages:

Pluripotent cells: They have the potential to generate virtually any somatic cell types.

Immortality: they can proliferate indefinitely, thus these cells can profoundly expanded before differentiation and transplantation.

They are suitable for genetic modification including gene correction and selection of the modified cells.

It is relatively easy to establish them from blastocysts.

Disadvantages:

It is difficult to control their differentiation potential, it is poorly defined their differentiation conditions.

The major concern of ES cell therapy is the possible risk that the transplanted cells could form tumors (teratomas) in vivo.

There are ethical concerns about utilization of ES cells because early embryos are disrupted upon ES cell generation. However, there is no ethical concern if one uses iPS cells.

ES (iPS) based transplantation medicine:

ES or iPS cells have the potential to generate virtually any differentiated cell type. These pluripotent cells offer the possibility to create new sources of cells for regenerative medicine. However in vitro it is difficult to provide optimal conditions for efficient generation of a particular cell lineage. In addition, differentiated ES cells usually form a mixed cell population.
Figure 9.1. ES (iPS) based transplantation medicine

Example for an iPS cell based gene correction:
Correction the defect of Falcoanemia using iPS derived blood cell progenitor.

Figure 9.2. iPS cells based gene correction

Transgene delivery strategies:
Direct gene delivery.
Cell based gene delivery.

Figure 9.3. Transgene delivery strategies
ES/iPS based cell therapy:

Genetically modified ES derived blood cell progenitors confers a definitive hematopoietic engraftment potential (HSC with long term repopulating activity) in a mouse model.

Mouse skeletal muscle was regenerated with administration of ES derived muscle progenitors: intramuscular or systemic transplantation of these cells into dystrophic mice results in engraftment of adult skeletal muscle with enhanced contractile function.

Human ES based cell therapy is still in the research phase, one concern of this therapy is the possible risk that the transplanted ES derived cells could form tumors (teratomas).

Important note:

Recently, a pediatrics patient has been identified who was treated by repeated transplantations of fetal neural stem cells and who developed a donor cell derived multifocal brain tumor.

Pharmacological application of ES cells

The therapeutic potential of stem cells has been widely considered, but stem cells also represent an important system suitable to the identification of new molecular targets and the development of novel medicines.

Human ES/iPS cell lines are important tools to the development of safer and more effective drugs for human diseases.

ES-derived systems are of special importance for the investigation of embryotoxic properties of teratogenic agents.

ES-derived progenitors could be also used as delivery vehicles for the regulated release of drugs or therapeutic proteins.

2. 9.2 Clinical application of stem cells

Clinical application of adult stem cells

Advantages:

Adult stem cells normally develop into a narrow set of cell types (multipotency), directing them to a desired fate is easier.

Autologous adult stem cells would not be rejected by the immune system.
Some adult stem cells are relatively easy to harvest and purify.

Disadvantages:

Adult stem cells are rare in mature tissues and it is difficult to expand them.

They have a limited survivor capacity upon cell culture or freezing.

These cells are less plastic, only specific cell lines can be obtained from them.

They might harbor somatic mutations.

Clinical application of hematopoietic stem cells (HSC)

Many blood cells are short-lived and need to be regenerated, the continued production of blood cells depends on the presence of HSCs, the ultimate and only source of all blood cells.

HSCs have been used clinically since 1959 and are used increasingly routinely for transplantations. Recently more than 40,000 transplants are performed annually world-wide.

HSC transplantation is carried out in the case of hematopoietic cancers (leukemias and lymphomas); other indications include diseases that involve genetic or acquired bone marrow failure.

HSC transplantation can be performed in two different settings, autologous and allogeneic. Autologous transplantations employ a patient’s own cells/tissues and thus present no tissue incompatibility between the donor and the host. Allogeneic transplantations occur between two individuals who are not genetically identical; allogeneic grafts must match most of the major HLA antigens between host and donor.

Curative potential CD34+ hematopoietic stem cells (HSC) to treat disorders of the blood system:

CD34+ hematopoietic cells are can be obtained from bone marrow, these cells are able to repopulate the whole blood system.

**Figure 9.4. Curative potential 34CD+ hematopoetic stem cells (HSC) to treat disorders of the blood system**

Cord blood cell banks: biological life insurance?

Human umbilical cord blood cells (placental blood cells) can be frozen and stored for a long period of time. They are a readily available stem cell source for regenerative medicine, however it is debated whether cord blood cell storage is necessary and useful for autologous cell therapy.
Cord blood contains various stem cell populations: Besides the well characterized CD34+ hematopoietic stem cells (HSC), mesenchymal stromal cells and unrestricted somatic stem cells can be found in freshly obtained cord blood.

Cord blood cells are routinely used for allogeneic transplantation to treat various blood cell disorders (e.g., leukemia). However, it is arguable whether cryopreserved cord blood cells are useful for autologous stem cell therapy since non HSC stem cells are poorly recovered after unfreezing.

Are there any spontaneous lineage conversions (trans-differentiation) in vivo?

Donor derived stem cells which undergo functional trans-differentiation has been questioned over the years. Recent findings suggested that probably cell fusion is responsible for the in vivo detected lineage changes.

### Figure 9.5. Are there any spontaneous lineage conversions (trans-differentiation) in vivo?

Possible beneficial mechanisms of autologous stem cells transplantation:

1. Trans-differentiation: The engrafted stem cells might be converted to other cell types in vivo (e.g., HSC transforms into muscle).

2. Paracrine effects: Numerous murine and human studies demonstrated that stem cells transplantation into infarcted heart has improvement in cardiac function. The mechanism is still controversial but several observations suggests that these cells act as a ‘feeder’ and produce paracrine factors (cytokines, chemokines) which help on tissue regeneration, cell survivor moreover these factors enhance angiogenesis (blood vessel formation).

In summary, autologous stem cell transplantation is not ‘the fountain of eternal youth’ but stem cell based therapy has a reproducible, beneficial effect in various disease models.

### 3. 9.3 Stem cell therapy to cure various diseases

Application of stem cell therapy to treat heart disease The heart is one of the least regenerative organs in the human body, after birth human heart regeneration becomes limited to very slow cardiomyocyte replacement.
The human left ventricle contains 2–4 billion cardiomyocytes, and a myocardial infarction can kill 25% of these cells in a few hours. A resident population of cardiac progenitor cells (CPCs) were identified in heart, these cells are c-KIT positive.

A clinical trial is under way, testing autologous c-KIT+ cells as an adjunctive treatment for patients undergoing coronary bypass surgery.

The ‘stemness’ of CPCs has recently been questioned, and it found that these cells are principally cardiac fibroblasts and not real progenitors of cardiomyocytes.

Clinical trials have also focused on the delivery of bone marrow mononuclear cells by the coronary circulation. These trials indicate that the delivery of bone marrow cells through the coronaries is feasible and safe, but the benefits are modest.

Application of stem cell therapy to treat neurogenerative disease Alzheimer disease (AD) and Huntington disease (HD) develops over a long period of time and result in widespread loss of neurons in the brain, whereas Parkinson disease (PD) involves the specific loss of dopamine producing cells in the substantia nigra.

Neurodegenerative diseases create an enormous burden on society due to their devastating nature, cost and lack of effective therapies.

The NIH has sponsored two large clinical trials in which cells from aborted fetuses were transplanted into the striatum of patients with Parkinson’s disease. The patients’ progress was followed for up to eight years. Both studies showed that the transplants offered little benefit to the patients as a group, however some patients showed improvement.

The ability to produce iPS derived, patient specific dopamine producing cells has recently been demonstrated. Transplantation of these cells into a rodent PD model improved functional deficits and demonstrated cell integration in the host tissue.

Application of stem cell therapy to treat diabetes

Diabetes can be defined as a deficiency of insulin-producing beta-cell mass. Transplantation-based therapeutic approaches aimed at restoring the insulin producing capacity of the impaired beta cells.

Islet grafts have demonstrated that patients with diabetes would benefit greatly by beta-cell therapy. However, the paucity of available islets for transplantation as well as the immunological reactions faced in allogeneic transplantation represents a tremendous barrier to this approach.

A type of trans-differentiation currently being studied by several labs is the conversion of liver cells to pancreatic beta cells. This is because reprogramming of hepatocytes could act as an innovatory method of generating transplantable insulin producing beta-cells.

An alternative approach involves the combination of in vitro ES/iPS cell-derived pancreatic progenitor generation, followed by in vivo differentiation, and maturation into glucose- responsive beta-cells. It was described that committed pancreatic progenitors, when injected into mice tissue can give rise to glucose responsive, insulin-secreting cells.

Summary

Stem cells have the capacity to self-renew and to sustain cell turnover in vivo. There are two basic stem cell types: embryonic stem (ES) cells and adult (tissue specific) stem cells. Both cell types have an important role in regenerative medicine. Adult blood stem cells (hematopoietic stem cells) have been used in the clinic for almost 40 years to treat patients with leukemia and hereditary blood cell diseases. Recently bone marrow derived adult stem cells have been applied to treat other ailments using their inherent plasticity. ES cells have demonstrated their potential to develop into all types of somatic cells which make up the body, therefore these cells have an enormous potential for cell therapy in the future. Researchers think that ES cells may hold cures for conditions such as Alzheimer’s disease, spinal cord injuries, heart failure or diabetes. In addition, ES cells are important research tools to study cell differentiation and embryonic development. Moreover patient derived stem cells can be used to establish disease models and serve as in vitro assays for screening and testing potential drugs.
Chapter 10. 10. Cell Cycle and Cancer Therapy, p53 I.

1. 10.1 Cell Cycle

The eukaryotic cell cycle is a highly regulated series of events leading to the duplication and division of cellular compartments resulting in formation of daughter cell. During the cell cycle, most of the cell’s components - organelles, proteins and RNAs - are replicated resulting in approximately doubling of cell size in M phase. Single copies of the chromosomes are present in a cell, which must be duplicated in a highly regulated manner in a discrete stage called synthetic or S phase only once per cycle. The distribution of duplicated components into daughter cells takes place in the final stage of cell cycle called mitotic or M phase. The eukaryotic cell cycle consists of additional phases, known as gap phases, occurring between S and M phases. The first gap phase, G1, happens before S phase, whereas G2 occurs before M phase. Gap phases provide both for time for cell growth and regulatory transitions, in which progression to the next cell-cycle stage can be controlled by intracellular and extracellular signals.

Figure 10.1. The functional cell cycle

The conventional cell cycle is modified to indicate:
- G1 activities, those preparatory for S phase, may begin during the previous cycle, concurrently with G2 and mitotic events.
- early preparation for mitosis (G2) may overlap with S
- exit from G1 into the G0 quiescent state and reentry into the cycle

The critical points:
C (indicating competence), V (end of entry)
R (restriction point, end of progression)

In the cell-cycle control system, a family of enzymes called the cyclin-dependent kinases (Cdks) catalyzes the covalent modifications of protein substrates by phosphorylation resulting in changes in the substrate's enzymatic activity or its interaction with other proteins. Through the cell cycle the Cdk activities rise and fall as the cell division progresses, which lead to cyclical changes in the phosphorylation of components of the cell-cycle machinery. Cdks are activated by binding to regulatory proteins called cyclins. During the cell cycle the fluctuations in Cdk activity due to changes in the amounts of cyclins. Different types of cyclins are produced at various cell-cycle phases, resulting in the periodic formation of distinct cyclin-Cdk complexes that trigger different cell-cycle events.

Figure 10.2. Cyclin-CDK regulators of cell cycle
Three regulatory checkpoints direct the cell cycle events. The first checkpoint is called Start or the G1/S checkpoint. When conditions are ideal for cell proliferation, G1/S- and S-phase cyclin–Cdk complexes are activated, resulting in the phosphorylation of proteins that initiate DNA replication. These Cdns also encourage activation of M-phase cyclin–Cdk complexes, which drive progression through the second major checkpoint into the beginnings of mitosis (G2/M checkpoint). M-phase cyclin–Cdns phosphorylate proteins that promote spindle assembly up to metaphase. The metaphase-to-anaphase transition is the third major checkpoint, which initiates the sister-chromatid segregation and then the completion of mitosis and cytokinesis.

Cdk inhibitor proteins (CKI) are proteins which interact with Cdkns or Cdk–cyclin complexes to block their activity, usually during G1, G2 or in response to inhibitory signals from the environment or damaged DNA.

**Figure 10.3. Constitutive and inducible cell cycle kinase inhibitors**
2. 10.2. Mitogenic Signaling in Eukaryotic Cell Controls the Rate of Cell Division

Entry into a cell cycle occurs only when the cell is exposed to the mitogens. Mitogens are generally soluble peptides or small proteins secreted by neighboring cells. The best understood mitogens include platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), both of which are soluble polypeptides that control the rate of division in many different cell types. Their binding to transmembrane receptors which causes the dimerization of two receptor molecules is a first step in cascade of events which subsequently ensue. This initial event activates the protein kinase domains of the receptors, causing phosphorylation of multiple tyrosine residues within the cytoplasmic domains of the transmembrane receptor generating new binding sites that recruit intracellular signaling proteins as Grb2 (growth factor receptor-bound protein 2), which then binds the protein SOS (guanine nucleotide releasing factor). These protein interactions brings SOS in close proximity to RAS, which it activates by stimulating it to exchange its GDP for GTP. Ras can exist in an inactive GDP-bound state in quiescent cells or an active GTP-bound state by mitogenic signalization. Ras promotes cell division by the stimulation of a three-component MAP kinase cascade. The first kinase in this cascade, Raf, is activated at the cell membrane by binding to activated Ras. Raf phosphorylates and activates the second kinase, MEK, which then activates the third kinase, ERK (MAP kinase). ERK relays the mitogenic signal to the nucleus, where it phosphorylates multiple targets including gene regulatory proteins which are activated by the phosphorylation and induce the expression of a set of genes that are called the immediate early genes. Fos transcription factor is one of the most important immediate early genes, which promotes assembly and activation of the transcription factor complex called AP-1.

Figure 10.4. Mitogen-Activated Protein (MAP) kinase cascade
AP-1 triggers the expression of a second wave of genes which encode proteins such as the G1 cyclin, cyclin D1, providing a link to the activation of G1–Cdkks and thus the stimulation of cell-cycle entry. The key to the regulation of G1/S genes is that the phosphorylation of pRB proteins results in their dissociation from E2F to ensure that G1/S genes are expressed only at the appropriate time in the cell cycle. In quiescent cells, RB inhibits the E2Fs, in this manner the function of the major E2Fs is controlled primarily by binding to the pRB proteins. Re-entry into the cell cycle from quiescence, therefore, requires removal of the inhibitory effects of the pRB proteins. The phosphorylation of pRB proteins is catalyzed primarily by G1 cyclin–Cdk complexes - cyclin D and its partners Cdk4 and Cdk6. E2F triggers the expression of dihydrofolate reductase (DHFR), thymidine kinase (TK), thymidylate synthase (TS), DNA polymerase-α (POL), cdc2, cyclin E and possibly cyclin A, and E2F-1 itself. This establishes a positive feedback loop promoting RB phosphorylation by cyclin E-CDK2, contributing to the irreversibility of the restriction point transition. Cyclin E-CDK2 may oppose the inhibitory action of p27 by phosphorylating it. This allows cyclin A-CDK2 and possibly cyclin E-CDK2 to start S phase. Once cells enter S phase, cyclin A-CDK2 phosphorylates E2F and inhibits its binding to DNA. Like p27, p53-inducible p21 can induce G1 arrest by inhibiting the cyclin D–, E–, and A-dependent kinases.

Figure 10.5. Transcriptional events in G1 phase of cell-cycle
Modification of chromatin structure for the regulation of gene expression is an important mechanism. In quiescent cells, RB-E2F complexes actively repress gene transcription by interacting with nucleosome-modifying enzymes (such as histone deacetylases, HDCA). In the promoter regions of E2F responsive genes, RB proteins initiate local formation of a chromatin structure that suppresses gene transcription.

Figure 10.6. Mechanisms of gene-suppression by the Retinoblastoma Protein
modifying enzymes (such as histone deacetylases, HDCA). In the promoter regions of E2F responsive genes, RB proteins initiate local formation of a chromatin structures that suppresses gene transcription.

Figure 10.7. Biochemical events of cell-cycle in M-phase

Figure 10.7. Biochemical events of cell-cycle in M-phase

3. 10.3. Biochemical Events of Cell-cycle – in M Phase

During the S phase of the cell cycle, the amount of cyclin B increases. As cyclin B–Cdk1 complexes are being formed, they are inactivated by phosphorylation to keep Cdk1 activity at a minimum. At the G2/M checkpoint the sensor mechanisms check a number of factors to ensure the cell is ready for mitosis. The key molecular event in this checkpoint is the activation of cyclin B–Cdk1 complex activity by CDC25 phosphatase, which removes the inhibitory phosphates present within cyclin B–Cdk1 complex. If the cell passes these checkpoints, many molecular processes will initiate the beginning of mitosis. The key substrates of cyclin B–Cdk1 complex include H1 histone, lamins, centrosomal proteins, and other proteins which need to be displaced from chromatin to allow chromosomal condensation. In the third major checkpoint, in metaphase, checkpoint systems sense that all chromosomes have aligned at the mitotic plate correctly and the accurate segregation of them will occur. This sensing mechanism activates the anaphase-promoting complex (APC) that triggers the metaphase-to-anaphase transition. Although the APC catalyzes the proteolysis of many proteins, degradation of two major substrates leads to destroying the sister-chromatid cohesion and thereby allows the sister chromatids to be drawn to opposite ends of the dividing cell. The second includes the degradation of S and M cyclins that inactivates S– and M–Cdk5, thereby allowing the completion of mitosis and cytokinesis.

4. 10.4. Protooncogenes

Protooncogenes code proteins are involved in signal transduction and transmit mitogenic signals from extracellular matrix though the cytoplasm into the nucleus. There are 7 different classes of proteins that are typically found to be mutated in cancerous cells; all of these classes affect the proliferation of cells in one way or another. These include growth factor proteins, growth factor receptor proteins, signal transduction proteins, transcription factors, DNA repair enzymes, cell-cycle control proteins (like cyclins, cyclin-dependent kinsases, or cyclin kinase inhibitors), and apoptosis regulating proteins (like Bcl-2 or p53).

Possible biochemical mechanisms of protooncogene - oncogene conversion are the following: promoter insertion, enhancer insertion, chromosomal translocation, gene amplification and single point mutation or
deletion. Of the 5 mechanisms described above, the first 4 involve an increase in the amount of the product of an oncogene due to increased transcription but no alteration of the structure of the product of the oncogene. Thus, it appears that increased amounts of the product of an oncogene may be sufficient to push a cell becoming malignant. The fifth mechanism, single point mutation, involves a change in the structure of the product of the oncogene, but not necessarily in its amount. This implies that the presence of a structurally abnormal key regulatory protein in a cell may be sufficient to tip the scale toward malignancy.

There are two ways in which a mutations can alter the function of a protein called gain-of-function mutations and loss of function mutations. Gain-of-function mutations activate or overactivate some process that is normally not active or is tightly controlled in cells. Usually proteins with gain-of-function mutations are activators of cell division, that is, proteins encoded by oncogenes. These kinds of mutants normally affect growth factors, receptors, signal transduction pathways, transcription factors, or some kinds of cell cycle control proteins. These include overproduction of growth factors (such as platelet derived growth factor, PDGF); growth factor receptors which can be activated in the absence of growth factors (such as neu receptor or erb-B (EGF receptor); intracellular signal transduction proteins like Ras, which lead to constitutive activation of the MAP kinase pathway; transcription factors such as myc or jun/fos, that when overproduced or converted to a constantly active state promote transcription of proteins necessary for DNA replication and cell cycle progression (these transcription factors are normally activated by phosphorylation by MAP kinase, and so in a sense they are, like Ras, part of the MAP kinase signal transduction pathway); and overproduction of cell cycle promoting proteins, like cyclin D. These are all common gain-of-function mutations found in cancers.

Loss-of-function mutations results in the inactivation of some protein necessary for the cell to function normally and are usually in proteins that regulate the processes of cell growth or division, and it is said that these proteins are encoded by tumor suppressor genes. Loss-of-function mutations mostly affect inhibitors of cell division or proteins that repair damaged DNA; such as some cell cycle control proteins, anti-apoptosis proteins, or DNA repair enzymes.

**Figure 10.8.** Cancer causing genes in mitotic signal pathway

**Figure 10.9.** Proto-oncogenes and oncogenes
5. 10.5. ErbB/HER Receptors

Most Receptor Tyrosine Kinases (RTKs) are single subunit receptors, (epidermal growth factor, EGF receptor, platelet-derived growth factor, PDGF receptor, vascular endothelial growth factor, VEGF receptor, human epidermal growth factor receptor, HER), but some (like the insulin receptor, IGF) exist as multimeric complexes. Each monomer has a single transmembrane (TM) spanning domain. The NH2-terminal ends of such receptors are extracellular and comprise a very large domain for binding the growth factor or hormone. The COOH-terminus are intracellular and comprise the domains responsible for the catalytic activities of the receptors. When a growth factor binds to the extracellular domain of an RTK, it triggers dimerization with adjacent RTK subunits, which leads to rapid activation of the cytoplasmic kinase domain. The first protein substrate for this activated tyrosine kinase is the receptor itself. The intracellular part of the receptor becomes autophosphorylated on multiple tyrosine residues. Each of the phosphorylated tyrosine residues then acts as recognition or anchoring sites for other proteins that are substrates for the RTK.

The epidermal growth factor (EGF) receptor was the first with intrinsic tyrosine kinase activity to be identified. This catalytic receptor is one member of a family of four related proteins, termed the ErbB/HER receptors. Over-expression of the human ErbB1 gene, which encodes the human EGF receptor (HER1), characterizes several cancers. A mutant ErbB1 gene produces a receptor lacking the extracellular EGF-binding domain. The ErbB2 gene that encodes the HER2 protein that lacks the ability to bind any known extracellular growth factor. Rather, HER2 receptors possess a basal conformation that permits them to form homodimers with other unliganded HER2 proteins or heterodimers with growth-factor-occupied HER1, HER3, or HER4 receptors. Because dimerization is the critical first step for activating the intrinsic tyrosine kinase activity of this protein, even modest over-expression of HER2 can alter normal cell growth regulation. Significantly, ErbB2 gene expression is amplified by up to two orders of magnitudes in 20–30% of human subjects with invasive breast cancer.

6. 10.6. Therapeutic targets

The aberrant expression of ErbB/HER protein in multiple human cancers has prompted the development of several drug therapies that target these receptors. One group of therapeutic agents includes monoclonal antibodies that bind to functionally significant extracellular domains of different HER subtypes. Trastuzumab (Herceptin® from Genentech) is an anti-HER2 antibody that has been used since 1998 for the treatment of those breast cancers characterized by over-expression of ErbB2/HER2. The mechanism underlying the anti-tumor actions of Trastuzumab/Herceptin® involves both the antibody-dependent recruitment of immune factors that kill the tumor cells and the attenuation of the HER2 ectodomain proteolysis (by native metalloproteases) which further potentiates the constitutive dimerization of these receptors. Cetuximab (IMC-C225 or Erbitux® from ImClone) is an antibody that interacts with the EGF-binding domain of the ErbB1/HER1 protein and
thereby prevents ligand-induced activation of the receptor. In addition to these antibody-based therapies, a variety of small molecule drugs has been designed to target the intracellular tyrosine kinase domains of the ErbB/HER proteins. These reagents are 4-anilinoquinazoline-based compounds that act as competitive inhibitors of the ATP-binding sites of the kinases, particularly the ErbB1/HER1 subtype. As a result of that, the message is blocked and the cells undergo G1 arrest, or they undergo apoptosis because they are less able to survive the stress.

**Figure 10.10.** Different families of Receptor Tyrosine Kinase recognize a diverse set of different ligands

![Diagram showing different families of RTKs and their ligands](image1)

**Figure 10.11.** Ligand binding activates RTKs by dimerization

![Diagram showing ligand binding and receptor dimerization](image2)

**Figure 10.12.** Proto-oncogenes are normal genes that can become oncogenes

![Diagram showing the conversion of a proto-oncogene to an oncogene](image3)
Figure 10.13. Neu, EGFR targeting methods

Figure 10.14. Expression of HER2 receptor on the surface of normal and tumor cells
Figure 10.15. Figure 10.15. Therapeutic targets

1. Blocking signalization on cell surface receptor
2. Inhibition of TK activity of TKR
3. Inhibition of protein kinases phosphorylation cascade
4. Activation of growths-inhibiting pathway (Rb) or restoration of p53 function
5. Inhibition of angiogenesis
6. Inhibition of methastasis

In addition to the classical form of cancer treatment like surgery, chemotherapy and radiotherapy, there are new therapeutic targets at molecular level against cancer cells.

Figure 10.16. Figure 10.16. Blocking of oncoproteins of EGFR and Mitogen-Activated Protein Kinase signalization via monoclonal abs and specific inhibitors
Philadelphia chromosomes is a specific chromosomal abnormality. It is the result of a reciprocal translocation between chromosome 9 and 22 that is associated with chronic myelogenous leukemia (CML). The BCL-ABL protein, a fusion protein, functions on a cancer cell to phosphorylate a substrate or a target protein. The way it does that, it takes a phosphate group from ATP, which binds to it, and transfers that phosphate group to its target substrate protein. Then the shape of the target protein changed and it goes on to stimulate cell growth of leukemia cells. Gleevec mimics ATP and binds in substrate site of BCL-ABL, where normally binds ATP, and prevents ATP from binding, thereby prevents phosphorylation of substrate target protein, and in this manner, prevents cell growth.

Doxorubicin is an anthracycline antibiotic and used in cancer chemotherapy as it works by intercalating DNA, blockage of topoisomerase II, generating oxygen radical production. One of the most useful cancer drugs – new formulations extending uses. Doxorubicin is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas.

Alkylating agents

All are electrophilic molecules that covalently modify nucleophilic molecules in cells, e.g. DNA. Cisplatin is a chemotherapy drug. It is used to treat various types of cancers, including sarcomas, some carcinomas (e.g. small cell lung cancer, and ovarian cancer), lymphomas, and germ cell tumors. It was the first member of a class of platinum-containing anti-cancer drugs, which now also includes carboplatin and oxaliplatin. These platinum complexes react in vivo, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis (programmed cell death).

Metabolites and their antimetabolites

Antimetabolites resemble cellular metabolites and act to interfere with DNA synthesis or the synthesis of DNA precursors. General drug classes are antifolates and antinucleotide analogs. As with most antimetabolites, methotrexate is only partially selective for tumor cells and is toxic to all rapidly dividing normal cells, such as those of the intestinal epithelium and bone marrow. Folate antagonists kill cells during the S phase of the cell cycle and are most effective when cells are proliferating rapidly.
5 FU is a prodrug that needs to be transported into the cell and activated (metabolized) into the nucleotide pool. There are several parallel pathways to achieve this, but ultimately 5 FU is metabolized to FdUMP. FdUMP is a substrate for thymidylate synthase, an enzyme essential for the synthesis of thymidine (a DNA precursor). FdUMP inhibits thymidylate synthase and starves the cell for thymine, the so-called thymine less death.

Cytosine arabinoside; araC is also a prodrug that, like 5 FU, needs to enter the nucleotide pool. The ara-C triphosphate is a substrate for DNA polymerase. It inhibits the enzyme and causes termination of DNA synthesis. Antimetabolites are cell cycle specific drugs (S-phase).
Chapter 11. 11. Cell Cycle and Cancer Therapy, p53 II.

1. 11.1. Tumor Suppressor Genes and p53

Products of tumor suppressor genes suppress the cell division cycle or promote apoptosis. For a tumor suppressor gene to contribute to cancer, it must lose activity, in contrast to the oncogenes that are active and promote the cancer. Thus p53, required for normal apoptosis initiated by a variety of stimuli including DNA damage, is inactive in the majority of cancer cells. When active, it suppresses the cell cycle through its regulation of p21 expression and as an inducer of apoptosis in cells that contain damaged DNA, so that its functions inhibit development and propagation of cancer cells. Rb protein is a prominent tumor suppressor as it sequesters E2F and inhibits entrance into S phase. The CKIs, such as p27, p16, p19, p27, and p21 inhibit progression through the cell cycle and are tumor suppressors.

Wild-type p53 functions as a tumor suppressor. The wild-type protein helps to control the checkpoint between the G1 and S phases of the cell cycle, activates DNA repair, and, in other circumstances, leads to programmed cell death. Thus, the biochemical actions of p53 serve to keep cell growth regulated, maintain the information content of the genome, and, finally, eliminate damaged cells.

Figure 11.1. Figure 11.1. Transcriptional events in G1 phase of cell-cycle

Figure 11.2. Figure 11.2. Tumor suppressor genes: retinoblastoma and P53
The p53 transcription factor can either induce growth arrest or apoptosis in response to a variety of cellular stresses, including exposure to DNA damaging agents, hypoxia, and inappropriate pro-life signals. DNA damaging agents and UV irradiation stabilize p53 through phosphorylation of p33 at its N-terminal and activate its DNA binding through dephosphorylation and acetylation of its C-terminal region. Hypoxia and hypoglycemia stabilize p53 through both phosphorylation dependent and independent mechanisms. Inappropriate oncogenic stimulation leads to p53 stabilization through the p19ARF pathway. Binding of hdm2 to p53 inhibits its transcriptional activity and leads to its degradation. ARF overexpression leads to p53 stabilization by binding to hdm2 and preventing the hdm2 mediated p53 inhibition and degradation. Disruption of hdm2 and p53 interactions appears to be critical for the stabilization of p53. Stabilized and activated p53 can then transactivate its target genes.

Figure 11.3. Regulation transcription factor of P53 I.

Figure 11.4. Regulation transcription factor of P53 II.
Figure 11.5. Primary structure of transcription factor p53

Figure 11.6. Restoration of p53 function in tumor cells I.

Nutlin3s act by blocking interaction of Mdm2 with p53, therefore prevents its destruction leading to more of the the stable form of p53

Figure 11.7. Restoration of p53 function in tumor cells II.
The p53 transcription factor acts as a tumor suppressor by inducing growth arrest or apoptosis in response to a variety of cellular stresses including DNA damage, hypoxia and inappropriate proliferative signals.

Figure 11.8. Loss of p53 is observed in 50% of all human cancers
2. 11.2. Biochemical Pathways of Apoptosis and its Therapeutic Utilization

Apoptosis is the Greek word for “falling leaves,” and apoptosis describes an often natural biochemical process of cell death. Apoptotic death is required in developmental processes as well as to maintain homeostasis of a whole organism. As new cells are generated in the human, death of a similar number of cells is required on the same time scale to maintain a steady state. Apoptotic death differs from necrotic cell death. In the latter, lysis of a cell membrane leads to the release of the cell’s contents into extracellular space and an inflammatory response, as often occurs in bacterial or viral infections and in trauma. In contrast, apoptosis is often unobservable and does not induce an inflammatory or immunological response. It can be initiated by a variety of signals, including those from damaged DNA, entrance of a cell into the S phase under improper conditions, lack of proper contacts of a cell with extracellular matrix, lack of necessary growth factors, or presence of death signal proteins in the environment of a cell. These signals activate cytoplasmic protease enzymes called caspases. The caspases hydrolyze specific peptide bonds in target proteins that, after peptide bond hydrolysis, contribute to cell death by either a gain or loss of its activity. Apoptosis is characterized by changes in the plasma membrane, cytoskeleton, and nuclear DNA. The plasma membrane becomes blebbled and sheds membranous particles that encapsulate intracellular contents. Endonucleases are activated that degrade the chromosomal DNA into nucleosome-size fragments. The remains of an apoptotic cell are engulfed and digested by phagocytic cells such as macrophages.

Figure 11.9. Morphology of apoptosis

The death receptor pathway (left half of figure, extrinsic pathway) is activated by ligand binding (eg. TNF, FasL). Ligation to the receptor results in sequential binding of adaptor molecules (e.g. FADD (Fas associated death domain)) and procaspase-8 through homophilic interaction domains, like death domains (DDs) and death effector domains (DEDs), respectively. Ligand-induced assembly of this complex ultimately causes activation of procaspase-8, which then proteolytically activates caspase-3 or it can cleave the pro-apoptotic Bcl-2 family...
member Bid, which then translocates to mitochondria where it facilitates cytochrome c release. The mitochondrial pathway (right half of figure) involves release of cytochrome c and other mitochondrial polypeptides. Once free in the cytoplasm, cytochrome c— in the presence of ATP/dATP — promotes assembly of Apaf-1 (apoptosis activating factor) and procaspase-9 into a macromolecular assembly called the ‘apoptosome’, which induces the activation of caspase-9. Active caspase-9 is capable of proteolytically processing procaspase-3 and other effector caspases. In addition to cytochrome c, the mitochondrial proteins apoptosis inducing factor (AIF) and endonuclease G (endo G) are also thought to play a role in apoptotic events after their release, most likely by exerting effects on nuclei, whereas SMAC/Diablo sequesters caspase inhibitors XIAP (IAPs, inhibitor of apoptosis proteins) away from caspases, thereby facilitating caspase activation and action.

**Figure 11.10.** Biochemical pathways of caspase activation dependent cell death

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent endogenous activator of the cell death pathway and functions by activating the cell surface death receptors. TRAIL is nontoxic in vivo and preferentially kills neoplastically transformed cells over normal cells by an undefined mechanism. Radiotherapy is a common treatment for breast cancer as well as many other cancers. Ionizing radiation can sensitize breast carcinoma cells to TRAIL-induced apoptosis. This synergistic effect is p53-dependent and may be the result of radiation-induced up-regulation of the TRAIL-receptor. Importantly, TRAIL and ionizing radiation have a synergistic effect in the regression of established breast cancer xenografts. This provides support for combining radiation with TRAIL to improve tumor eradication and suggest that efficacy of apoptosis.

**Figure 11.11.** Killing tumors by induction of apoptosis
Hypoxia-ischemia (H-I) in the developing brain results in brain injury with prominent features of both apoptosis and necrosis. An intracerebroventricular injection of caspase inhibitor blocks caspase-3 activation and is neuroprotective against neonatal H-I-induced brain injury. The selective inhibition of caspase-3 reveals that this caspase contributes to a significant component of brain injury after H-I.

Figure 11.12. Figure 11.125. Cell death prevention after Stroke
Summary

Biochemical pathways that regulate the cell cycle and cell death have opened up new possibilities for treatment of cancer by molecular therapeutics. Knowing the molecular components of both the cell cycle regulation and cell death pathway, which transmit signals from extracellular matrix up to the end of the cell division or cell death by protein-protein interactions, protein kinases, phosphatases or protease activities, have given several ideas on how to cure cancer more specific and selective manner. However, we should keep it in our mind that the molecular therapeutics is unlikely to lead to a “one-off” cure for cancer.

Definition: Gene silencing is the inhibition (or rather partial inhibition) of the expression of a selected gene specifically.

1. 12.1 Introduction

It must be emphasized that gene silencing must be specific for the targeted gene. It is not just a general inhibition of a certain function or functions of a cell. Usually, the target gene is a protein coding gene and the successful silencing process prevents the formation of the protein of interest. Specific inhibition of a protein by an inhibitor is not considered to be a gene silencing process, although the inhibited protein is not functioning, seemingly resulting in a similar effect as the silencing of the protein’s gene. However, other, not inhibited, functions of protein can still be active inducing biological responses. The specific inhibition of non-protein coding genes (like tRNA or miRNA coding genes) may also be considered as a form of gene silencing.

The gene silencing may be performed in vitro or in vivo. The in vitro gene silencing usually performed for experimental proposes, mainly in cell culture. The in vivo gene silencing may be performed also for therapeutic proposes, perhaps in the near future.

Since gene silencing must be specific, by definition, most of the gene silencing methods utilizes oligonucleotides or nucleic acid derivatives, taking the advantage of the high specificity of double or triple helix formation of nucleic acids. Most of the gene silencing methods are based on natural regulatory processes.

The gene expression can be inhibited at several levels from transcription to protein synthesis. Thus, the gene can be silenced by oligonucleotides and nucleic acids by

1. Inhibition the function or processing of mRNA by antisense oligonucleotides,
2. Inhibition the transcription by triple helix forming (antigene) oligonucleotides, or RNA mediated methylation of promoters.
3. Inhibitory action of ribozymes
4. Inhibition the function of mRNA by siRNA or miRNA.

All of these methods exploit the specificity of nucleic acid-nucleic acid interactions.

The gene silencing compounds (mainly oligonucleotides) are useful research tools and potential therapeutic agents. Therefore, intensive studies are pursued in research and industrial laboratories to develop specific and highly active gene silencing compounds.

2. 12.2 Action of antisense oligonucleotides

The design and synthesis of a biologically active antisense oligonucleotide is not an easy task. A number of chemical modifications must be introduced to increase the stability and cellular uptake of the molecule, and appropriate target site must be selected. Since these problems are very similar in case of other oligonucleotide-based gene silencing molecules, we will discuss some possible chemical modifications and biological considerations in detail, here.

Antisense molecules are short (8-30 nucleotides) single stranded oligonucleotides, usually deoxyoligonucleotides, chemically modified deoxyoligonucleotides or ribooligo-nucleotides, that are complementary to a target mRNA or a precursor of mRNA.

Figure 12.1. Figure 12.1. The basic concept of antisense activation
Since the mRNAs have complex secondary structures the target site, where the antisense binds to, must be selected carefully. A target sequence of the mRNA (which is freely accessible for the antisense oligonucleotide) may be selected by theoretical considerations using computer generated models, or may be determined experimentally. Binding an antisense oligonucleotide to the target mRNA may inhibit gene expression (protein synthesis) by

1. steric hindrance, or
2. activation of RNase H (degradation of the target).

**Figure 12.3. Stability, cellular uptake of antisense oligonucleotides, accessibility to the target**
The natural (unmodified) deoxyoligonucleotides are not stable in biological environment due to the presence of nucleases. Therefore, chemical modifications are required to increase their stability. Certain modifications may also increase the rate of cellular uptake of the oligomers.

Since the target mRNA has a tight secondary structure, only certain stretches are accessible for antisense oligonucleotides.

The mode of action of a specific antisense oligonucleotide depends on its structure, i.e., on the various chemical modifications introduced into the inhibitory molecule. Without any chemical modifications, the DNA/RNA hybrid is an excellent substrate of the RNase H. Some of the chemical modifications do not affect significantly the action for RNase H. In this case, the mRNA is hydrolyzed by the RNase H activity, and the released antisense molecule may interact with another mRNA causing its degradation.

When the antisense-mRNA complex is not a substrate of RNase H, the protein synthesis is inhibited by steric reasons. The firmly attached antisense can inhibit the binding of mRNA to the ribosomes, or may inhibit the move of the ribosome along the mRNA.

In both cases, the protein synthesis is inhibited.

A third possible mode of action of antisense oligonucleotides is the inhibition of the maturation of the mRNA precursors. The capping, splicing or polyadenylation may be inhibited by appropriately designed oligonucleotides.

The haploid human genome contains 3 x 10^9 nucleotides. In a random sequence of this size, any sequence that is 17-nucleotide long may be present only once indicating high specificity of the antisenses. However, a 20-mer contains eleven 10-mers and each 10-mer would be present 3000 times in the human genome. A 10-mer is long enough to activate the RNase H.

### 3. 12.3 Chemical modifications of gene silencing oligonucleotides; general considerations

The natural (unmodified) deoxyoligonucleotides are not stable in biological environment due to the presence of nucleases. Therefore, chemical modifications are required to increase their stability. Certain modifications may also increase the rate of cellular uptake of the oligomers. It must be noted that the localization of the chemical modifications and the number of modified nucleotides also alter the activity of the gene silencing oligonucleotides. For example, the introduction a phosphorothioate internucleotide linkage to the 3’ end increases the stability of the oligonucleotide in a biological system. The same modification at the 5’ end is less effective, because the exonucleases present in the cell are mostly 3’ exonucleases (attacking the oligonucleotide at the 3’ end). The number of modifications (e.g., the number of phosphorothioate linkages) may also have a strong effect on the activity of the oligonucleotide inhibitor. In sum:

- Chemical modifications of oligonucleotides are often used to affect the nuclease resistance, cellular uptake, distribution in the body and thermal stability of the double or triple helixes.
- The chemical modifications may hit the internucleotide linkage, the pentose or base residues, and may be combined within a single oligonucleotide.
• Chemical modifications of the oligonucleotides are usually required for potent gene silencing, independently of the method (antisense, antigen, ribozymes and siRNA).

**Figure 12.4. Chemical modification of gene silencing oligonucleotides I.**

**Phosphorothioate linkage**
The most often utilized chemical modifications. This modified internucleotide linkage quite resistant to nucleases; able to activate RNase-H. It somewhat decrease the Tm of the double strand. When synthesized by automatic DNA synthesizer a diastereomeric mixture is formed. Its main drawback is that it tends to interact nonspecifically with proteins, like DNA polymerases or proteins of the cytoskeleton.

**Figure 12.5. Chemical modification of gene silencing oligonucleotides II.**

**2’-O-methyl RNA**
This modification on the pentose residue increases the Tm of the double helix. It cannot activate RNase H. It increases the stability of the oligonucleotides against nucleases, and also increases the cellular uptake of the modified nucleotides. It must be noted that other modifications in the 2’ position have also been applied, like introduction of methoxyethyl and allyl group.

**Figure 12.6. Chemical modification of gene silencing oligonucleotides III.**

Figure 12.7. Chemical modification of gene silencing oligonucleotides IV.

**N3’→ P5’ phosphoramidite internucleotide linkage**
Highly stable against enzymatic hydrolysis and has a high affinity for single stranded DNA or RNA and readily forms triple helixes.

Figure 12.8. Chemical modification of gene silencing oligonucleotides V.

**Locked nucleic acids (LNA)** are ribonucleotides containing a methylene bridge that connects the 2’-oxigen of ribose with the 4’ carbon. Introduction of locked nucleotides into a deoxy-oligonucleotide improves the affinity for complementary sequences and significantly increases the melting temperature. The locked nucleotides are not toxic.
Some common chemical modifications

Phosphorothioate linkage

The most often utilized chemical modifications. This modified internucleotide linkage is quite resistant to nucleases; able to activate RNase-H. It somewhat decrease the Tm of the double strand. When synthesized by automatic DNA synthesizer, a diastereomeric mixture is formed. Its main drawback is that it tends to interact nonspecifically with proteins, like DNA polymerases or proteins of the cytoskeleton.

2'-O-methyl RNA

This modification on the pentose residue increases the Tm of the double helix. It cannot activate RNase H. It increases the stability of the oligonucleotides against nucleases, and also increases the cellular uptake of the modified nucleotides. It must be noted that other modifications in the 2’ position have also been applied, like introduction of methoxyethyl and allyl group.

N3’→P5’ phosphoramidite internucleotide linkage

Highly stable against enzymatic hydrolysis and has a high affinity for single stranded DNA or RNA and readily forms triple helices.

Locked nucleic acids (LNA)

Locked nucleic acids are ribonucleotides containing a methylene bridge that connects the 2’-oxigen of ribose with the 4’ carbon. Introduction of locked nucleotides into a deoxy-oligonucleotide improves the affinity for complementary sequences and significantly increases the melting temperature. The locked nucleotides are nontoxic compounds.

Peptide nucleic acid (PNA)

The backbone of PNA carries 2’-aminoethyl glycine linkages in place of the regular phosphodiester backbone of DNA. The PNA is highly stable, and forms high Tm duplexes and triplexes with natural nucleic acids. The cellular uptake of PNA is poor, therefore often hybridized with normal nucleic acids. The natural nucleic acid component of the hybrid is degraded in the cell after uptake.

Figure 12.9. Half lives of natural DNA, phosphorothioate (PS) and end-blocked oligonucleotides with 2’-OCH3 or locked nucleotides (LNA in human serum
A large number of base modified nucleotides were synthesized and incorporated to gene silencing oligonucleotides. The 5-position of pyrimidine nucleotides is one of the most favored substitution site, because substitution at this position is expected neither to interfere with base pairing nor to influence the general structure of double helix. Propynyl group at this position significantly increase the Tm of the double helix.

The extension of half lives of oligonucleotides with various chemical modifications. Results of a gene silencing experiment with phosphorothioate antisense oligonucleotide are presented in Fig. 12.10.

**Figure 12.10. Figure 12.10. Gene silencing in the laboratory for experimental purposes**

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Number of end blocks</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>PS</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>LNA a</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>LNA b</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>LNA c</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>LNA d</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>LNA e</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>OMe</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

**Conclusion:** The antisense oligonucleotide inhibited the synthesis of BCL-2 protein. The effect was dose and time dependent. The primary cell line isolated from a 5-years old girl was also sensitive to the antisense oligonucleotide.

**4. 12.4 Inhibition of transcription by triple helix forming oligonucleotides**
Certain sequences of the natural double stranded DNA are able to interact with short oligonucleotides forming stable triple helixes. The proper localization of the triple helix forming sequence may be utilized to silence that gene by directly inhibiting the transcription by steric hindrance or inhibiting the initiation of the transcription. This strategy is also called anti-gene inhibition of gene expression. The antigens seem to be more effective than antisenses, because a single oligonucleotide, at the target site, may be able to inhibit the gene expression.

**Figure 12.11. Schematic representation of triple helix**

Stable triple helix formation requires a poly-purine/poly-pyrimidine double helix sequence.

Parallel triplets: the third pyrimidine containing strand runs parallel to the purine strand of the duplex and are stabilized by the formation of Hoogsteen base pairs. The formation of C+.GC requires low pH.

**Figure 12.12. A comparison of the anti-gene and anti-sense strategy**

The antigens seems to be more effective than antisenses, because a single oligonucleotide, at the target site, may be able to inhibit the gene expression.

12.5 Gene silencing by ribozymes

Ribozymes are catalytically active RNAs. They are able to catalyze many biochemical reactions, including the hydrolysis of internucleotide bonds. This activity (discovered originally by Cech) may be utilized to silence gene expression, by degrading mRNA, mRNA precursors and viral RNA.

Large catalytic RNAs: Group I and Group II introns and RNase P.

Small catalytic RNAs: hammerhead, hairpin, hepatitis delta.
For experimental and therapeutic proposes (so far, this is also experimental) mainly small catalytic RNAs are produced with appropriate chemical modifications in order to increase the stability and cellular uptake of the molecule.

**Figure 12.15.** General structures of ribozymes often utilized for gene silencing

![Ribozyme structures](image)

**Figure 12.16.** Mechanism of self-splicing of the rRNA precursor of *Tetrahymena*

![Splicing mechanism](image)

*Two exons of rRNA are denoted in red. Catalytic functions reside in the intron (brown). This splicing function requires an added guanine nucleoside or nucleotide.*

**Figure 12.17.** Construction of hammerhead ribozymes against M1 RNA
The ribozymes are potential antitumor and antiviral agents: mRNAs encoding oncogenes can be targeted. Growth factors and their receptors are also feasible targets to inhibit the proliferation of cancer cells. Synthesis of viral proteins may also be inhibited by ribozymes attacking the virus specific mRNAs.

There are two main difficulties for the use of ribozymes for gene silencing either for experimental or therapeutic use:

- Nuclease sensitivity
- Cellular uptake

These problems may be solved by chemical modifications and/or use of effective carrier systems. Those chemical modifications which are described for antisens oligonucleotides may be applied for ribozymes, too.
6. 12.6 Gene silencing with short RNA fragments

Short RNA fragments, 19-23 nucleotides long, are able to inhibit specifically the protein synthesis by interacting with the targeted mRNA. Thus, they are very powerful tools for experimental gene silencing and promising potential therapeutic agents.

There are two distinct classes of gene silencing RNAs, microRNAs (miRNA) and small interfering RNAs (siRNA).

Both miRNAs and siRNAs are produced primarily as partly double-stranded RNAs synthesized by RNA polymerase II. They are processed in the nucleus by DROSHA then transported to the cytoplasm, where they are further processed by DICER to short (21-23 nucleotides) double stranded or partly dsRNAs. The antisense strand (guide strand) of both miRNAs and siRNAs associates with effector assemblies, known as RNA Induced Silencing Complexes (RISC), forming siRISC and miRISC, respectively. The antisense strand guides the RISC to the target mRNA to inhibit the protein synthesis mainly without significant degradation of mRNA (miRNA) or cleaving the mRNA (siRNA).

The main function of miRNAs is the regulation of gene expression.

The miRNAs are endogenous noncoding RNAs. The antisense strand of miRNAs does not form a perfect double helix of the target mRNA. Usually multiple binding sites exist for miRISC at the 3’ untranslated region of the target mRNA.

**Figure 12.19.** The miRNAs are endogenous noncoding RNAs. The antisense strand of miRNAs does not form a perfect double helix of the target mRNA. Usually multiple binding sites exist for miRISC at the 3’ untranslated region of the target mRNA.

The function of the siRNAs is mainly the protection against the expression of foreign genes (e.g. viral gene).

siRNA or its precursor can be introduced exogenously; the antisense strand in the siRISC complex forms a perfect double helix with the target mRNA, leading to the selective cleavage of mRNA by the nuclease domain of the Agronaute protein, a component of the RISC complex. The hydrolyzed mRNA then further degraded by cellular nucleases.
As it was mentioned above, a 21 nt long dsRNA with 3’ overhangs can be introduced into cells to induce gene silencing. A great advantage of the method is that a relatively low molecular weight drug can be designed and produced to silence genes specifically and efficiently. It may have the utility as a systemic experimental or therapeutic agent.

**Figure 12.20.** Figure 12.20. miRNA and siRNA pathways

![miRNA and siRNA pathways diagram](image1)

**Figure 12.21.** Figure 12.21. miRNA and siRNA biosynthetic pathway

![miRNA and siRNA biosynthetic pathway diagram](image2)

In vitro produced shRNAs (small hairpin RNA) or dsRNAs (double stranded RNA) may also be used to transfect cells directly. The pre-made shRNA and long dsRNA processed by Dicer to 21 nt long siRNA with 2 nt 3’ overhang, then the short siRNA is phosphorylated.
Figure 12.22. miRNA and siRNA pathways, and various methods to induce RNA interference

The two strands of siRNA complexes have different functions: the antisense (guide) strand will be a component of RISC directing it to the target site of the mRNA, the sense (passenger) strand is degraded, without any further function.

Function of argonaute protein.

Argonaute proteins are the catalytic components of the RNA-induced silencing complex (RISC), with endonuclease activity. Argonaute proteins are evolutionarily conserved and can be phylogenetically subdivided into the Ago subfamily and the Piwi subfamily. Ago proteins are ubiquitously expressed.

Figure 12.24. Roles of Ago proteins in gene silencing induced by siRNA and miRNA
Methods to introduce functionally active siRNAs to silence genes for experimental or therapeutic proposes:

1. The use of plasmid or viral vectors (the expressed products must be processed).

**Figure 12.25. Figure 12.25. Plasmids expressing functional shRNA**

2. The use of dsRNA or shRNA (must be processed by dicer).

3. The use of short double stranded (~21 nt) RNA oligonucleotides, usually with chemical modifications.

Use of plasmid vectors for shRNA production. Small Hairpin RNA (shRNA) are precursors of siRNA. shRNA producing plasmids are commercially available. The shRNA requires cellular processing by Dicer to obtain the siRNA (dsRNA, ~21 nucleotide long). Then it is involved in the formation of the active RISC by its antisense (guide) strain. The sense strain is degraded.

**Figure 12.26. Figure 12.26. Action of shRNA-plasmid gene silencer**
Use of viral vectors for shRNA production. Viral vectors can effectively be utilized to produce shRNA in those cells that difficult to be transfected by other methods and even can be used in nondividing cells. The viral vectors can transduce cells naturally, and very efficiently. The most widely used viral vectors for shRNA delivery: Adenovirus, Adeno associated virus (AAV), Lentivirus, Retrovirus, Herpes and Baculovirus vectors.

**Figure 12.27.** Gene silencing with 21 nt long dsRNA oligos

**Figure 12.28.** Lentiviral delivery of shRNA and its mode of action
Epigenetic gene silencing. Epigenetic modification of DNA can be achieved by specific siRNA. The specific methylation of dC in DNA can be directed by siRNA. The methylation process involves the action of RNA polymerase, which produces a short RNA, called scaffold RNA, forming a double strand with the siRNA. In the transcription bubble a complex is formed containing RNA polymerase, dsRNA (scaffold RNA/siRNA) methylase enzyme and some other proteins. This complex methylates specific sequences. The RNA directed DNA methylation is an example for specific epigenetic gene silencing. The specificity of the methylation is determined by the sequence of the siRNA.

The RdDM is able to inactivate promoter regions, thus, inhibiting the transcription of specific genes. This type of gene silencing was mostly studied in plants.

Possible chemical modifications of siRNAs. In order to increase the efficacy of the siRNA a number of chemical modifications may be introduced into the oligonucleotide strands.

The 3’ overhangs, the sense strand and the 3’ ten nucleotides of the antisense strand may be modified without significantly decreasing the silencing activity of the construct.

The seed region, 6-7 nucleotide at the 5’ end of the antisense RNA strand, is more sensitive to chemical modifications.

Effects of chemical modifications on the activity of siRNA.

• It may increase the resistance against various nucleases and diesterases, thus, could increase the half life of siRNA.

• It may improve the cellular uptake.

• It may target specifically the siRNA molecules.

• It may increase the overall activity of the molecule by the combination of the above mentioned improved features.

7. 12.7 Important final note

The above described gene silencing molecules are nucleic acids, ribo- or deoxyribo- oligonucleotides, often with chemical modifications. For many years, the main obstacle to the widespread use of these agents, either for some experimental or therapeutic use, was the high price of the chemically synthesized oligonucleotides. Today, automatic oligonucleotide synthesizers are available for synthesis of kg quantities of crude oligonucleotide in a single run with most of the desired chemical modifications at acceptable prices. Now, the avenue is open for the discovery and largescale production of oligonucleotide drugs for specific gene silencing.