Kunert

Teil 1

Products from animal cells

- vaccine production: virus propagation on host cells (polio, influenza, pertussis)
- production of pharmacological substances in immortalised cells and recombinant cell lines (ab, hormones, cytokines, vaccine antigens, enzymes)
- cells as products (skin transplantations, drug screening, toxicology test, organoids)
- gene therapy (in vivo gene-therapy, ex vivo gene therapy)

Complex pharmaceutica for:

- regenerative medicine
- life style medicine
- chronic diseases
- disease of common people – civilisation diseases

Properties of medical products to prevent or cure diseases:

- safe
- effective
- economical
- yield (500 kg/year)

Efficient processes of biopharmaceutical

- continuous vs. batch production
- processmodelling and – control
- down stream
- integral processes
- Innovation: new cells, new cell lines and molecules; sustainability by improved cells and process-engineering

Animal cell cultures as tools for:

- model system (testing pathways, pathogens, drugs, cell-aging, nutrition research)
- toxicology (liver and kidney cells)
- cancer research
- virology
- cells as production vehicle (cell based manufacturing; virus or proteins as products, cell cultures for production of tissue and organs)
- genetical investigations (amniozellen aus fruchtwasserpunktuation)
- gene therapy (in vivo, in vitro)

Growth conditions

a) Fermentation
   adharent cells grow on micro-carrier
   suspension cells
b) perfused micro-capillars
   cells grow on surface of plastic-micro-cpaillars; nutrient and dissolved gases are able to pass; cells grow as multiple layers
c) three dimensional growth
   organ-cultures

Scale up

- small scale for investigation and diagnostic (ng/ml - µg/ml)
- vaccines and therapeutic products require large scale (pfu/ml)
- monoclonal antibodies – in 20.000 liter reactors
- early cell lines (diploid cells) used for vaccines are attachment dependent requiring medium supplemented with serum and surfaces on which to grow

High efficiency cell culture processes

- efficiency is measured by specific productivity of the cultures – amount per volume per day (µg/mio.cells/day)
- depends on the cell density and productivity per each cell
- specific production rate is measured – how much product is secreted per unit of time
- monoclonal ab – 10-20 pg/cell/day minimum; up to 100 pg/cell/day???

Teil 2

Cell cultures derive from animal tissue or blood: primary cells, diploid cell lines or continuous, immortalised cell lines

Reasons for **immortalisation**: spontaneous mutations, virus infections or viral oncogenes

Characteristics of transformed cell lines

- growth
  - infinite life span
  - matrix independent growth
  - loss of contact inhibition
  - increased plating efficacy
  - higher growth saturation
  - changed (lower) serum requirements
  - independence of growth factors
  - shorter doubling times
- genetic properties
  - higher spontaneous mutation rates

    - aneuploidy/heteroploidy
    - over-expression of oncogenes
    - deletion of suppressor-genes
- structural changes
    - morphology and cytoscelet
    - surface associated fibronectin
    - increased lektin-agglutination
    - modified extracellular matrix
    - change in cell adhesion molecules
    - destruction of cellular polarity
- neoplastic (krebsartig) properties
    - tumor induction in nude mice
    - angiogenetic properties
    - invasive

primary vertebrates grow predominantly (überwiegend) as monolayers on artificial surfaces; inhibition of proliferation in suspension. e.g. glass, disposable materials (Polypropylen, polystyrene,...), micro-carrier
Improved growth by coating of substrates (surfaces) with collagen-nerve-cells, fibronectin, gelatine, poly-D-Lysin

- contact is necessary for specific interactions – with different extracellular components or adjacent tissue cells

Basal-Media

- Basal-Medium Eagle BME
- Dulbecco's modified Eagle Medium DMEM
- F-12 Nutrient Mixtures HAM for cloning of CHO cells developed
- Minimal essential Media MEM
- RPMI 1640 Media with higher concentrations of nutrients – developed for suspension cells
- media are additionally supported with complex and undefined/definded supplements
- selection factors if required
- L-aa, vitamins (biotin, vit B12, folic acid, riboflavin,...), organic ingredients (acetate, adenine, pyruvate, thymidin), inorganic salts ($CaCl_2$, KCl, KNO_3, $MgCl_2$,...), trace elements ($CuSO_4$, $FeSO_4$, $MnSO_4$,...), buffers and indicators (HEPES, Phenole red,...)

Gas-Phase

- oxygen: demands varies according to cell density and on tissue type
- CO_2: dissolved, regulates the pH with HCO_3, alternative: HEPES or TRICIN buffers, pyruvate increases the endogenous CO_2 production; 5-10 %; addition of CO_2 in closed systems
- at high concentrations: cultures produces enough CO_2, O_2 have to be inserted

Energy use and energy source

- 60 % for maintenance
    - Na/K- ATPase: Ion gradient
    - Transport processes

    - general metaboli and regulation activities
- growth
- production
- sugars as energy source: glucose, fructose, galactose, mannose
    - unnaturally high concentrations in media (prüfungsfrage!)
    - Blood: 0.6 – 1.4 g/L
    - Basalmedia: 1 – 4.5 g/L

Metabolic Flux

- Glucose
    - 15-20 % pentose-cycle
    - 80 % lactate via pyruvate (highly toxic)
    - 0-5 % via AcetylCoA into lipid synthesis
- Glutamine
    - highly involved in glycosilation
    - 95-100 % TCA cycle
    - 35 % oxidised completely
    - 10-15 % pyruvate into lactate or alanine
    - 50 % into aspartate via oxal acetate
    - release of highly toxic ammonia

Serum as supplement

Serum – why and how it is controlled?

From where?
Production from fetal calves – fetus during 3.-7. month of pregnancy, blood clotting (gerinnen lassen), then centrifuged, activation by thrombocytes increases release of growth factors, sterile filtered and packaged.
Storage and usage:
at -20 or -80°C, let thaw slowly in refrigerator, inactive complement and virus for 30 min at 56°C, portion and use within 1-2 months
Controlling:
Every serum-charge must be quality tested! On recombinant cell lines for volumetric productivity (µg/ml, mg/l), specific productivity (pg/c/d), cell viability and max. cell density. Also test cloning efficacy!
Why serum?
Serum contains proteins (sometimes function unknown; binding of minerals, fatty acids and hormones), albumin, globuline, fibronectin, alpha-macroglobulin and polypeptide like the PDGF as important growth factor. Also insulin (supports uptake of sugar and aa), hydrocortisone (for adhesion and cell proliferation), transferring (for iron uptake) and lipoproteins!
Also metabolites and nutrients (aa, glucose, fructose,...), minerals (bound to serumproteins), inhibitors (destroyed during heat inactivation), vitamins A and E, immunoglobuline (cause purification problems) and enzymes (alkaline phosphatise, lactad dehydrogenase, trypsin inhibitor, prolaktin,...)
Inhibitors in serum:

- Trypsine-Inhibitor
- Chalones
- some growth factors are inhibiting to some cell types

Adhesion factors in serum:

- in adherent cell lines required for growth and stimulation
- large surface area
- intracellular signals transferred by 3D-structure
- may influence differentiation
- e.g. fibronectin, fetuin
- proteins require pos. or neg. charge on surface of culture vessels, cells attach to adhesion factors via Ca2+ and ionic bonds

Problems

- variability (high testing requirements)
- expensive, sometimes availability
- potential source of contamination, undefined animal origin (prions)
- toxins, special inhibitors
- changes in cell surface and membrane fluidity
- undefined culture conditions
- purification
- in primary cell lines fibroblasts grow preferentially
- influence on bioassays (adsorption of toxins in toxicity assays)
- high protein content hinder protein purification

General considerations for **serum-free media**

- sensitive cells
- higher requirements in media optimisation
- adaptation of cells to serum-free conditions may necessary

Selection criteria – what are the objectives? Proliferation, maintenance/murvival, differentiation or productivity?

Reduced Serum/serum-free media

- Lipids (structural components for cell membranes, source of metabolic energy are fatty acids, phospholipids, lecithin)
- cholesterol
- growth factor extracts (Fibroblasten-Growth-factor, Epidermal-growth-factor, Nerve growth factor, transforming growth factor, PDGF, IL-6)
- defined hormone cocktails
- HINTES (hydrocortisone, insulin, transferring, ethanolamine, selenit)
- ITES (insulin, transferring, ethanolamine, selenit)

Classification of cell culture media

- Serum-containing media: basal medium with serum of different sources

- Serum free media: basal medium with proteins like insulin, transferring, growth factors and chemically defined substances
- protein free media: basal media with plant derived protein hydrolysates and chemically defined substances
- chemically defined media: basal media only with animal component free and chemically defined substances

CHO-cells in serum media and proteinfree media are adherent or in suspension.

Gene expression in **mammalian cell lines**

- disulfide bonds between cystein residues
- addition of complex sugar structures to asparagin, serin or tryosin
- blocking of N-terminus by N-formy-, N-acetyl- or N-acylresidues
- Addition of Glycolipid-anchor for stabilisation of protein in the cellular membrane
- Modification by farnesylation, myristinylation for membrane association
- Hydrolysis-processes for heterogeneous molecule populations

Co- and post-translational modifications

- have influence on different protein properties (like activity, half life, receptor-binding,...)
- change in tertiary structure, charge and hydrophobicity
- no general rules

Describe 5 post-translational modifications except glycosilation!

Which groups are important for membrane anchoring? At which aa? How are proteins anchored on the membrane?

Glycolipid-Anchor

- to anchor glycoproteins on the outside of the plasmamembrane. Anchor = Glycosylphoshpatidylinositol
- in every eukaryotic cell
- GPI-anchor is added to the terminal carboxylgroup of glycoprotein in the ER (before: signal sequence for ER-Import is spliced off)
- protein-**Cystein**-phosphoethanolamine-3 mannose – N-Acetyl-Glucosamin- Inostiolphosphat – Glycerin – fatty acids
- increase the mobility of some proteins on the membrane and support the signal-transduction and cellular transporting
- important in terms of cell identity
- parasite Trypanosoma brucei has variable membrane proteins (variant surface glycoproteins – VSG) and is not recognized by the immunesystem of the host.

Protein-farnesylation

- via farnesyltransferase adding a farnesyl group (isoprenoid) to proteins
- type of prenylation
- targets include members of the Ras superfamily of small GTP-binding-proteins critical to cell cycle progression → FTase inhibitors testing as anti-cancer agents

- farnesylated proteins become membrane-associated due to the hydrophobic nature of the farnesyl group
- Protein- Lysine- S - Farnesyl

protein-myristoylation

- irreversible, protein lipidation modification
- myristoyl group is covalently attached by an amide bond to the amino-group of an N-terminal **glycine** residue
- co-translationally or post-translationally
- N-myristoyltransferase NMT catalayzes the myristic acid addition reaction in the cytoplasm of cells
- for protein-protein- and protein-lipid interactions and membrane targeting
- for signal transduction
- Protein-Serine-Glycin-Myristat

Influence of glycosylation on the protein?

N-glycosylation in ER

- sugar binds to N of free acidamidgroup of asparagin
- influences protein structure
- biological activity of protein
- transport from the ER
- Solubility of the protein
- clearance from the blood
- antigenicity

O-Glycosylation in GOLGI

- starts in the late ER and in the golgi
- sugar binds at the hydroxygroup of serin, threonin, hydroxyprolin or hydroxylysin
- o-glycane are often clustered on the protein – first N-Acetylgalactosamin (GalNAc) is added via an O-Glycosidic-bond to Ser or Thr
- some carbohydrates have a Gal residue as initial sugar, bound to Hydroxy-lysin
- o-glycans often are built from only 1-4 sugar residues
- they are rather sensitive to alkali and O-Glycosylases and divergent in structure
- maximum are 2 sialic acids

Which sugars are added?

- L-Fucose
- D-Galactose
- D-Mannose
- D-Glucose
- D-Xylose
- D-N-Acetylglucosamine
- D-N-Acetylgalactosamine
- D-N-Acetylneuraminacid

Acylation
by fatty acids to cystein allow the protein to anchor to membranes

GPI-anchor

Sulfatation
occurs by cleavage of the sugar acetyl-moiety and binding of the sulphate residue

Gamma-Carboxylation
enables Ca2+ binding and change in biological activity

Especially blood coagulation factors show distinct modifications:

- gamma-Garboxylation of glutamic acids residues
- beta-Hydroxylation of aspartic acid and asparagines
- Proteolytical modifications

Collagen

- polypeptide chain; consists of glycin, prolin and hydroxyprolin
- triplehelix is stabilized by 5-Hydroxylysin und 4-Hydroxyprolin
- Glycosylation on 5-Hydroxylysin
- additional crossings → insoluble in water
- skin, bones, cartilage, blood vessels, teeth
- collagen helps to connect the cells of organs
- usage: structure, texture, stabilization, film formation, protective colloid, water binding, adhesion/cohesion

Blood clotting factor VIII

- example of PTMs
- 330 kDa
- Glycoprotein with 20 % sugar content
- 25 potential glycosylation sites, not all of them are modified
- 23 of the 2332 amino acids are cysteins, and 14 of them a bridged
- modification is important for biological activity

Differences between N-Terminus of eukaryotic and bacterial expression systems?
in mammalian cells blocking of N-Terminus by N-Formyl, N-Acetyl or N-Acylresidues
in bacteria: ???

PTMs and effects on protein properties (example collagen and bcf VIII)

- Glycosylation (N/O)
- Acylation
- Y-Carboxylation
- Sulfatation

- S-S-bridges
- Blocking of N-Terminus by N-Formyl, N-Acetyl
- beta-Hydroxylierung of aspartat
- proteolytical modifications
- prenylation (Farnesylation, Myristylation)
- PTMs have effects on e.g. collagen or blood clotting factor VIII

Demands to the expression cell line

Which properties of expression cell lines for production of recombinant proteins?

- Efficient expression of Transgenes
    - effective transcription
    - correct post transcriptional processing
    - efficient translation
    - secretion into the medium
- Posttranslational modification
    - correct folding (secondary and tertiary structure)
    - establishing of disulfide bonds
    - attachment of N- and O-Glycosides
    - Phosphat-, Sulfat-, Acetyl-residues
    - Farnesylation, Myristinylation, Glycophosphatidylation
- Stability of the expression (genotypic)
- No contamination and contaminating agents
- growth requirements
- up stream properties

Expressionvectors

shuttle-vectors:
can propagate in 2 different host species, include plasmids that can propagate in eukaryotes and prokaryotes, contain Origin of replication from E.coli and a resistance gene for propagation in bacteria and eukaryotic transcription unit, gene of interest, resistence gene for eucaryotes; used to quickly make multiple copies of the gene in E.coli and for in in vitro experiments and modifications, e.g. adenovirus shuttle vector can propagate in E.coli and mammals

Like the promoters also the terminators are selective for the regulation machinery of the production system. Signal-Sequences are compatible in the species, they lead the product to the compartiment, the organelle or to the secretion machinery.

Promotors

- in animal cell culture
    - Cellular p.: Elongationsfactor-1-alpha-gen, Ubiquitin p
    - viral p.: CMV P, SV40 P
    - consecutive P.: s.o.
    - inducible P.: Tetrazyklin-inducible
- in plant cells

    - viral P.: CaMV 35 S promoter
    - cellular P.: Ubiquitin P, Actin P, Gt1 P, Glucocorticoid P
- in insect cells
    - Polyhedrin P. of virus surface protein
- in yeast
    - GAPDH P, GAP P, AOX P, XYL1 P
- in bacteria
    - Lac P, trp P, T7 P

Table to fill in promoters of bacteria, yeast, animal cell culture plus usage

Promoters	Bakteria	Yeast	Insect cells	plant cells	animal cells
constitutive	trp	GAPDH P		Ubiquitin P Actin P Gt1 P	Ubiquitin P
inducible	Lac P	AOX P		Glucocorticoid P	EF-1alpha
viral	T7 P		Polyhedrin P	CaMV 35 S P	SV40 P CMV P

Function:

- trp-Promoter: intracellular synthesis of tryptophan in bacteria; only if aa is not in the medium; for high level production of proteins, easy to activate
- Lac P: important for transport and catabolism of lactose in bacteria
- T7 P.: for expression of recombinant proteins with vectors with T7-System; lacUV5-promoter is induced with IPTG or lactose
- GAPDH P.: GAPDH is a catalytic enzyme for glycolysis, promoter is induced with insulin or hypoxia
- AOX P.: induced by methanol and repressed by glucose, for recombinant protein production in yeast

Enhancer and silencer influence the transcription activity and interact with the promoter by proteins bound to the regulatory regions.

Heterologous gene expression

a) transient gene expression
b) stable gene expression

stable gene expression:
An integral host-vector system is generated, in which the transgenic DNA is co-replicated with the genome. The generated population has genetically identically cells. The introduced DNA is either randomly integrated (**heterologous recombination**) or introduced by site directed integration (**homologous recombination**). Distinct viral sequences replicate independently from the genome and provide the basis for episomale expression vectors BPV. BPV = bovine papilloma virus; episomal plasmids are usually based on sequences from DNA viruses

transient gene expression:

is used for fast protein generation, it is therefore not necessary to develop a stable clone. The expression vector might be propagated in an episomal manner. The genotype of the population is not necessarily homogenous. The gene is performed often by transduction or infections.

Reportersystem – Chloramphenicol Acetyltransferase CAT

how to control beta glycosidase?
which types of reportergenes for beta-galactosidase?
How to monitor gene expression?

- CAT is used as a reporter system to measure the level of a promoter or its tissue-specific expression
- the reportergene (in this case the cat-gene) shows effects of other genes e.g. with fluorescent reporter protein or reporterenzyme or detectible antigen expression; to monitor gene expression
- CAT is a reporter gene
- is responsible for chloramphenicol resistance in bacteria (is able to detoxify chloramphenicol)
- CATenzyme covalently attaches an acetyl group from acetyl-CoA to chloramphenicol, which prevents chloramphenicol from binding to ribosomes
- if enzyme is in the cells, after cell disrupton→ CA is changed to Diacetoxy-CA, which can be analyzed with Dünnschichtchormatographie (thinlayer chrom.)
- CAT activity is determined by looking for the acetylated forms of chloramphenicol, which have significantly increased migration rate as compared to the unacetylated form

Reportersystem – beta-Galactosidase

- catalyses the hydrolysis of glycosidic binding of beta-galactopyranosides into monosaccharides
- gene for beta-Galactosidase = lacZ is used for a reporter gene
- in bacteria: blue-white-selection
- in yeast: yeast-2-hybrid-system
- or in knockout mice
- 3 substrates for beta-galactosidase
    - hydrolysis of X-Gal to Galactose and blue Indigo-Colour shows the gene expression of lacZ; distinguishing the presence or absence of cloned product in a plasmid
    - hydrolysis of ONPG shows the activity of beta-Galactosidase quantitative
    - MUG-Assay: 4-MUG is cleaved by beta-Galactosidase and the product is then fluorescent active
- beta-galactosidase has a high turnover rate and is absent in mammalian cells it serves as a very useful and sensitive reporting tool for gene expression (to see if the gene of interest, which is under control of the lacZ-promoter, is active)

Reportersystem – Luciferase

- luziferase-gene as reporter gene from photinus pyralis and renilla reniformis, coding for a biolumineszenz-enzyme
- Luziferase catalyzes following reaction:

    - luciferin + ATP → luciferyl adenylate + PPi
    - then: luciferyl adenylate + O2 → oxyluciferin + AMP + LIGHT
- to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest

Teil 3

Cell-lines from animal organisms

cell-line	**species**	**properties**	**requirements for high expression**	**longtime-production**
CHO dhfr-	hamster	dhfr-	geneamplification	+
BHK-21	hamster	efficient, transient and stable expr.	no geneampl.	+
C127	mouse	no replication of BPV-vectors	BPV-depending episomal replicating vectors	+

Storage of cell cultures

- -80°C - -196°C
- 2-5 *10^6 cells/mL in growth medium plus 5-10 % DMSO or Gylcerol (stabilizing cells and proteins)
- factors effecting cell survival
    - water permeability
    - cell size
    - cell cycle stage
    - equilibration time of cryo-preservative
    - level of cryo-preservative
    - method of addition
    - storage temperature
    - cooling method
- dispensed in glass or plastic ampoules (1ml/vial)
- cooling 1°C/min to -80°C than quickly in liquid nitrogen to -140°C
    - at slow cooling rates (1°C/min) the ice is formed outside the cell and the water escapes from the cell
    - at high cooling rates the cells are filled with ice crystals → damage cells

Monitoring of cell death

- cell cycle analysis as a performance predictor
- increasing cell number and biomass – visible with DNA fluorochroms
- determination of cell number and viability: trypan blue dye, MTT assay, reduction assay,...
- morphological assessment of cell death
- apoptotic cells: cell shrinking, nuclear condensation and nuclear fragmentation
- necrotic cells: nuclear swelling, chromatin flocculation (ausfällung), loss of nuclear basophilia
- changes in cell size and granularity
- forward scatter indicates cell size (in flow cytometry; fluorescence activated cell sorting FACS)

- side scatter reveals the degree of granularity
- cell cycle profile determines DNA content → position in the cell cycle, subdiploid cells are apoptotic
- Annexin V binding to phosphatidylserine assay → detection of apoptosis

Mycoplasma (class of Mollicute)

What are mycoplasma and how can they be detected and eliminated?

- mycoplasma are a genius of bacteria that lack cell walls → M. are unaffected by most antibiotics that target cell wall synthesis
- grow mostly associated with the mammalian cell membranes
- low GC-content
- infect per definition only vertebrates
- alternative genetic code where UGA encode for tryptophan instead of an stop codon
- pathogenic in humans
- complex growth requirements
- m. contaminations → problem to the culture of mammalian cells!
- alter cellular parameters like:
    - proliferation
    - virus susceptibility (Anfälligkeit)
    - production
    - unreliable experimental results
    - unsafe biological products
- Detection:
    - routine testing using special techniques
    - by DNA staining
    - by the toll-like receptor 2 (TLR-2), that initiates the signalling cascade leading to the activation of NF-kappaB and other transcription factors → expression of SEAP (secreted embryonic alkaline phosphatase)
    - via PCR with mycoplasma specific primers
- Elimination:
    - antibiotics acting on the protein synthesis machinery
    - antibiotics acting on the DNA replication (both acting on targets only found in mycoplasma)
    - dispose (entsorgen) the whole content in the incubator

Introduction of foreign DNA

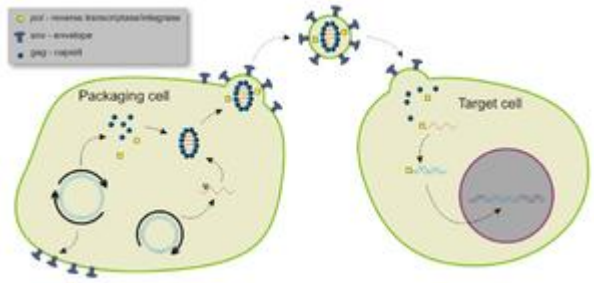
- cell-fusion (yeast, fungi, animal cells)
  chemical substances or electric pulses or viruses induces cell fusion and gene rearrangement
- virus-infection (animal cells)
  recombinant viruses as transport vehicles are used for efficient transfer of foreign DNA
- transduction (bacteria, animal cells)
  transfer of propagation incompetent (recombinant) viruses
- conjugation (bacteria, plant cells)
  transfer from donor to acceptor cell

- transformation, Transfektion (bacteria, yeaset, fungi, animal cells)
  gene transfer by free DNA without vector or cell contact
  by electroporation (bacteria, higher cells)
  by laser
  by gene gun
  Ca-Phosphat Transfektion
  Lipofektion

Receptor-mediated gene transfer
Adenovirus-Polylysin-DNA-Complexes – Adenovirus-capsid is modified in the endosome by the pH-gradient to release the DNA.

Virus-mediated gene transfer
the target gene is cloned in a vector that contains the viral LTRs and packaing signals. The final step to generate the DNA vehicle is done in a packaging cell-line: this cell line constitutively expresses envelope proteins which pack the transgene to generate an artificial proliferation incompetent virus. The infection but not augmentable (verstärkungsfähig) virus-particles are used for gene transfer. Several plasmids are transfected into a so called packaging cell line eg HEK293. One encode the virion proteins, the other contains the genetic material to be delivered by the vector.

Packaging and transduction by a lentiviral vector
lentiviral → ability to integrate into the genome of non-dividing cells
the packaging cell-line has integrated one or two genes of the viral genome. The vector is integrated, transcribed, RNA is packed and released from the cell. Recombinant viral particles infect the cell: viral RNA exits from the virus and is reverse transcribed by retroviral reverse transcriptase into DNA, which is integrated into the genome of the infected cell. After DNA transcription, the RNA cannot be packed again. But: site of integration is unpredictable. For safety reasons, lentiviral vectors never carry the genes required for their replication!



Packaging cell lines: Properties, explanation, usage?
Packaging cell lines are needed to extablish a whole virus out of a virus vector. Because the genes for capsid or coating protein are not present in the vector. The packaging cell line carry 1 or more viral genomes – so they provide it for the cell. The cell line does the packaging and transporting. These viruses infect other cells, where RNA is transcribed to DNA and DNA is integrated into the genome. DNA is transcribed and translated, but no capsid or coating is formed because the genes aren't there.

SV40 Virus

What happens if a permissive/non-permissive cell is infected by SV40 virus?

The SV40 Virus genome consist of an early region and a late region separated by the ORI. The early region encodes large T and small t protein, essential for viral infection and alterations of cellular control processes. The late region encodes viral coat proteins necessary for virus replication.
SV40 was identified as contamination of the poliomyelitis vaccine but no case of tumor induction is known. SV40 has a 5200 bp circular dsDNA which can be defined as early and late regions. The viral DNA is covered by histones and recognized as mini-chromosome; the region of the ori is free (no nucleosome) and also there the early promoter/enhancer is identified. The structure of the SV40 minichromosome is enveloped by ikosahedral structure proteins.

Lytic infektioncycle in permissive cells:

permissive cells are cells, where after virus infection the whole replication cycle of the virus with building of offsprings (nachkommen) is possible (virus uses the synthese system (replication system) of the cell). When virusparticle leave the cell or the membrane is ruptured → cell lysis
Large T-antigen (protooncogen of SV40) is transported back into the nucleus and binds as tetramer to the ori of the viral DNA. Thereby the replication is initiated. The expression of the early genes is inhibited (repression of LT too). Stimulation of DNA-Polymerase and cellular proteins to generate proteins for virus-replication. Small-T-antigen (ST) accumulates at the nuclear membrane in the cytoplasm and increases the function of LT. In parallel to viral DNA-replication the expression of late genes (eg capsid genes) starts. The expression of cellular genes is not affected.

Infection of non permissive cells:

no replication of the virus in the cell possible! → no cell lysis
Infection occurs similar to the lytic cyle. Host-cell-factors for replication are available and therefore the virus is not able to replicate. However, the virus may integrate into the cellular chromosome. The expressed LT is able to induce mutations on p53 which can cause morphologically visible cell transformation.

Expression of SV40 large T antigen extends the life span of normal human fibroblasts, followed by a stage of cessation (crisis). In rare cases a focus of immortalized cells appears, which can be subcultured indefinitely. → induced cellular immortalization

Non-replicating SV40 vectors

- pSV2 expression vector
- only cis-acting elements of the early genes are used
- recombinant DNA is introduced by transfection
- yield depends of different factors like membrane transfer of the DNA, but also integration of the DNA
- stable clones can be generated

Replicating SV40 vectors

- make use of the viral ori for multiplication of DNA
- LT for the replication can be provided by different methods:
    - co-Transfektion of LT via helper plasmid (similar to pSV2)
    - use of the whole SV40 virus for LT expression (helper virus → cells die quickly)

- lytic cycle of virus propagation can be circumvented by temperature sensitive mutants
- use of helper cells (COS-cells are CV1 cells, transformed with an ori deficient SV40 virus)

SV40-COS-Expression-System

COS-cells: properties and for what used?

COS = fibroblast-like cell line; COS-cells are obtained by immortalizing CV1-cells with a version of the SV40 virus that can produce LT but has a defect in genomic replication. So COS-cells express SV40 LT. LT binds to the ori of SV40 genome → initiate replication of viral DNA (plasmid has the ori of the SV40 virus). Cells grow adherent in single layers. COS-cells are supporting the expression of transgenes from plasmids.

+ high yields
- no stable production cell lines, only transient expression

Vaccinia-System

Describe Vaccinia-System with gene transfer and selection

Vaccina-virus is used as a vector. Causes Smallpox (Pocken). They belong to the Pox-virus containing a huge dsDNA-genome (similar to BPV) and are able to infect many vertebrates and invertebrates. The virus replicates in the cytoplasm and not as most others in the nucleus. The early viral genes are transcribed by the virus owned transcription complex. The viral genome consist of 185 kbps which is not entirely used as vector.
The expression vector contains a selectable marker, a MCS, and a vaccinia homologous DNA for homologous recombination between the transfer vector and the vaccinia vector.
The most suitable system is selection with the thymidin kinase of the virus itself. Homologous recombination destroys the TK gene and integrates the gene of interest instead of. Afterwards the selection is based on TK-negative cells.

1) Gene is inserted in TK-gene of a plasmid
2) homologous recombination – TK is replaced by the gene of interest
3) then in vaccinia viruses packed

+ appropriate transport, secretion, processing, PM
+ relatively high level of protein synthesis
+ a wide host range
+ permits cling large fragments

Selection of TK negative cells – TK positive cells die
after gene replacement the selection of TK-negative cells can start which should be positive for the gene of interest (the transgene). The pro-drug Ganciclovir (virostaticum against herpes virus) is an analogous to thymidin and replaces thymidin in the DNA-chain. Since Ganciclovir has no 3'OH group, the DNA-chain breaks and cells are not able to survive!

Viruses as vehicles

- vaccinia, baculovirus, semliki-forest virus for protein expression
- flavivirus, poliovirus, hep B virus, influenzavirus as vaccines
- Adenovirus, adeno associated virus, herpes virus, HIV for gene delivery

Teil 4

Commonly used host cell lines

- MOPC21 and subclones NS0
- CHO cells and variants (hamster)
- HEK 293 and variants (human)

NS0 cell line from murine myeloma

The MOPC21 tumour secrete IgG1 and it was used to establish a continuous tissue culture line. They were maintained as ascites (Bauchwasser in bauchhöhle) in mice before they were cultured in medium. Then this heterogeneous population of cells, which grew in suspension, were called P3K cells. They synthesize and secrete IgG1 and were then cloned and gave rise to P3-X27. Some other cell lines did not secrete IgG1, synthesizing only the light chain but not the heavy chain. → cell line was renamed NSI/1. A further subclone which did not secrete or synthesize heavy or light chains of Ig was cloned and hence this murine myeloma cell line was named NS0/2 meaining non-secreting cells.

The glutamine synthetase (GS) system – biochemical selctable marker

Why is glutaminsynthetase helpful with NS0-cells? Development? Application?

Glutamine is a non-essential aa and provides a source of nitrogen for many biosynthetic pathways. Important in protein synthesis, purine, pyrimidine biosynthesis, ammonia formation, amino sugars, as cofactors and for the degradation of amino acids. The carbon chain of glutamine is an significant source of energy. Enzymatic synthesis of energy requires hydrolysis of ATP; this reaction is catalyzed by GS, a universal housekeeping gene. NS0 cells express a very low level of endogenous GS and have an absolute requirement for exogenous glutamine.
The GS-NS0-Systems relies on the fact, that cells containing very low levels of endogenous GS, when grown in glutamine-free media, require either an exogenous source of glutamine or exogenous GS in order to survive. The incorporation of a GS gene in a plasmid vector containing the gene of a heterologous protein allows selection of cells, in glutamine-free media, that have taken up the plasmid during transfection and are stably expressing the GS gene and hence the heterologous protein.

Human embryonic kidney cells (HEK)

How are they immortelized? How they look like genotypic and phenotypic? Which other forms of HEK cells exist and what are the properties? Advantages?

HEK-293 was developed by transformation of Human embryonic kidney-cells with a part of DNA of the human adenovirus 5.
4.5 kbs of the viral genome were introduced into the chromosome of the cells. HEK-293 are hypotriploid epithelial cells and grow adherently (fibroplast-like cells in single layer cultures). HEK-

293 are often used for the development of viral vaccines and to generate recombinant adenovirus-vectors. HEK cells are easily cultivated in serum free medium.
The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein. The cells support a transient and stable expression.
HEK-cells are good for virology because they were transformed with an adenovirus, they express certain adenovirus-genes – so they are also used for adenovirus proliferation.
other forms of HEK-cells:

- HEK.EBNA cells
  HEK 293 cells stably transformed with EBNA-1 gene, the cells express EBNA1
  + very high frequencies of transformation are obtained with vectors containing the Epstein-Barr virus (EBV) oriP
  EBNA-1 protein derives episomal replication of ori-P containing plasmid
  integrated Ad5 E1a/E1b fragement in HEK293 cells enhances transcription of CMV promoter driven transgen
  + from gene to protein within 4-6 weeks!
  + cells can be grown adherently and in serum free suspension cultures
  + to generate stable cell lines and in transient mode on small and large scale
  + secreted, membranebound and intracellular proteins can be expressed
- HEK293T cells
  + to propagate lentiviruses
  + stably express the SV40 Large T-antigen
  + allows episomal replicaton of transfected plamids containing the SV40 ori
  + high level expression
  + resistant to G418
- also similarly modified cell line like HeLa, COS and Chines Hamster Ovary cell

Immortalization

- by deregulation of the cell cycle
  - spontan mutation
  - virus infection
  - virale oncogenes
  - fusion with tumour cells

Small scale expression trails using HEK.EBNA cells work very efficiently using

- adherent cultures in DMEM + 10% FCS
- different well-plate formats
- pre-coating with Poly-D-lysine: facilitates attachment of cells and minimizes cell losses during transfection
- lipofectamine 2000^{TM} for over 90 % transfection efficiency
- also with serum free suspension cultures – but less efficient

Large scale transient transfection

- 1-10 liter
- Prerequisite (voraussetzungen):

- adaptation of cell culture to serum-free suspension
- several vendors (anbieter) offer cell culture media either specifically developed for HEK293 or for other cells
- suitable transfection reagent which is cost-effective, readily available in large quantities and gives rise to high transfection efficiencies
- commercially available reagents (cheap)
- transfection using CaPO4 precipitation or cationic polymers such as polyethylenimine – successfully used at multi-liter scale
- generation of sufficient plasmid DNA

CHO-cells

CHO-cells – properties? advantages? variants?

- derived from ovary of the Chinese hamster
- grow adherent
- from hamster
- morphology: epithelial-like
- small size, low chromosome number → + good model for tissue culture and radiation studies
  + good growth
  + proteinfree cultivation
  +no known retrovirus
  + high dynamic diversity
  + grow dense
- source: from ovary
- hypodiploid
- require aa proline in the culture medium
- used as mammalian host for industrial production of recombinant protein therapeutics
- CHO cells do not express the epidermal growth factor receptor (EGFR) (which makes them ideal in the investigation of various EGFR mutations)
- the **CHO-K1 cell** line was derived as a subclone from the parental CHO cell line initiate from biopsy of an ovary of an adult Chinese hamster; containing slightly lower amount of DNA than original CHO
- CHO-K1 mutagenized generating **CHO-DXB11**, a cell line lacking DHFR activity. These cells have a deletion of one dhfr allele and a missense mutation in the other.
- **CHO-DG44** (from CHO cell line by radiation and chemical treatment mutagenized) carry a double deletion (both dhfr alleles) for the dihydrofolate reductase gene (dhfr). They are widely used for the stable production of recombinant proteins since they exhibit the DHFR-selection and amplification system.
- CHO-DXB11 and CHO-DG44 require glycine, hypocanthine and hymidine (GHT) for growth. Used for experiments demonstrating stable transfection with an exogenous dhfr gene via selection in GHT-minus medium (standard methods to establish stably transfected CHO cell lines for the production of recombinant proteins)
- Process:

1) cloning of the gene of interest and the dhfr gene in a single or separate mammalian expression vector
2) the plasmid DNA carrying the 2 genes are then delivered into cells by transfection
3) cells are grown under selective conditions in GHT-minus medium
   ➔ each surviving cell will have one or more copies of the exogenous dhfr gene, usually along with the GOI, integrated in the genome. The integrated copy number varies widely. The growth rate and the level of recombinant protein production of each cell line also vary widely.

Teil 5

Selection mechanism to establish stable production clones

Demands for technological use of recombinant cell lines

- stable integration into the cellular genome
- no degradation of GOI
- stable and equal distribution of the gene to daughter cells
- qualitative and quantitative adequate protein expression

Co-integration of the selection marker

- dominant selection (can be applied in nearly all cell lines)
- recessive selection (the host cells need a specific deficiency in a distinct enzyme acdtivity)

Dominant selection marker

- Dominant resistance for antibiotics: Neomycin-Phosphotransferase, Puromycin-Acetyltransferase, Hygromycin-B-Phosphotransferase

Dominant biosynthetic selection marker

- AA which are essential for mammals can often be synthesized by bacterial enzymes (e.g. Tryptophan synthetase – catalyzes the 2 final steps of biosynthesis of tryptophan, Histidin Dehydrogenase – catalyzes the terminal step of synthesis of histidine)

Biosynthesis of Desocynucleodies

Purine-Synthesis and Pyrimidine-Synthesis

De novo synthesis of pyrimidiens – which enzyme can be used as selection marker affecting the corresponding salvage pathway?

Purine

The purine biosynthesis starts at the ribosephosphat. PRA is generated from PRPP. This step is catalyzed by the amido-phosphoribosyltransferase. Afterwards Glycin is added → AIR
Next step Inosinat is generated by adding of CO2, nitrogen, aspartat and formylation and condensation. Inosinat is the precursor of AMP and GMP.
The hydrolytic degradation of nucleic acids and nucleutides ends up in free purin bases which can be recycle (the salvage pathway).

Adeninphosphoribosyltransferase APRT: Adenine + PRPP → AMP +PPi

Hypoxanthin-Guanin-Phosphoribosyltransferase HGPRT: Hypoxanthine + PRPP → IMP + PPi
Guanine + PRPP → GMP + PPi

The purine-biosynthesis is regulated by feedback-inhibition

Pyrimidin

Pyrimidines are NOT synthesized on the ribosephosphat. Synthesis starts with the generation of Carbamylaspartat. After dehydration, ring closure and oxidation → Orotat
Afterwards it reacts with PRPP to generate Orotidylat (OMP). This reaction is catalyzed by the multifunctional enzyme CAD. Decarboxylierung of OMP leads to UMP. CTP is generated after amination of UTP.

Recessive selection-marker

recessive selection marker, 3 examples and function!

for cells with an decent defect (defect does not kill the cells); certain cells (with defect) can live on special culture

- thymidinkinase
  converts dU and dT tod UMP and dTMP; important when de novo synthesis is blocked by aminopterin
  Tk negative cells grow in the presence of brom-desoxyuridin
- HGPRT
  converts Hypoxanthin to IMP and Guanin to GMP, supplement in case of blocked de novo synthesis
- APRT
  converts Adenin to AMP, selected with Xyl-A, dCf inhibits ADA (Adenin → AMP savage pathway for purine biosynthesis)

Recessive amplificationmaker

DHFR – explain!

- converts dihydrofolat to tetrahydrofolat
- converts serin + FH4 to Glycin + Methylen-FH4
- converts dUMP to dTMP
- designs ribose to IMP (inosine monophosphat)
- Dihydrofolat-reductase (dhfr)
  converts dihydrofolat to tetrahydrofolat
  converts serin + FH4 to glycin + methylen-FH4
  converts dUMP to dTMP
  designs ribose to IMP
  no dhfr → no de novo snyhtesis if salvage pahtwy isn't working either → cell dies (like HGPRT and TK on HAT medium)
- dhfr is inhibit by methotrexat or aminopterin
- deficiency of cells is first supplemented via the medium (thymidin, glycin, hypoxanthin, nucleoside)
- selection by dialysed medium, nucleoside- free medium, no HT

- amplification by methotrexat (inhibits dhfr) and the gene (dhfr gene) is amplified and if the GOI is well placed, it also will be more amplified if no hypoxanthin and thymidin is in the medium (no salvage pathway possible)