Level 8 Cell Culture Processing (BIO08045)
Lecture 2 – "Cell Line Development & Characterisation"

- Dermot O' Sullivan
- Dermot.osullivan@nibrt.ie

Learning Objectives

Choice of cell lines for biomanufacturing

Mammalian cell lines – why they are preferred

Required characteristics of manufacturing cell lines

Genetic modifications for improved cell growth and viable cell density

Genetic modification of cell lines for optimal therapeutic protein expression

Recommended Reading

- Tihanyi, *et al*. Drug Discovery Today: Technologies (2020), 38, 25-34 *"Recent advances in CHO cell line development for recombinant protein production".*

  https://pubmed.ncbi.nlm.nih.gov/34895638/

- Feng Li *et al*. mAbs Volume 2 Issue 5: p.466-477 September / October 2010. *"Cell culture processes for monoclonal antibody production"*.

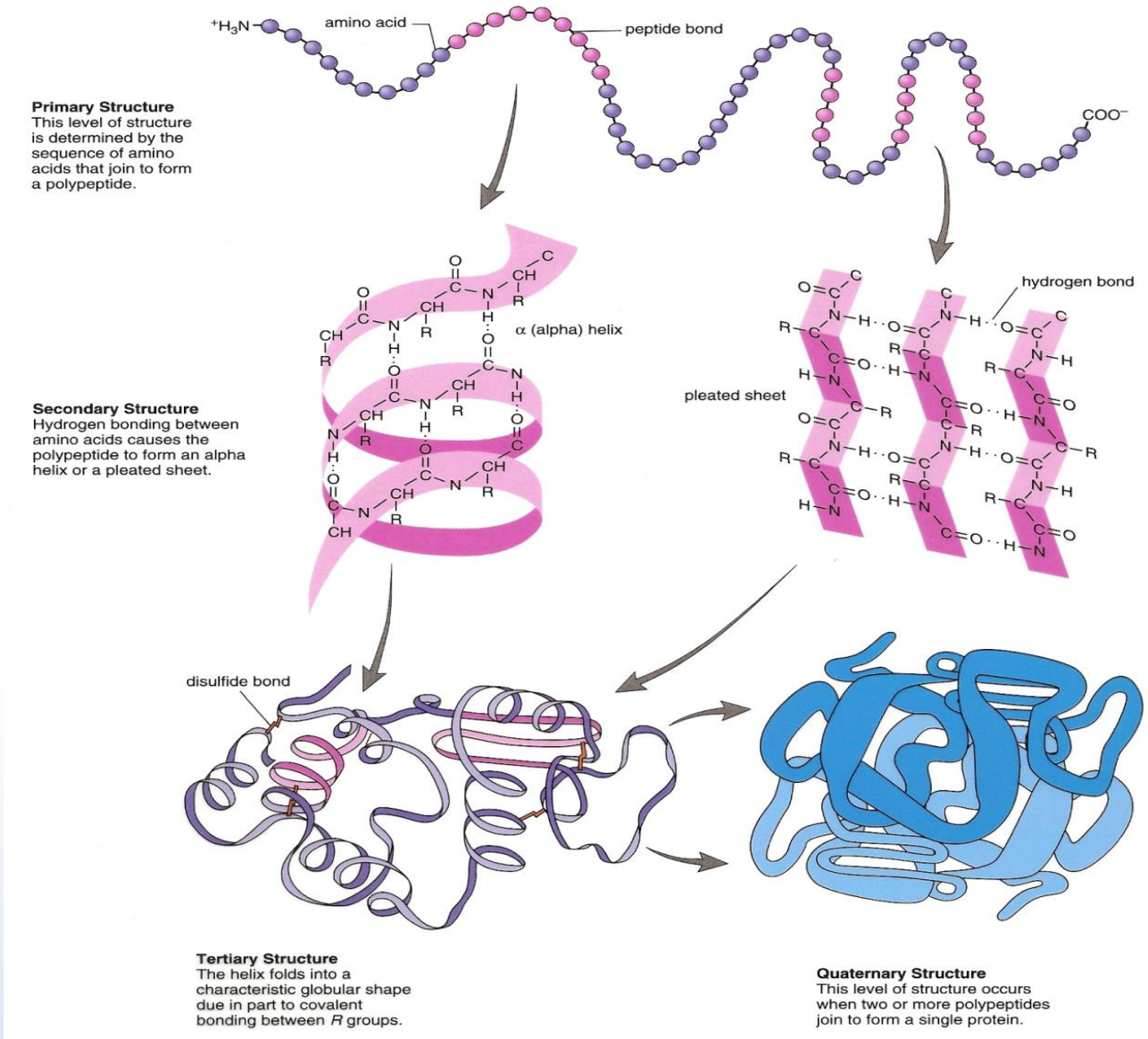
  http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2958569/

- AT Menendez, N Ritter, J Zmuda, D Jani, and J Goyal (2010) *"Recommendations for Cell Banks Used in GXP Assays"*. BioProcess International 10(1) January 2012 – while it relates to use of cells in bioassays, there is a very useful section of cell line characterisation which applies to all cell lines

Lecture Topics

- Choice of Cell for Biomanufacturing.
- Establishment of Cell Lines.
- Genetic Modification of Cell Lines for Protein Expression.
- Engineering Cell Lines for Optimal Growth Performance in Bioreactors.
- Impact of Genetic Engineering on Biomanufacturing

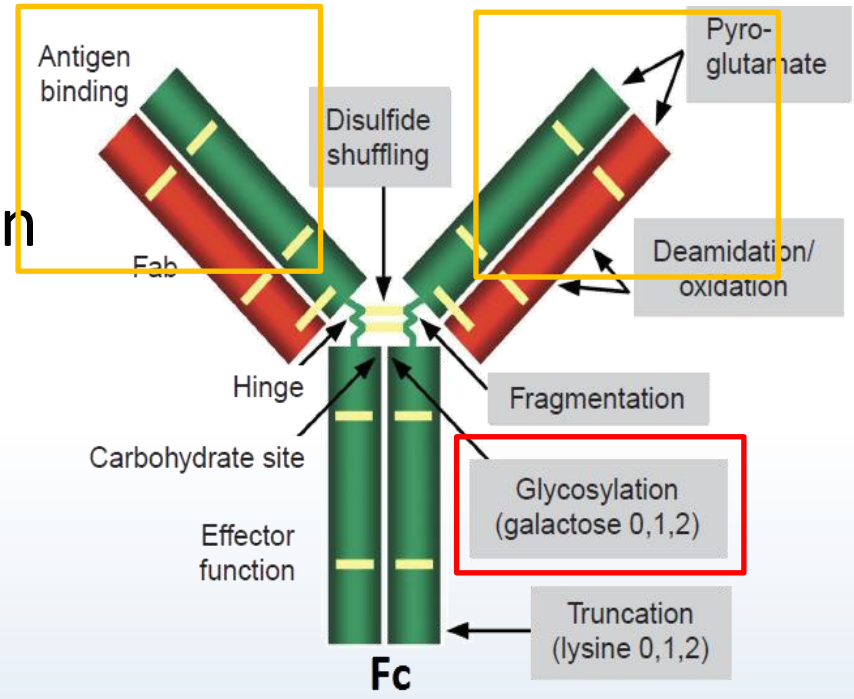
Protein Folding



- The function of a protein is determined by its shape.
- If the protein changes shape it can lose its function.
- Proteins can change shape due to changes in their environment and solution-pH, temperature, osmolarity etc.
- It is very important to control biopharmaceutical production processes to ensure the correct protein is being produced.

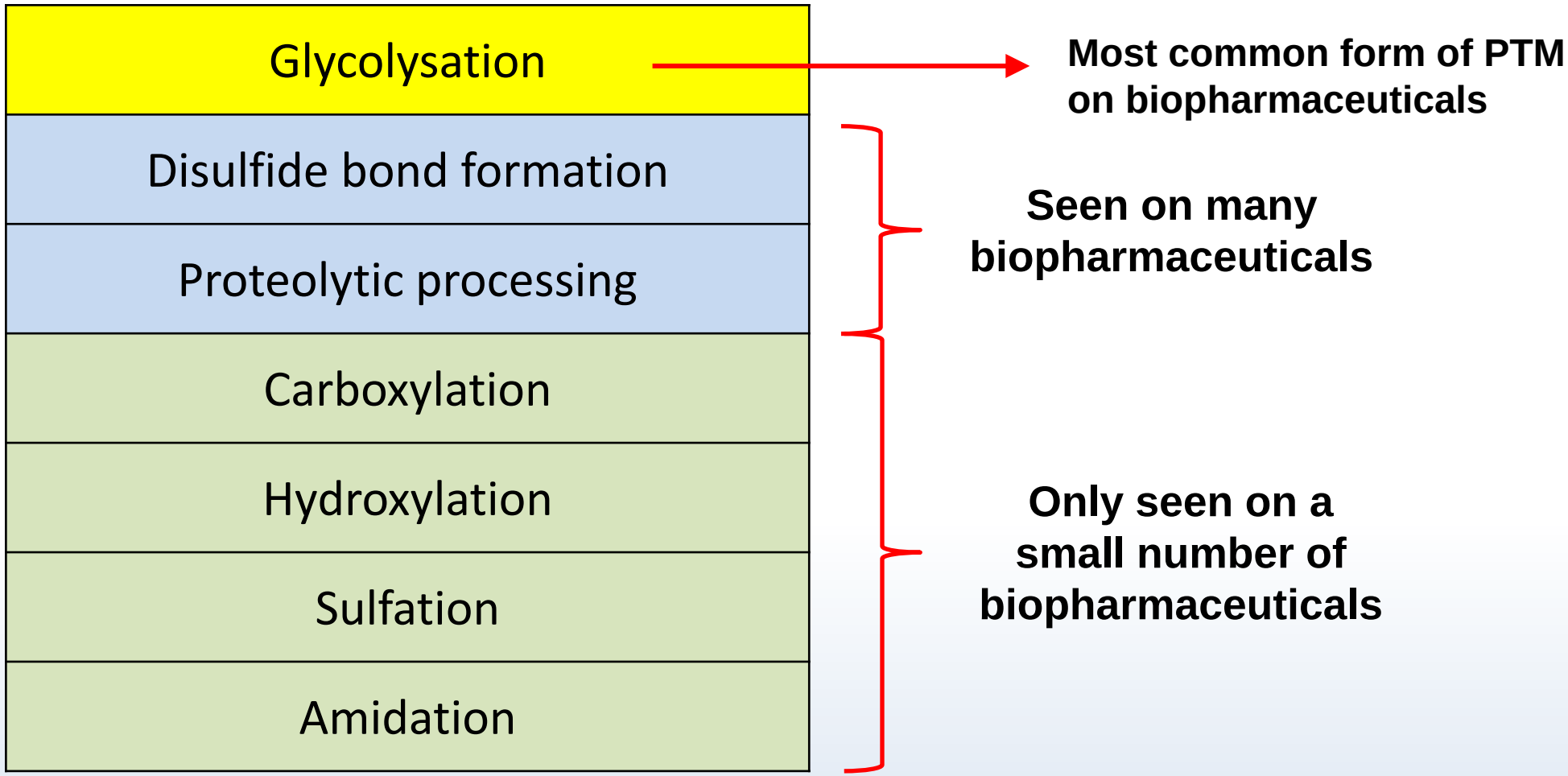
Post Translational Modification

- After a cell creates a protein (‘translation’) chemical modifications (PTMs) occur.
- Performed by enzymes in the cell
- Proteins can undergo 100s of PTMs
- PTMs influence the structure and thus the function of the protein
- PTMs are species specific:
  - Bacteria versus mammalian cells



Chirino and Mire-Sluis, 2004

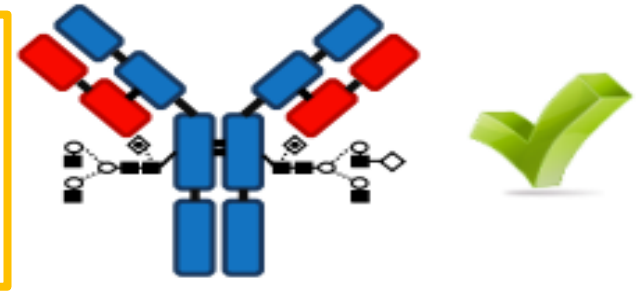
Important PTMs for Biopharmaceuticals:



The Process, the Product and the Patient

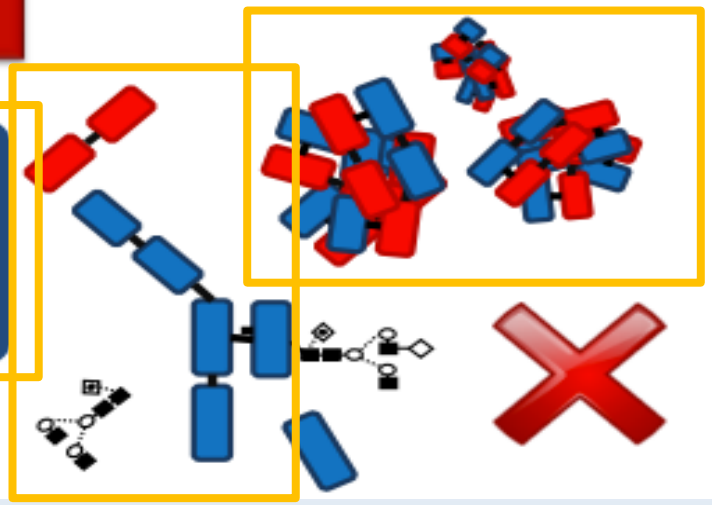
Safe & Efficacious Product

Correct Culture conditions → Correct Purification Conditions → Correct Filling, Shipping, Storage conditions



Potentially unsafe & Non-efficacious product

Incorrect media, mutations, scaling changes → Incorrect buffers, Column degeneration, Mechanical shear → Contamination during filling, environmental conditions during shipping/storage



Choice of Expression System

For Simple Proteins

- Simpler Fermentation
- Scalable
- Lower Cost of Goods

- Post-translational Modifications that are not human-like

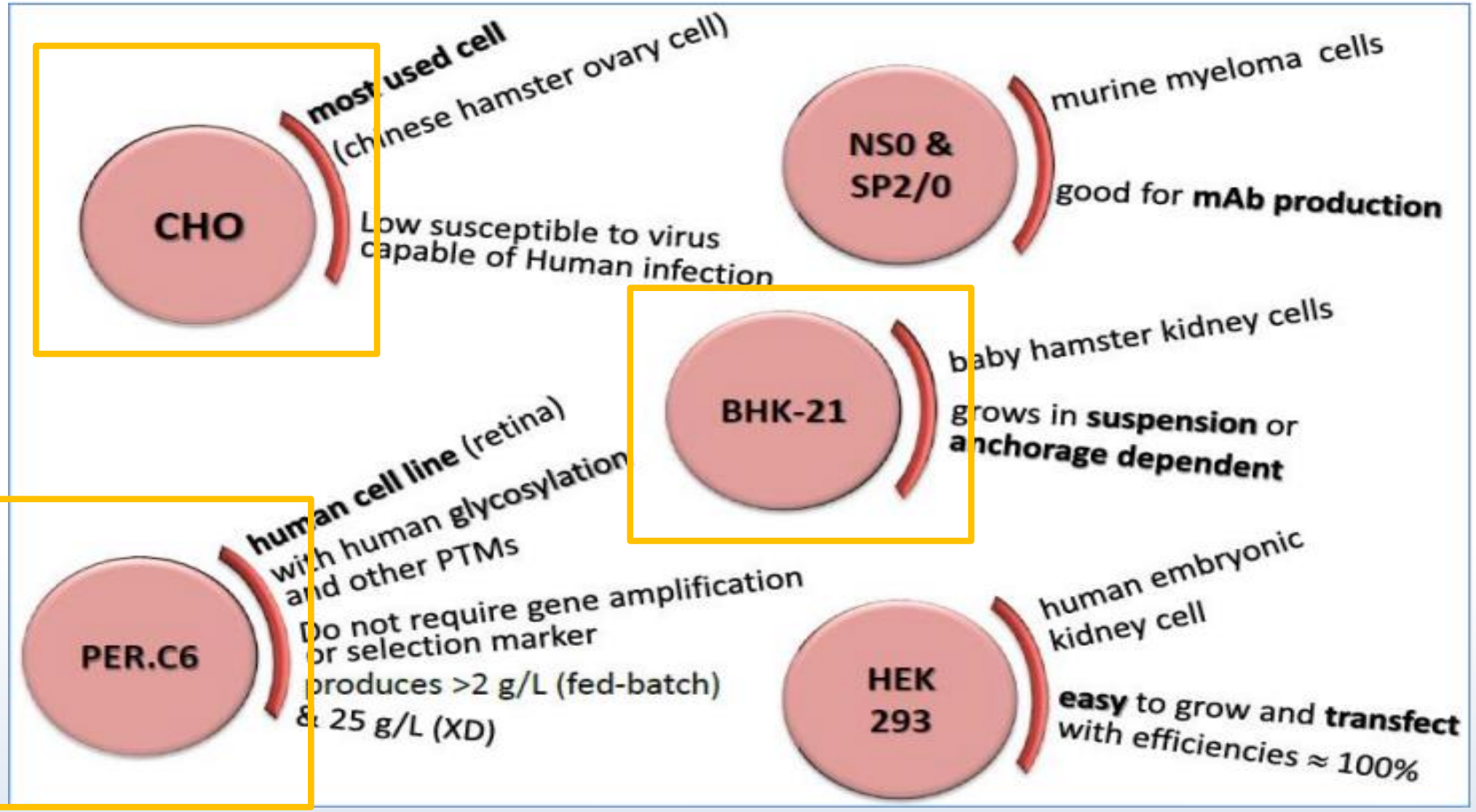
VS

For Complex Proteins

- Complex Culture
- Less Scalable
- High Cost of Goods

- Human-like PTMs

Cell Lines Used in Biomanufacturing



Why Mammalian Cells?

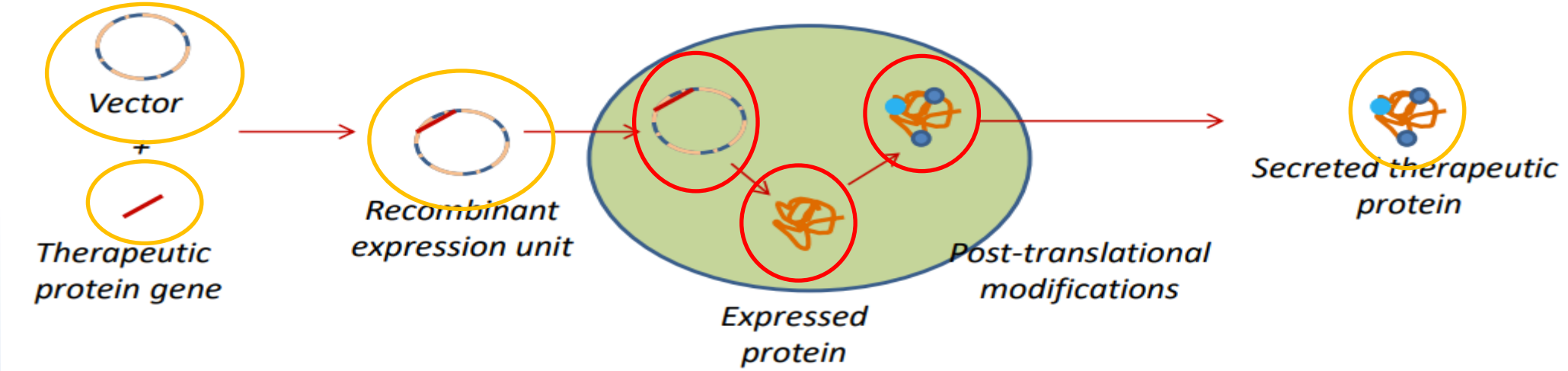
- Mammalian cells are frequently used as a host for expression of foreign genes because:

1. DNA cloned from higher eukaryotic cells (both cDNAs and genomic clones) is readily expressed since the signals for transcription, mRNA processing, and translation are conserved in higher eukaryotic systems.
2. Proteins are expressed in a stable functional form since the machinery to facilitate proper protein folding and assembly are conserved in higher eukaryotic cells.
3. Many post-translational modifications, especially for those proteins that transit the secretory pathway, are efficiently performed.

Why Mammalian Cells?

- Mammalian cells are frequently used as a host for expression of foreign genes because:

4. Many proteins are readily secreted from mammalian cells providing the ability to isolate the protein from conditioned medium that contains low amounts of protein when cells are propagated under serum-free conditions.



From: Kaufman, RJ. Overview of Vector Design for Mammalian Gene Expression. (2000) Molecular Biotechnology 16:151-160

Criteria for Cell Line Selection

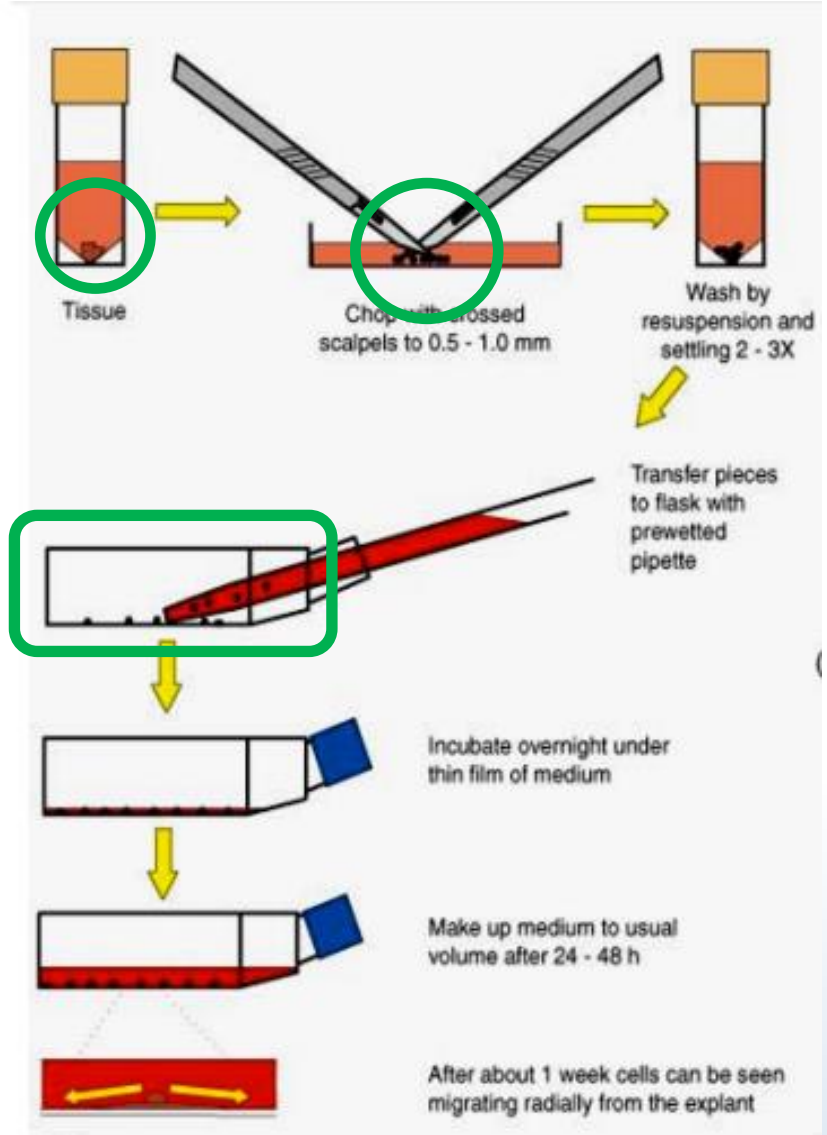
Before manufacture ever begins, some questions need to be asked;

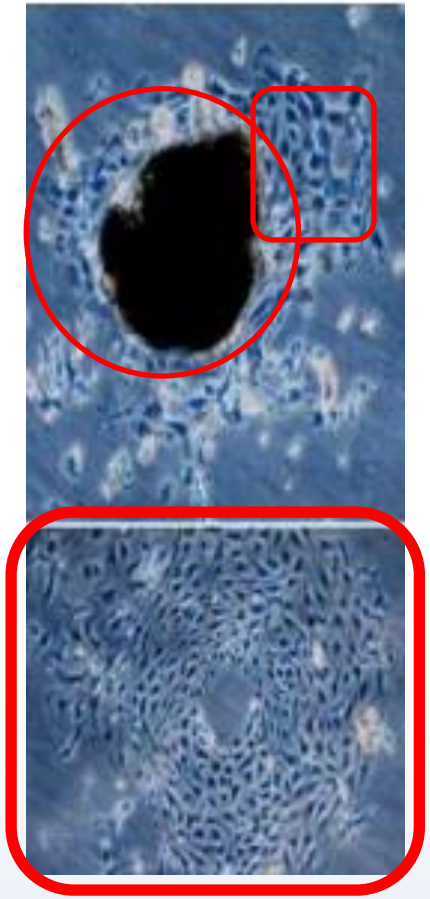
Product type?	• Simple recombinant protein • Monoclonal antibody • Fusion protein
Genetic Stability?	• Stability of transfection • Long-term genetic stability
Growth and productivity in large scale culture?	• Ease of selection of high producers • Adaptation to protein-free suspension culture • Apoptosis, proliferation rate, max cell number
Safety issues?	• Potential for endogenous viral contamination • Use of animal products in media (potential contamination)

Lecture Topics

- Choice of Cell for Biomanufacturing.
- Establishment of Cell Lines.
- Genetic Modification of Cell Lines for Protein Expression.
- Engineering Cell Lines for Optimal Growth Performance in Bioreactors.
- Impact of Genetic Engineering on Biomanufacturing

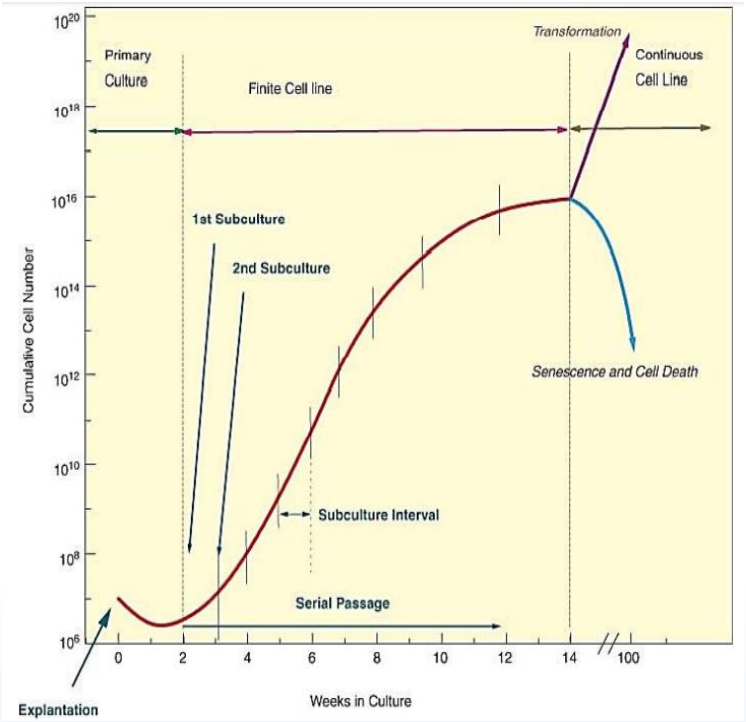
Explant culture





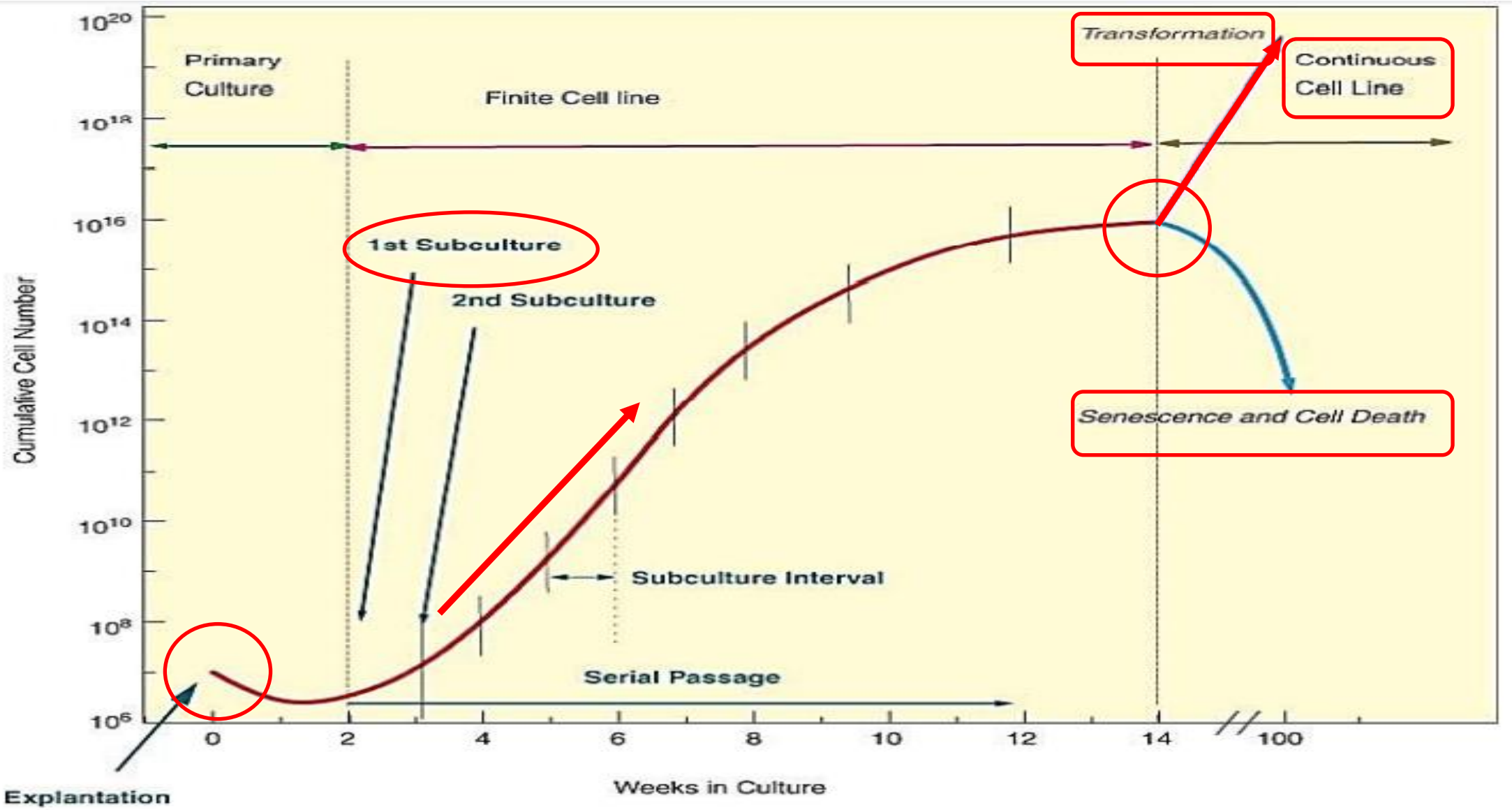
1. Finely chop up tissue into small pieces (explants) with scalpel in petri dish.
2. Transfer explants to culture flask, add media, incubate for 3 - 4 weeks.
3. Cells grow out from explant and become confluent when all flask is full the cultured is subcultured and referred to as cell line.

Evolution of a cell line



- Primary cells are growing in culture for number of passages.
- Begin to die off around passage 12-15 weeks : cell crisis.
- Related to senesence (age) and the onset of apoptosis – 'programmed suicide cell death'.
- The majority of cells die but small number survive and continue to grow.
- Referred to as 'cell transformation' event.
- Only a small number of cells lines form continuous cell lines: mostly tumour in origin.

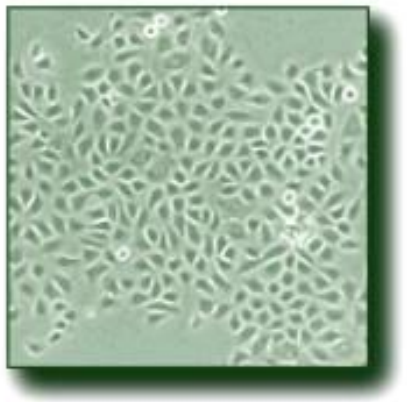
Evolution of a cell line



CHO Cells – An Industry Stalwart

- **Chinese hamster (Cricetulus griseus) ovary cells (CHO):**
  - Set up by Puck in 1957; currently the most widely used cell line in biomanufacturing.
  - 1980's: commonly used by molecular biologists.

- Many sub-clones, CHO-K1.
- Can grow **attached** or in **suspension** (preferred for bioreactors):
  - Easy to grow : robust, epithelial cells. Multiply quickly (~ 17 to 20 hrs).
- Similar post translational modifications to human cells:
  - Regulatory acceptance as 'normal' human cells.



Why CHO Cells?

- First approved for production of tissue plasminogen activator (tPA, Activase) in 1986.
- CHO cells are capable of adapting and growing in suspension culture - ideal for large scale culture in the industry.
- CHO cells pose less risk as few human viruses are able to propagate in them.
- CHO cells can grow in serum-free and chemically defined media ensuring reproducibility between batches of cell culture.
- CHO cells allow post translational modifications to recombinant proteins which are compatible and bioactive in humans. Specifically, glycosylation of glycoproteins produced by CHO cells are more human-like, with the absence of immunogenic α-galactose epitope.

Other Examples of Established Cell Lines

- **NS0**: a myeloma cell line derived from B lymphocytes of mice (Mus musculus) - Derived from NS-1 cells in 1981 – do not produce antibody:
  - Cells grow in suspension to high density in variety of media
  - Can be transfected readily and stably with genes for product
- **HEK293**: an epithelial cell line derived from human embryonic kidney cells transformed with adenovirus DNA.
- **BHK**: a cell line derived from the kidney cells of baby Syrian golden hamsters.
- **COS**: fibroblast cell lines derived from the kidney cells (SV40`transformed) of African green monkeys (Cercopithecus aethiopsis).
- **PER.C6**: a trademarked cell line (derived from a human retinal cell) developed and owned by Crucell Holland BV.

Lecture Topics

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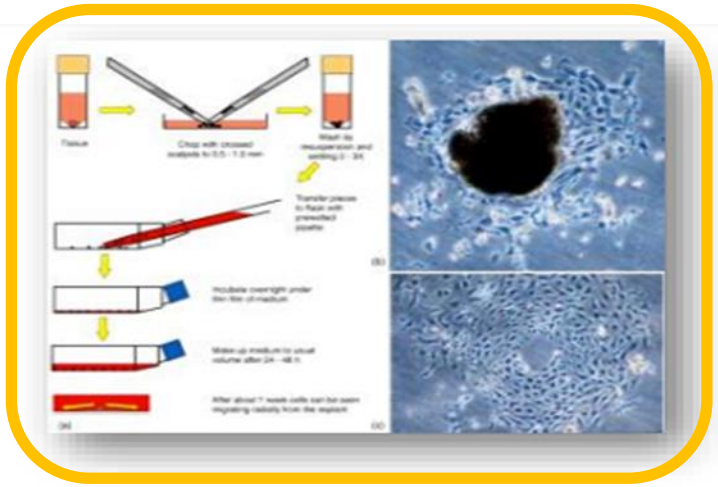
Stages in Cell Line Development for Biomanufacturing



Explant culture

Transformation

Continuous CHO cell line

Introduce the gene(s) for the biotherapeutic protein of interest

Transfection

Selection, Recovery & Expansion

Amplification

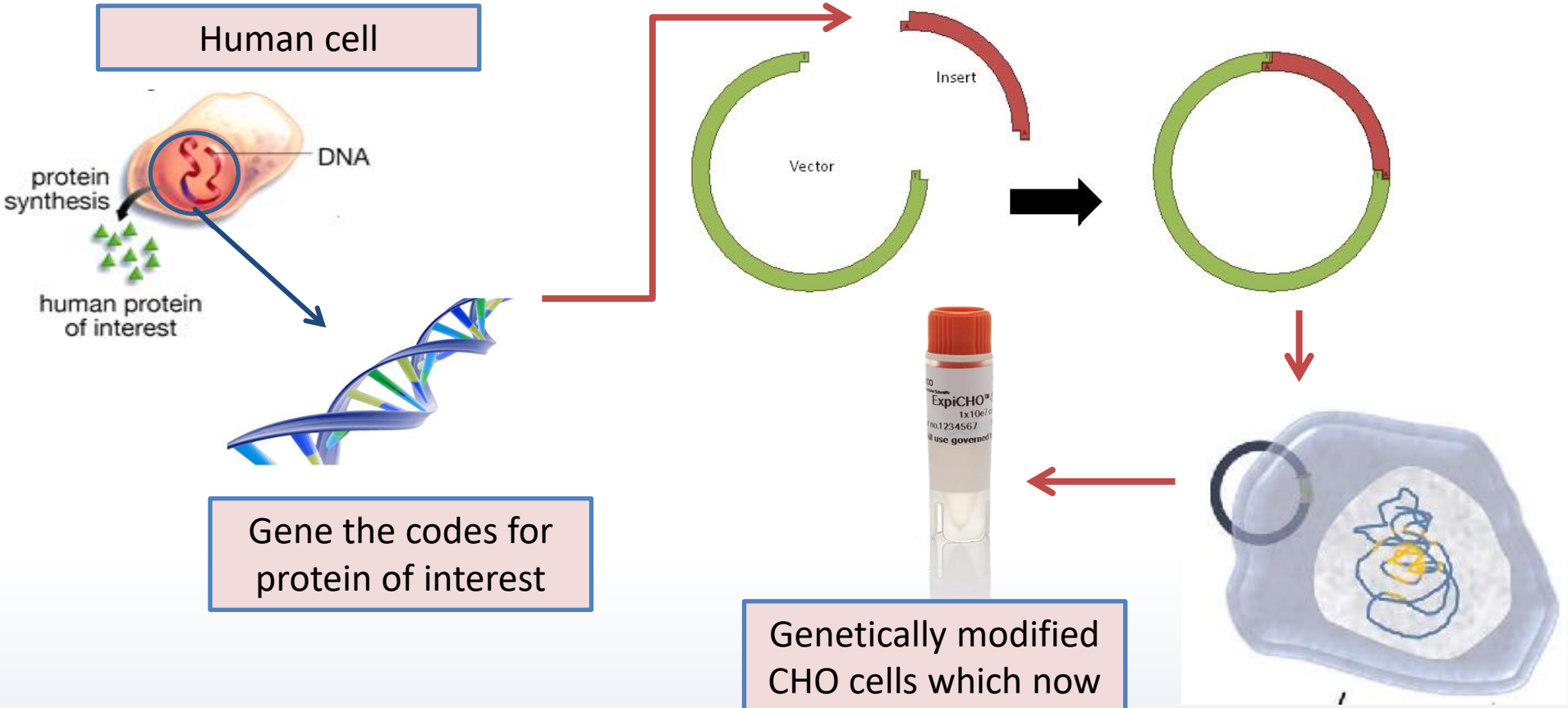
Screening, Expansion & Cell banking

Why CHO Cells?

- Several gene amplification systems are well established to make use of the genome instability of CHO cells to allow for gene amplification which ultimately result in higher yield of recombinant protein.
- Currently, recombinant protein titres from CHO cell culture have reached the gram per litre range which is a 100-fold improvement over similar process in the 1980s.
    - The significant improvement of titre can be attributed to progress in establishment of stable and high producing clones as well as optimization of culture process.

From: Tingfeng Lai, et al. Pharmaceuticals 2013, 6, 579-603 Advances in Mammalian Cell Line Development Technologies for Recombinant Protein Production

Transfection of Cell



Transfection Methods

How to we get the recombinant DNA vector into the cells?

Method	Mechanism
Calcium Phosphate	Precipitates of the + charged calcium and the - charged phosphate bind to DNA and enter nucleus
DEAE-Dextran	Binds and interacts with negatively charged DNA molecules
Electroporation	Electric field opens pores in membrane
Lipofection	Liposomes transport genetic material across the membrane

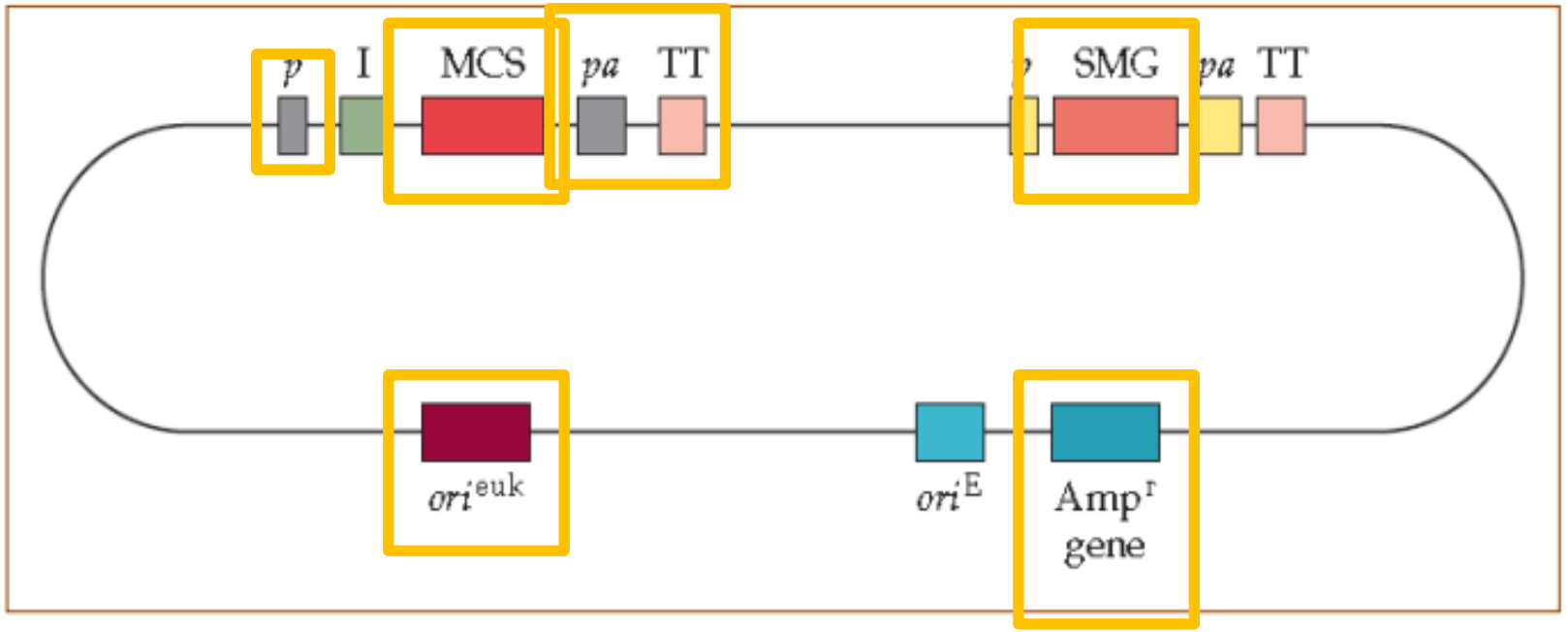
SV40 – Most Used System for Transient Expression

- Based on the small DNA tumour virus, simian virus 40 (SV40):
  - Cells transformed with an origin-defective mutant of SV40 express high levels of the SV40 large tumour (T) antigen required to initiate viral DNA replication.
  - T-antigen-mediated replication can amplify plasmid copy number to >10,000 per cell - results in high expression levels from the transfected DNA.
- SV40, Rous sarcoma virus (RSV), human cytomegalovirus (CMV), adenovirus - contain constitutive promoter elements that are very active in a wide variety of cell types from many species.

Features of Useful Vectors

- Most useful vectors contain multiple elements that include:
  - An **origin of replication** for amplification to high copy number in cells;
  - An efficient **promoter element** for transcription initiation;
  - **mRNA processing signals** that include intervening sequences and mRNA cleavage and polyadenylation sequences;
  - **Polylinkers** that contain multiple endonuclease restriction sites for insertion of foreign DNA; and
  - **Selectable markers** that can be used to select cells that have stably integrated the plasmid DNA.

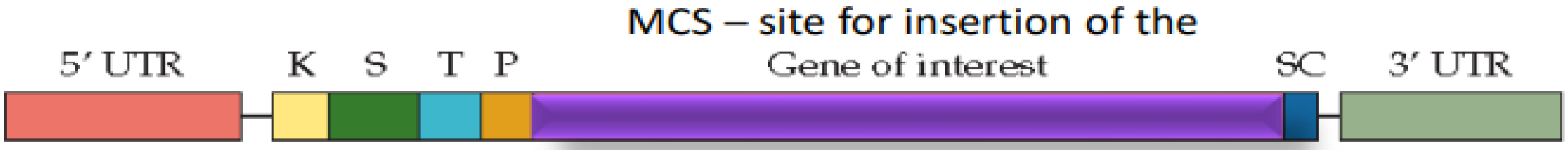
Generalized Mammalian Expression Vector



Key:

- Multiple cloning site (MCS)
- Selectable marker gene (SMG)
- Eukaryotic promoter(p)
- Polyadenylation (pa)
- Termination of transcription (TT) sequences
- Intron (I)

Translation control elements



K: Kozak sequence, e.g., GCCGCC(A or G)CCAUGG in vertebrates
S: Signal sequence to facilitate secretion
T: Protein sequence (tag) to enhance the purification of the heterologous protein
P: proteolytic cleavage sequence that enables the tag to be removed from the heterologous protein
SC: Stop codon
5′ and 3′ (UTRs): untranslated regions important for efficient translation and mRNA stability

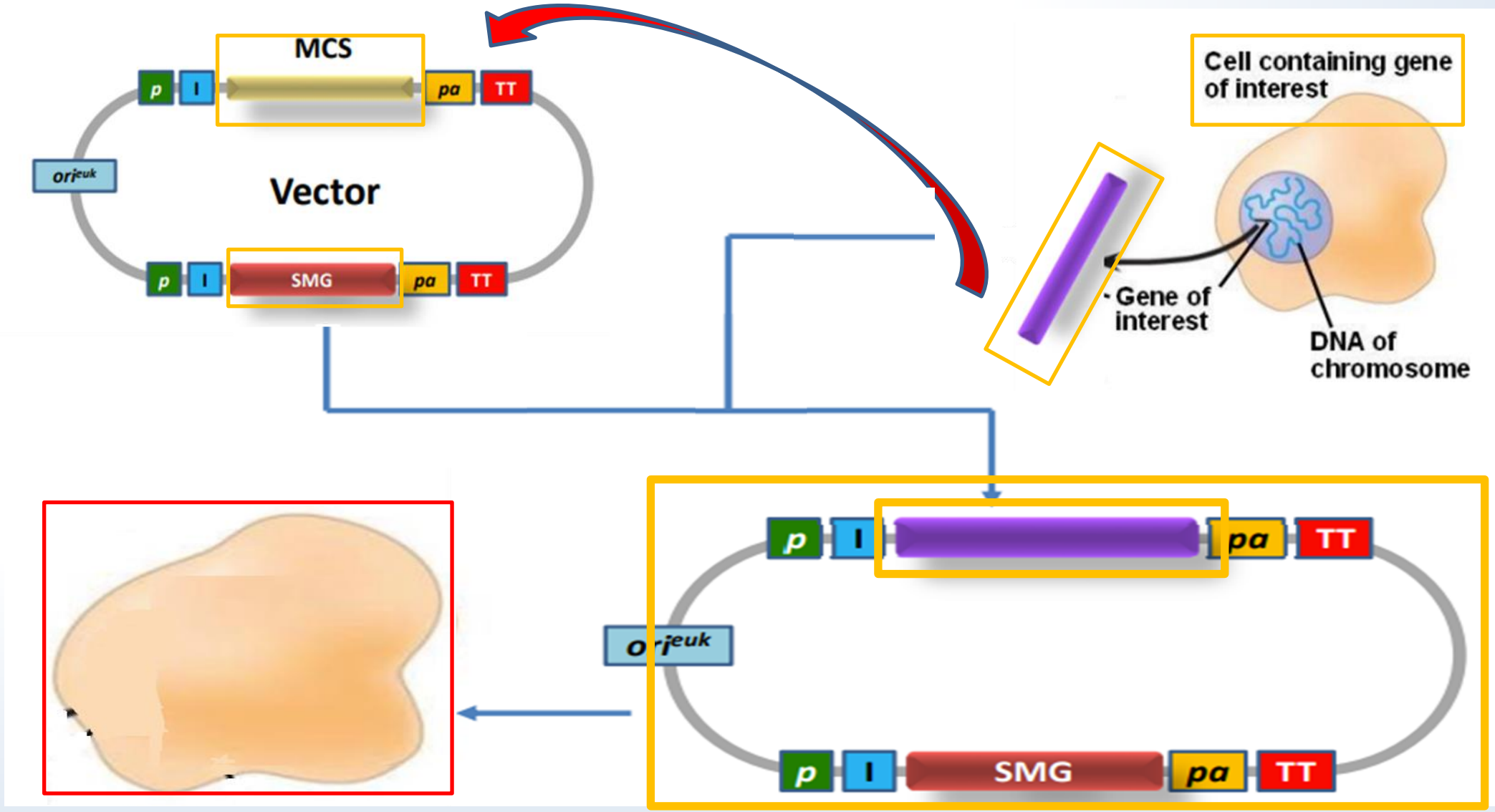
Selectable Markers for Mammalian Expression Vectors

TABLE 7.7 Selective marker gene systems for mammalian cells

Selective agent	Action of selective agent	Marker gene	Action of marker gene protein
Xyl-A	Damages DNA	Adenine deaminase *(ada)*	Deaminates Xyl-A
Blasticidin S	Inhibits protein synthesis	Blasticidin S deaminases *(Bsr, BSD)*	Deaminates blasticidin S
Bleomycin	Breaks DNA strands	Bleomycin-binding protein *(Ble)*	Binds to bleomycin
G-418 (Geneticin)	Inhibits protein synthesis	Neomycin phosphotransferase *(neo)*	Phosphorylates G-418
Histidinol	Produces cytotoxic effects	Histidinol dehydrogenase *(hisD)*	Oxidizes histidinol to histidine
Hygromycin B	Inhibits protein synthesis	Hygromycin B phosphotransferase *(Hph)*	Phosphorylates hygromycin B
MSX	Inhibits glutamine synthesis	Glutamine synthetase *(GS)*	Cells that produce excess glutamine synthetase survive.
MTX	Inhibits DNA synthesis	Dihydrofolate reductase *(dhfr)*	Cells that produce excess dihydrofolate reductase survive.
PALA	Inhibits purine synthesis	Cytosine deaminase *(codA)*	Lowers cytosine levels in the medium by converting cytosine to uracil
Puromycin	Inhibits protein synthesis	Puromycin *N*-acetyltransferase *(Pac)*	Acetylates puromycin

MSX, methionine sulfoximine; MTX, methotrexate; PALA, *N*-(phosphoacetyl)-L-aspartate; Xyl-A, 9-β-D-xylofuranosyl adenine.

Generalized Mammalian Expression



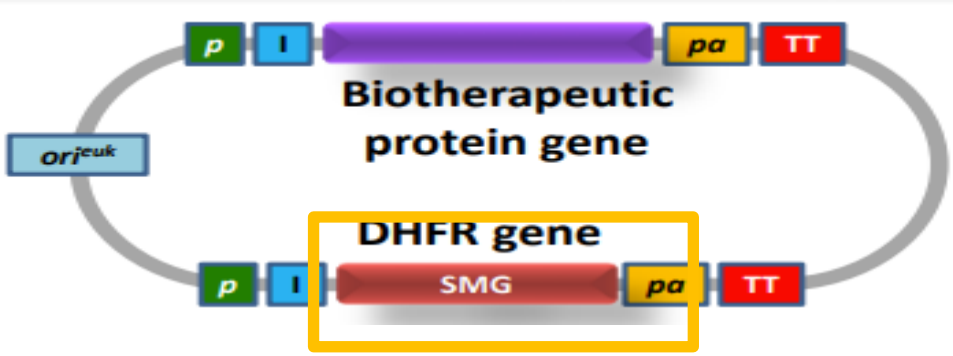
CHO Cell Transfection

- After transfection with expression vectors containing the expression cassettes for the recombinant protein and selection marker genes, the cells are selected and gene-amplified with the selection drug e.g. MTX or MSX.
  - MTX or MSX concentration can also be increased step-wise to further increase cell protein productivity by further gene amplification.
  - Single cell cloning or limiting dilution (a regulatory requirement) is then performed to ensure that the selected cells for further processing are producing the recombinant protein.
  - Analyses of protein titers are subsequently used to choose the clones for progressive expansions.
  - Finally, selected clones are evaluated in controlled bioreactors and banked for future use.

DHFR Selection

- DHFR catalyzes the conversion of folate to tetrahydrofolate (FH4). FH4 is required for the biosynthesis of glycine from serine, for the biosynthesis of thymidine monophosphate from deoxyuridine monophosphate, and for purine biosynthesis.
- Methotrexate (MTX) is a folic acid analog that binds and inhibits DHFR, leading to cell death. When cells are selected for growth in sequentially increasing concentrations of MTX, the surviving population contains increased levels of DHFR that result from amplification of the DHFR gene. Most frequently, the degree of gene amplification is directly proportional to the expression level of DHFR. Highly resistant cells may contain several thousand fold elevated levels of DHFR.

DHFR/MTX Gene Selection



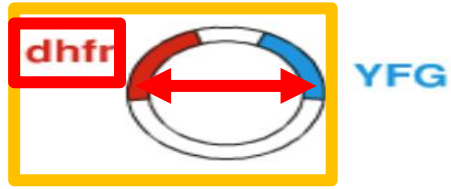
- Vector contains a MCS (to accommodate the inserted gene of interest i.e. biotherapeutic protein) AND a SMG – selectable marker gene:
  - SMG is used to selectively allow growth of transfected cells while non-transfected cells are killed off
- Using DHFR (dihydrofolate reductase) as the SMG:
  - MTX blocks nucleotide synthesis and therefore DNA / RNA synthesis.
  - DHFR+ cells are MTX resistant.
  - Growing cells in increasing concentrations of Methotrexate (MTX - an anti-cancer drug) results in co-amplification of the biotherapeutic protein expression.

DHFR/MTX Gene Amplification in Mammalian Cells

Principal system = dhfr⁻ CHO cells

Facilitated by the availability of DHFR-deficient mutant CHO cell lines

CHO dhfr- cell + vector with dhfr minigene + YFG



↓

-GHT medium

Most cells die

Transfectants live

↓ + gradually increasing conc. of MTX

Cells with gradually amplified dhfr transgenes survive

YFG is co-amplified along with the dhfr minigene

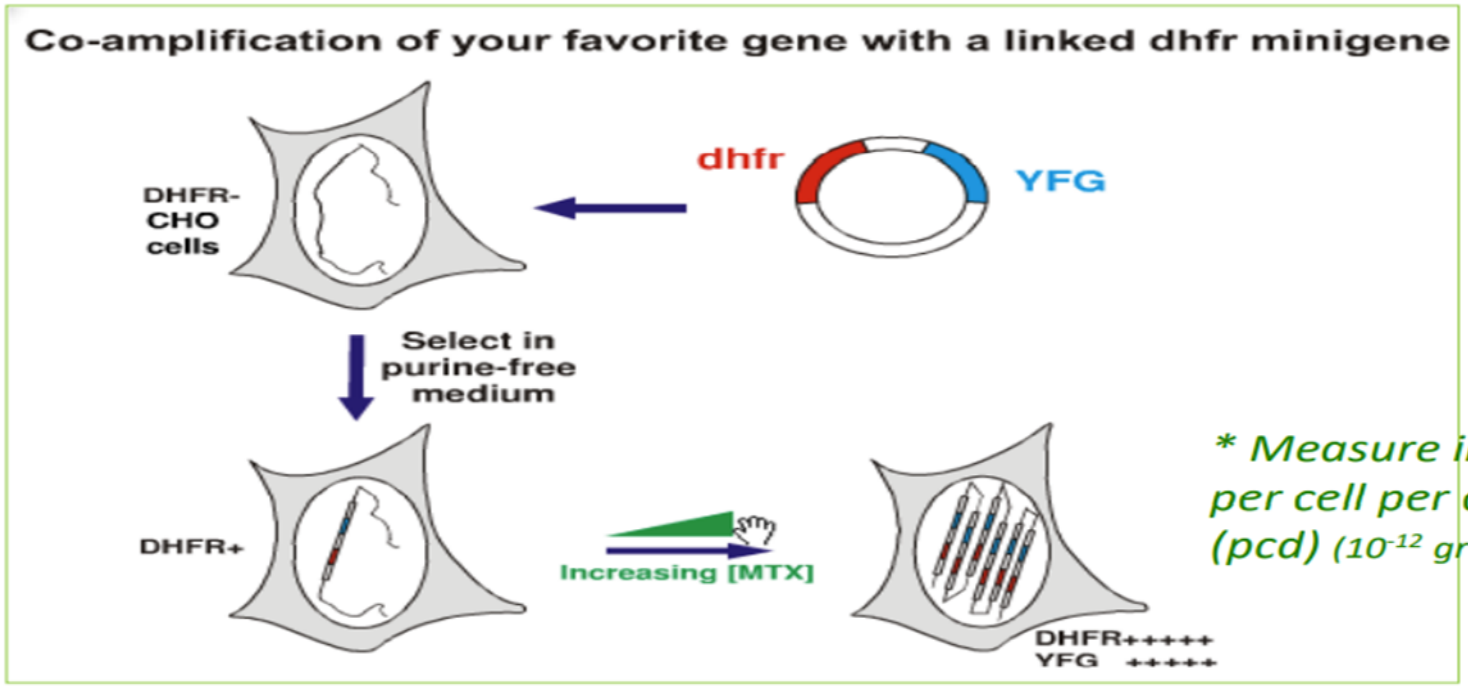
YFG = Your favourite gene –

GHT = without **g**lycine, **h**ypoxanthine (a purine source) and **t**hymidine

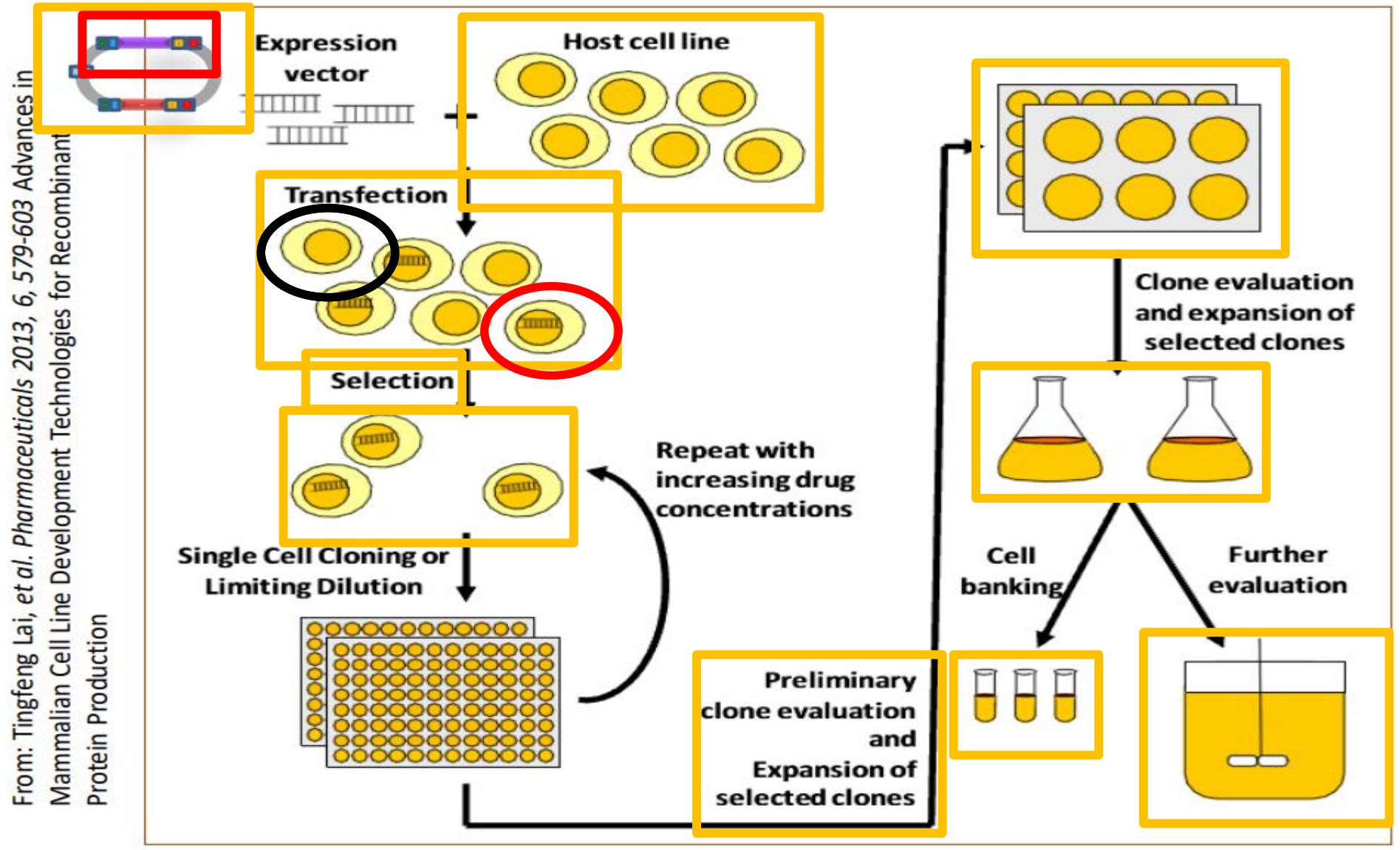
MTX = methotrexate

DHFR/MTX Gene Amplification in Mammalian Cells

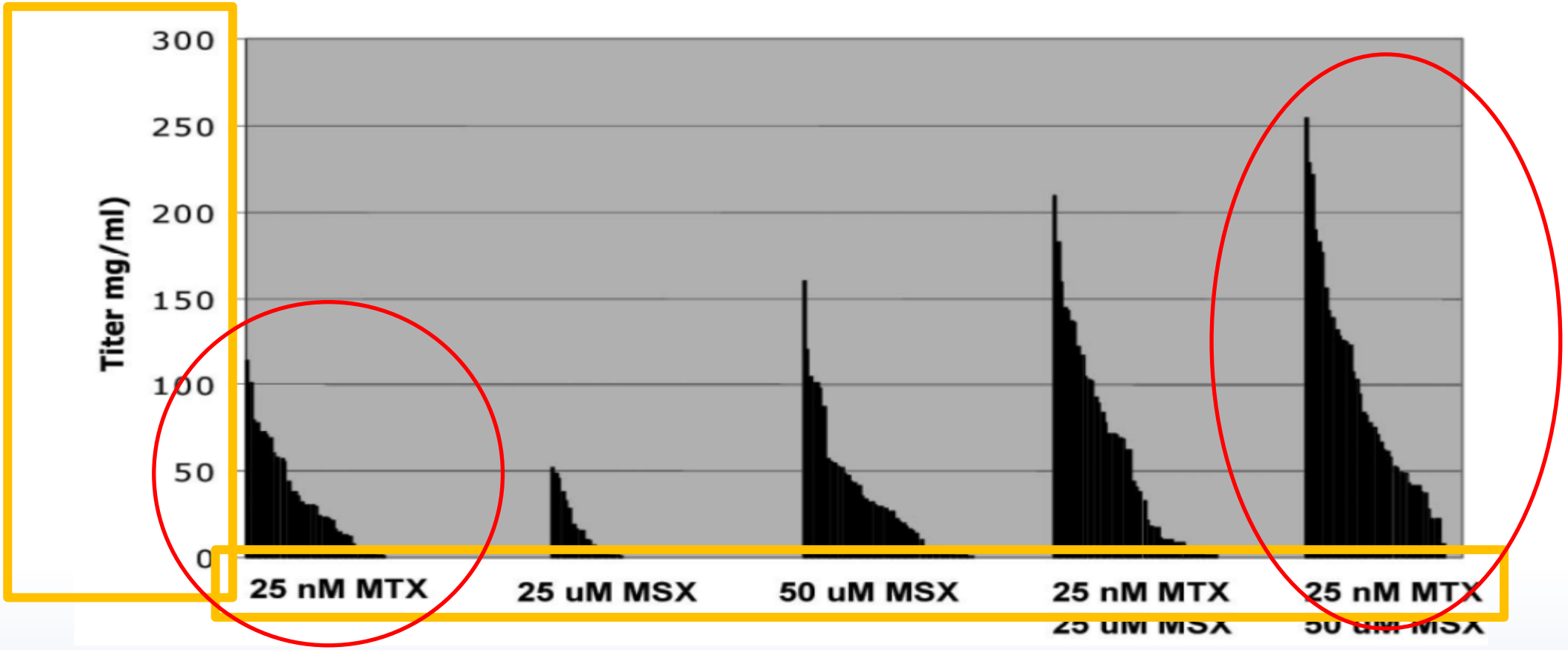
- Because the DHFR and biotherapeutic protein genes are linked within the same vector, strong expression of one means strong expression of the other:
  - i.e. the more resistant the cell is to MTX, the stronger the DHFR and therefore the biotherapeutic protein gene expression
  - > MTX resistance = > cell productivity (Qp = pg/cell/day)*



Genetic Modification of Cell Lines



DHFR Methotrexate Selection of High Producing Clones



96-well Elisa assay of clones from single and double selection. For each case, 72 clones were screened. [Notes: MTX, methotrexate; MSX, methionine sulphoximine].

From: Li, F. et al. Cell culture processes for monoclonal antibody production. mAbs (2010) 2(5):466-477

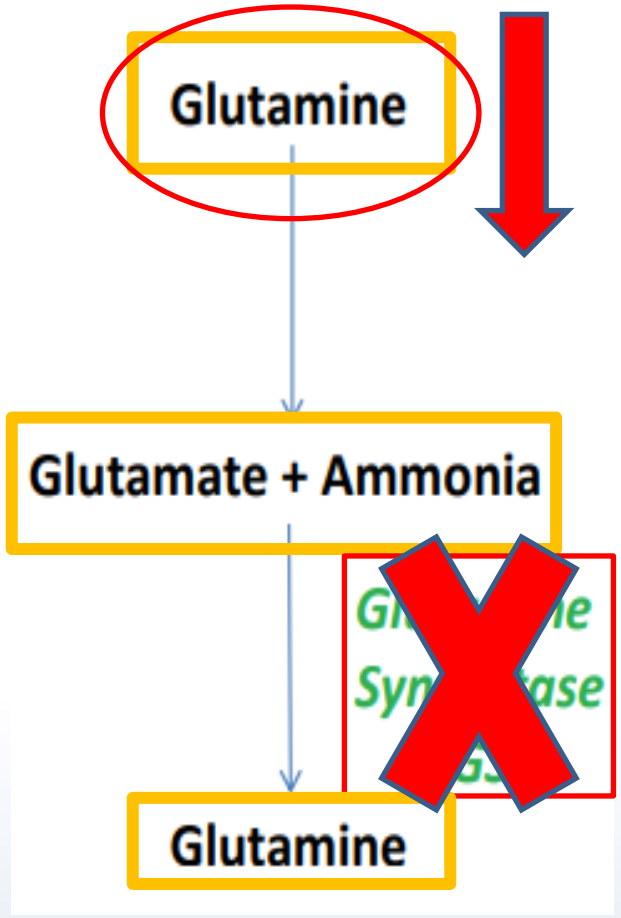
The Glutamine Synthetase (GS) System

- Currently, most cell line development technologies are based on either the methotrexate (MTX) amplification technology that originated from the 1980s, or the glutamine synthetase (GS) system (from Lonza).
- **The GS system uses the selectable marker gene Methionine sulphoximine (MSX) inhibits GS in the GS system:**
  - Developed by Lonza
  - Extensively used to modify the wild-type CHOK1 cell line, and its derivative CHOK1SV (developed by Lonza).

GS Modified Cell Lines

- Glutamine synthetase (GS) selection, capitalizes on the fact that, in the absence of an exogenous source of glutamine, cell survival is dependent on the GS enzyme to produce glutamine:
  - With host cell lines having low endogenous GS activity e.g. NS/0 cells, it allows a simple selection scheme when using a GS selectable marker in the expression vector and glutamine-free selection media.
  - On the other hand, CHO cells tend to have higher endogenous GS activity, making glutamine-free selection less efficient.
  - However, similar to the DHFR/MTX system, the GS competitive inhibitor methionine sulphoximine (MSX) can be added to the media to apply additional pressure and select for CHO cells that are driving high levels of expression from the integrated vector.
  - IP relating the GS technology is now expired making the approach more widespread.

Glutamine Synthetase(GS) system



- GS is an enzyme that converts glutamate and ammonia back into glutamine.
- Vector with GS enzyme transfected into CHO and NSO cell systems:
  - NSO cell do not express the enzyme and CHO have low levels
- **GS-NSO cells show 3 to 4-fold increase in productivity.**
- **Lonza have reported yields of up to 10g/L using the GS system**, with serum-free media and amino acid feed.

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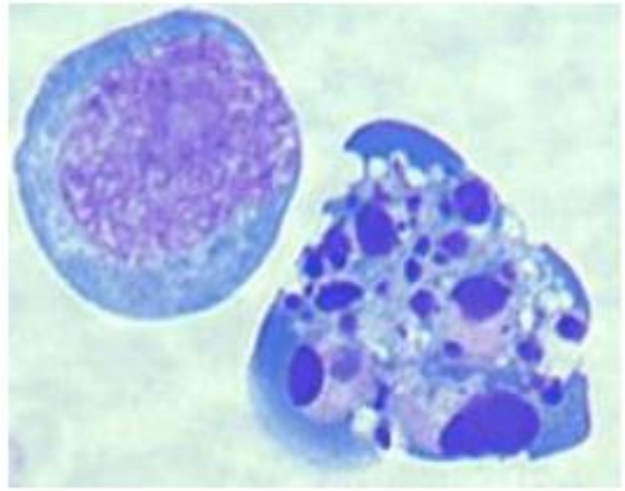
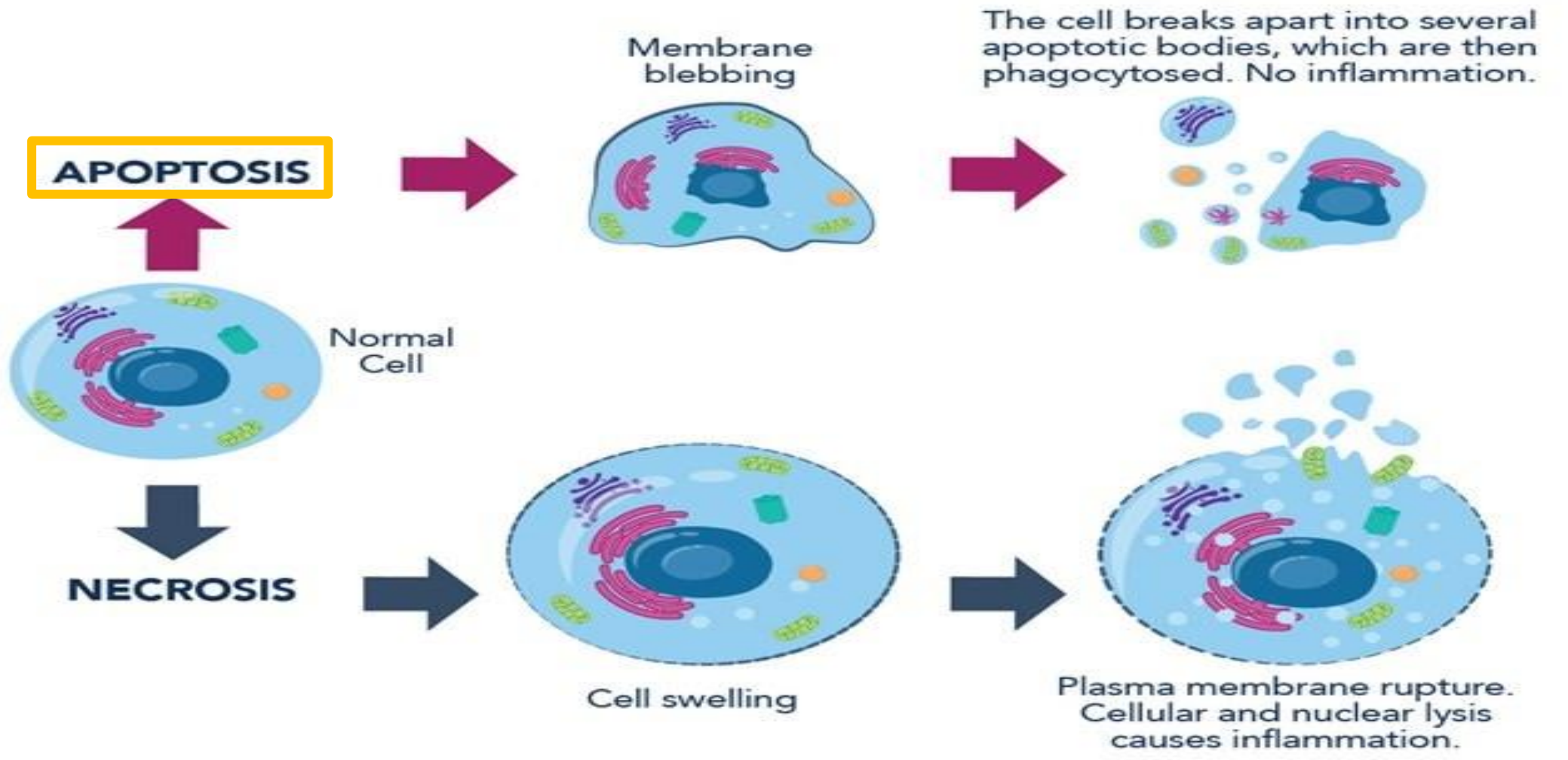
Cell Growth and Apoptosis

- Cell death is part of the aging process and also regeneration.
- **Necrosis:** results from injury or disease : Cell swells and bursts.
- **Apoptosis:** also referred to as programmed cell death or cell suicide:
  - A natural process.
  - Characteristic pattern, cells shrink, get formation of apoptotic bodies and break-up of DNA.
  - Removal of cells with damaged DNA.
  - Apoptosis is not reversible.



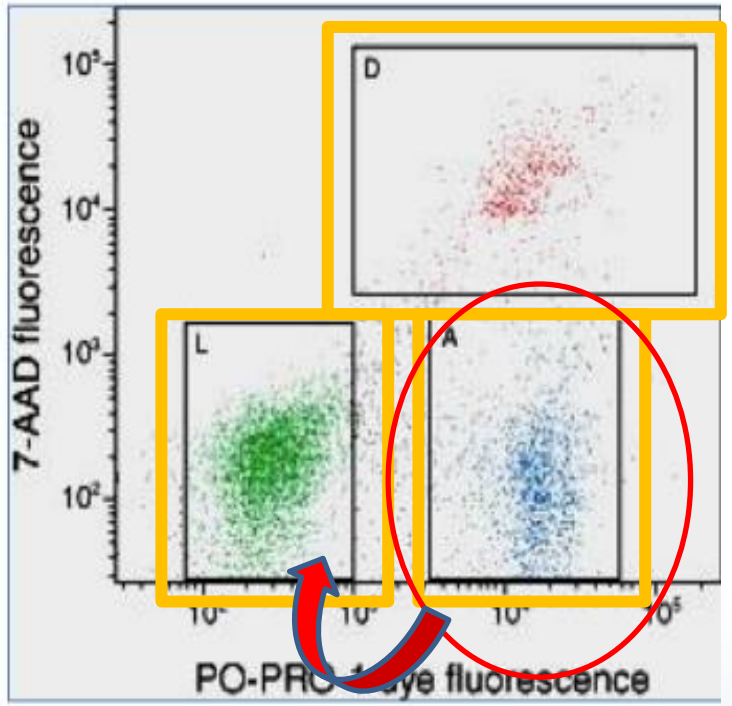
Healthy cell Apoptotic cell

Apoptosis –V- Necrosis



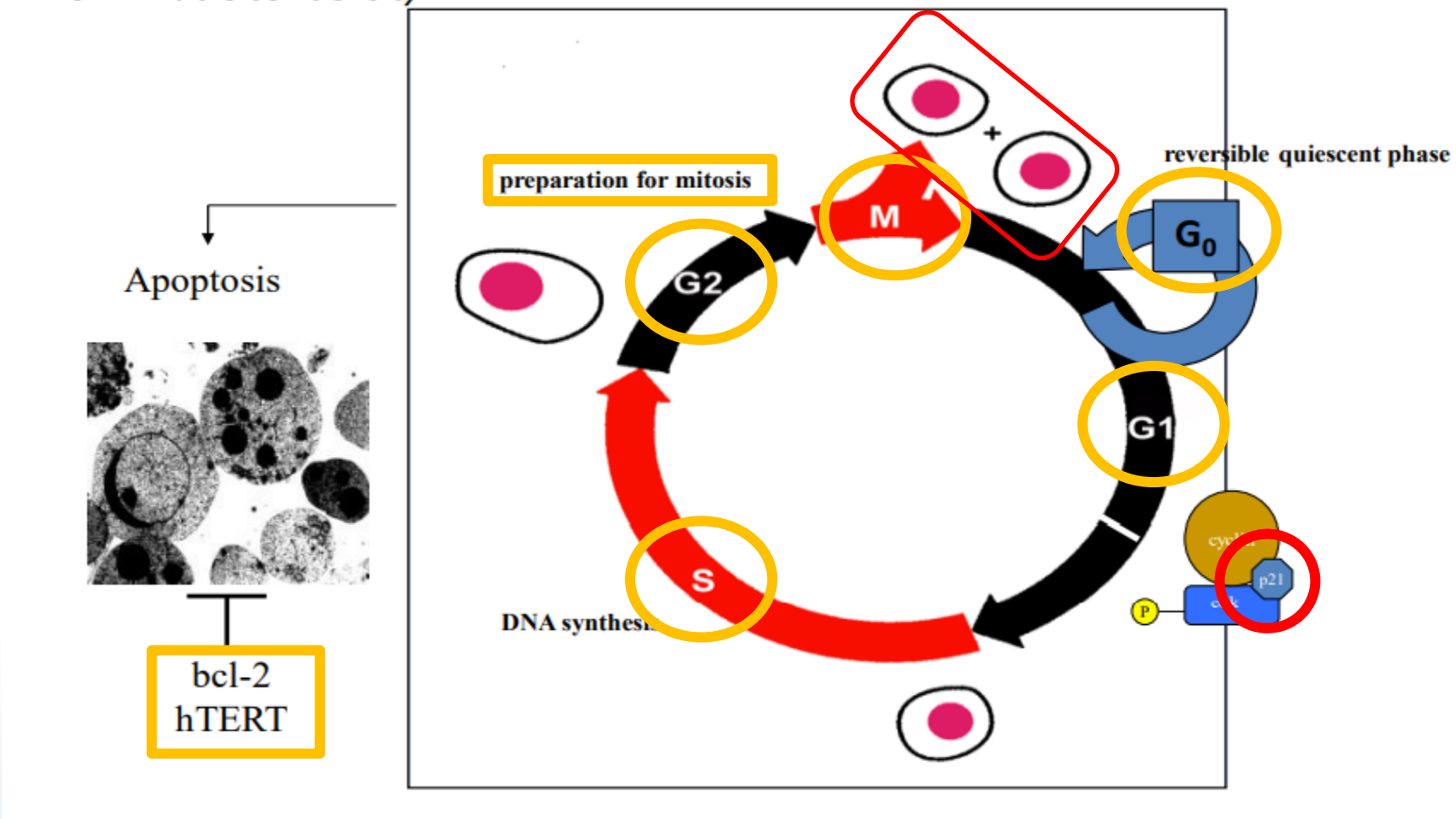
Apoptosis and Bioprocessing

- Apoptosis is a major problem in Bioprocessing.
- **Try to avoid apoptosis in production: to extend lifetime of culture (increase amount of product).**
- In bioreactor can be **triggered by lack of nutrients, O2 levels, agitation** (details in large-scale).
- Necrosis occurs due to 'gross' injury.
- Can monitor for apoptosis using fluorescent dyes and flow cytometry: picture shows L (live), D (dead) and A (apoptotic cells).



Optimising Cell Growth Kinetics and VCD

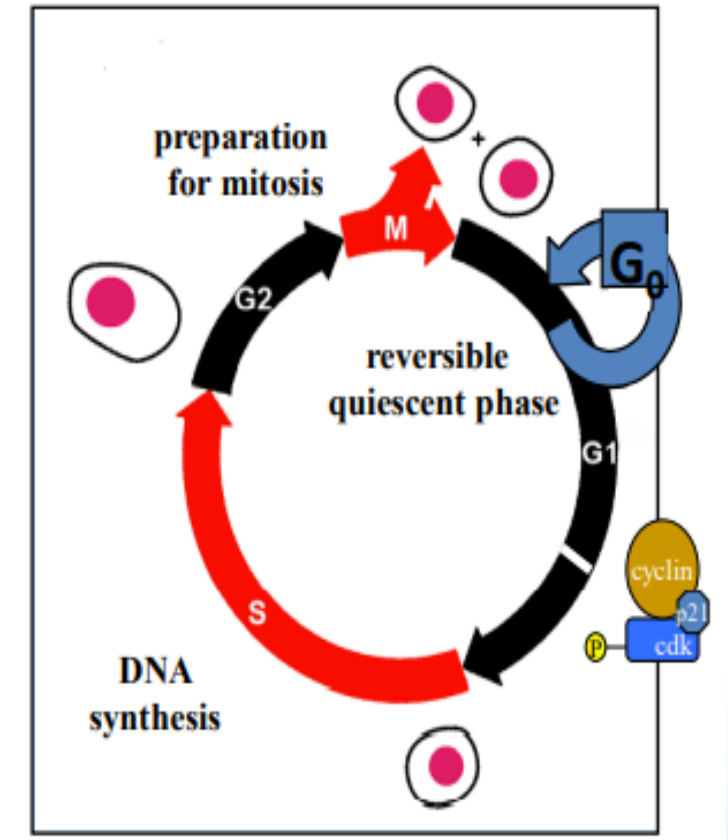
VCD = viable cell density



From: Al-Rubeai, M. Development of Cell Lines for Controlled Proliferation and Apoptosis. Presentation at 'Cell Line Development and Engineering' Meeting Zurich, May
https://slideplayer.com/slide/2303945/

Mammalian Cell Cycle

- Cells go through a repeating series of events called the cell cycle divided into 4 phases:
  1. **G1** phase (Gap 1), the cell grows and prepares for cell division;
  2. **S** phase (Synthesis) replication of DNA;
  3. **G2** phase (Gap 2) further growth takes place;
  4. **M** phase (Mitosis) where mitosis actually occurs.
- Also G0 phase where cells are non-dividing or resting - referred to as quiescence.



Optimising Cell Growth Kinetics and VCD

- **Key genetic elements:**
  - bcl-2: suppresses cell death
  - p21: arrests cell proliferation and enhances specific productivity
  - c-myc: enhances proliferation rates, reduces serum dependency and induces anchorage independence
  - hTERT: reduces apoptosis, enhances proliferation rates, and increases attachment tendency in serum free media

From: Al-Rubeai, M. Development of Cell Lines for Controlled Proliferation and Apoptosis. Presentation at 'Cell Line Development and Engineering' Meeting Zurich, May www.ucd.ie/ccerc/PowerPoint/Cell%20line%20development%20IBC.pps

Bcl-2 Over Expression

- Bcl-2 over-expression: the advantages:
  - Increases cell viability (VCD = viable cell density)
    - > VCD = > Qp pg/cell/day (productivity)
- Prolongs culture duration:
  - Therefore > Qp and < downtime
- Reduces serum dependency.
- Enhances adaptation in serum free media:
  - Favours serum-free media and less variability
- Improves nutrient metabolism:
  - Enhanced conversions rates for nutrient into product
- Protects cells in stressful conditions:
  - Supports > VCD and therefore >Qp

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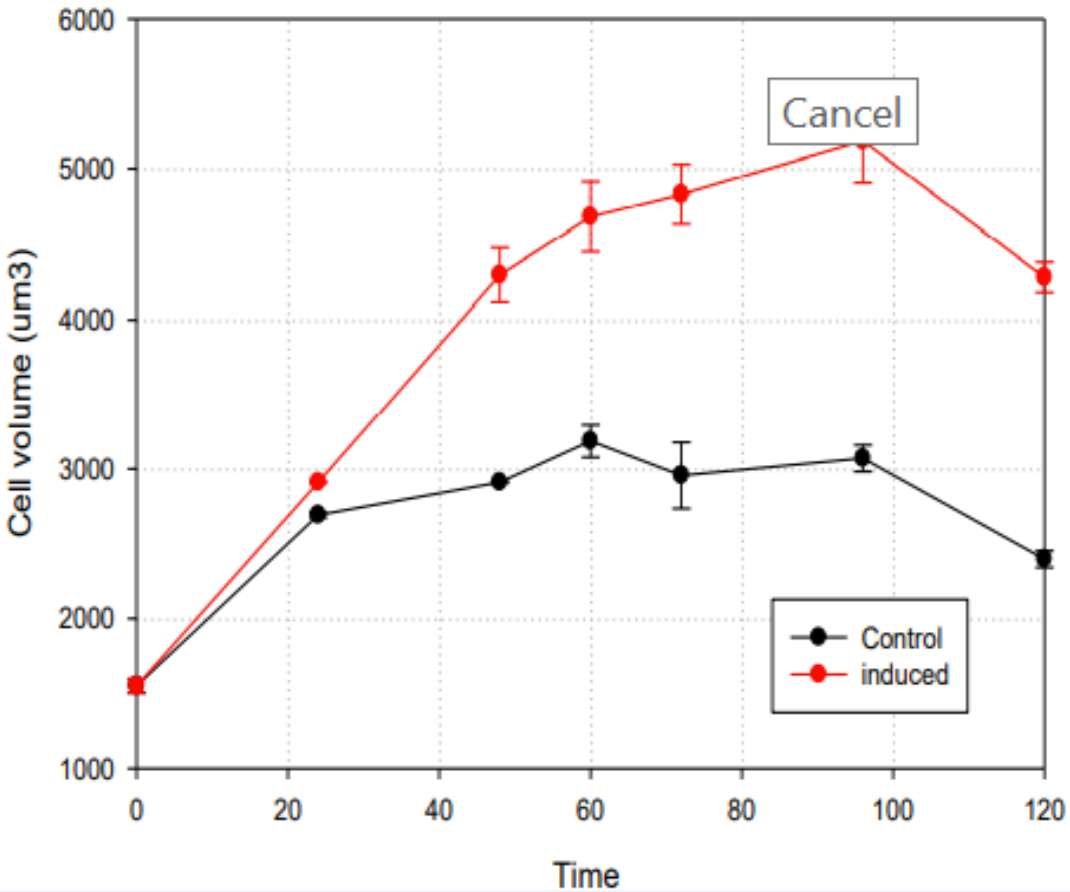
www.ucd.ie/ccerc/PowerPoint/Cell%20line%20development%20IBC.pps

Bcl-2 Over Expression

- P21 arrests cells in G1 phase:
  - G1 is more productive than S and G2
- P21 leads to increased:
  - mitochondrial activity;
  - oxygen uptake rate;
  - cell volume;
  - total cellular protein;
  - dry cell weight;
  - ribosomal biogenesis;
  - intracellular IgG;
  - H:L chain ratio.



- Larger cells are more productive than smaller cells.

From: Al-Rubeai, M. Development of Cell Lines for Controlled Proliferation and Apoptosis. Presentation at 'Cell Line Development and Engineering' Meeting Zurich, May
www.ucd.ie/ccerc/PowerPoint/Cell%20line%20development%20IBC.pps

Lecture Topics

- Choice of Cell for Biomanufacturing.
- Establishment of Cell Lines.
- Genetic Modification of Cell Lines for Protein Expression.
- Engineering Cell Lines for Optimal Growth Performance in Bioreactors.
- Impact of Genetic Engineering on Biomanufacturing

Cell Titre Improvements To Date

- After two decades of intensive development work in cell line, media and bioreactor condition optimization:
  - **Cell specific productivity of >20 pg/cell/day can be routinely achieved for production cell lines; some as high as 100pg/cell/day;**
  - **High titres up to ~10 g/L;**
  - **Cell densities of over 2 x 10^6 cells/mL in fed-batch processes and significantly higher in perfusion system.**
- Enhanced specific productivity per cell is achieved by selection of highly productive clones, optimization of culture media, and bioreactor operation conditions.

From: Li, F. et al. Cell culture processes for monoclonal antibody production. mAbs (2010) 2(5):466-477

Genetic Engineering of Cell Lines

- Bioreactor volumetric productivity is directly proportion to the cell density achieved related to:
  - Cell growth (μ) rates and maximal densities and death rates;
  - Media composition and exchange rate;
  - Aeration capacity.
- Cell density in:
  - Original media formulations: circa **1 – 2 x 10^6 cells/ml;**
  - Optimised media: circa. **10 x 10^6 cells /ml** (in batch culture);
  - Perfusion systems: circa. **20 – 50 x 10^6 cells /ml** due to high media exchange rates.

Cell Line Development

Abbreviation	Full Name	Origin
NS0	Mouse non-secreting myeloma	Mouse lymphocyte
CHO	Chinese Hamster Ovary	Fibroblast from hamster ovary

Cancel

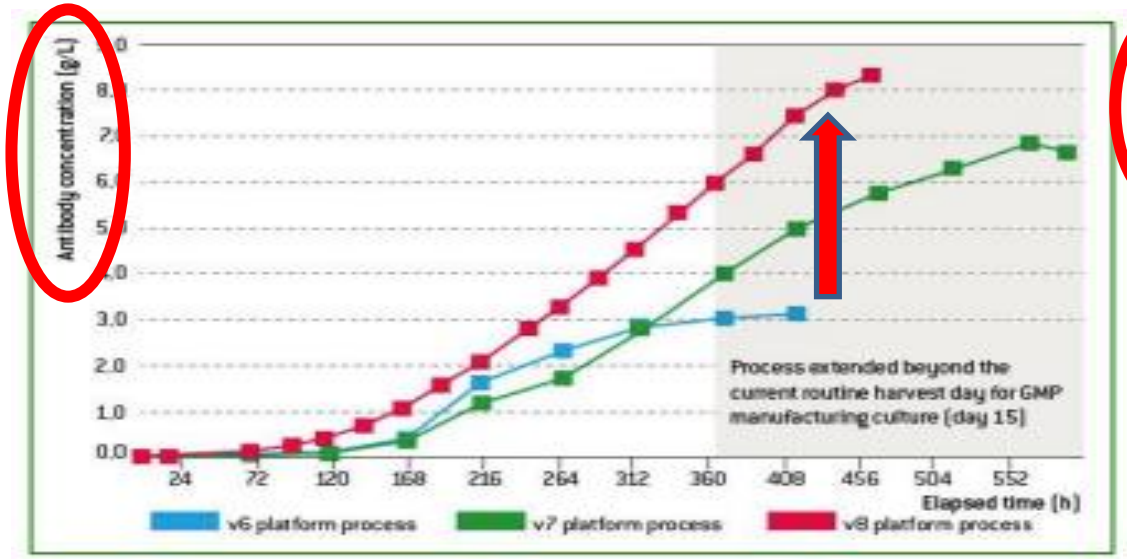
Industry standard production from stable CHO-derived cell lines

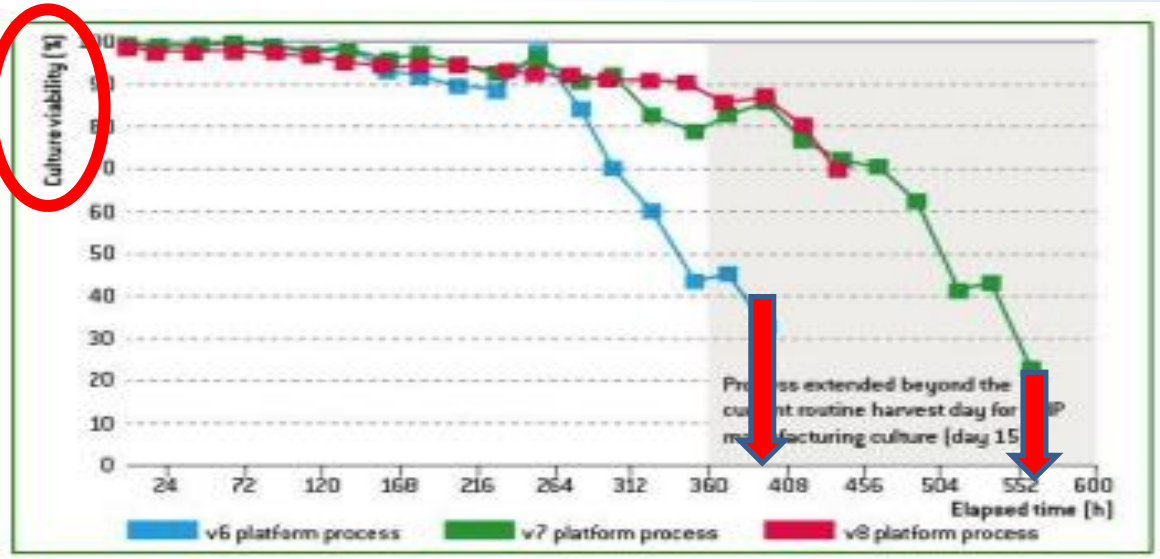
Parameter	1986	Today
Protein pg / cell / day	10	100
Titres	50 mg / L	10 g / L
Cell Density	2 x 10^6 cells/ml	10 x 10^6 cells/ml
Process duration	7 days	21 days

Commercial production of recombinant protein requires a minimum of 10 pg/cell/day

Optimised industrial cell lines: typically 20 to 40 pcd of recombinant protein with 65 pcd now common. Some have reported Qp's of up to 100 picograms per cell per day (pcd)

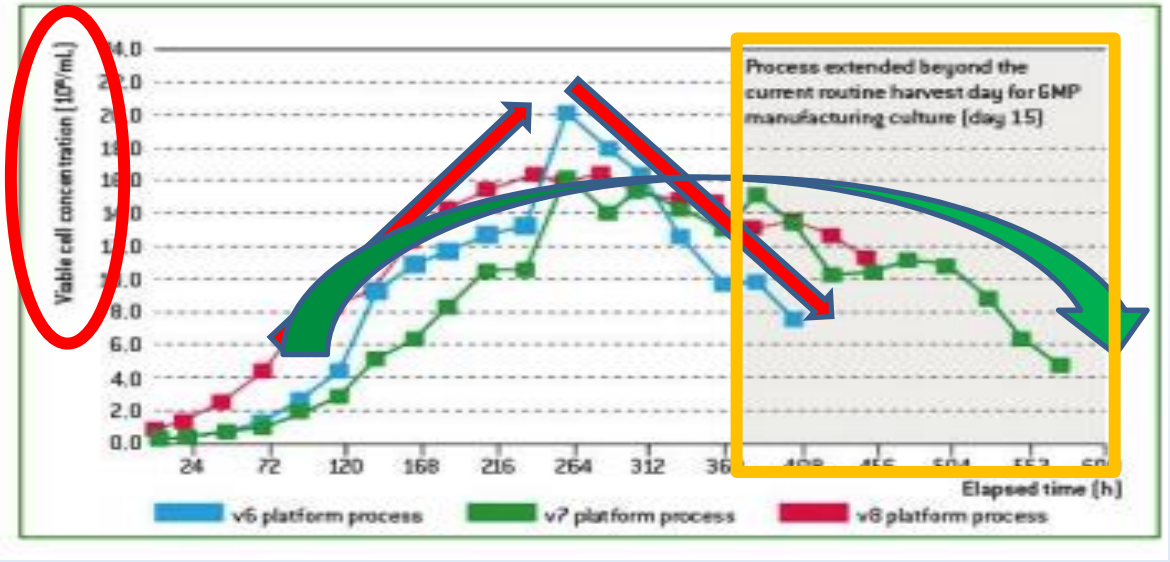
Growth of Mab-producing GS-CHO & GS-NS0 With and Without Anti-apoptosis Gene



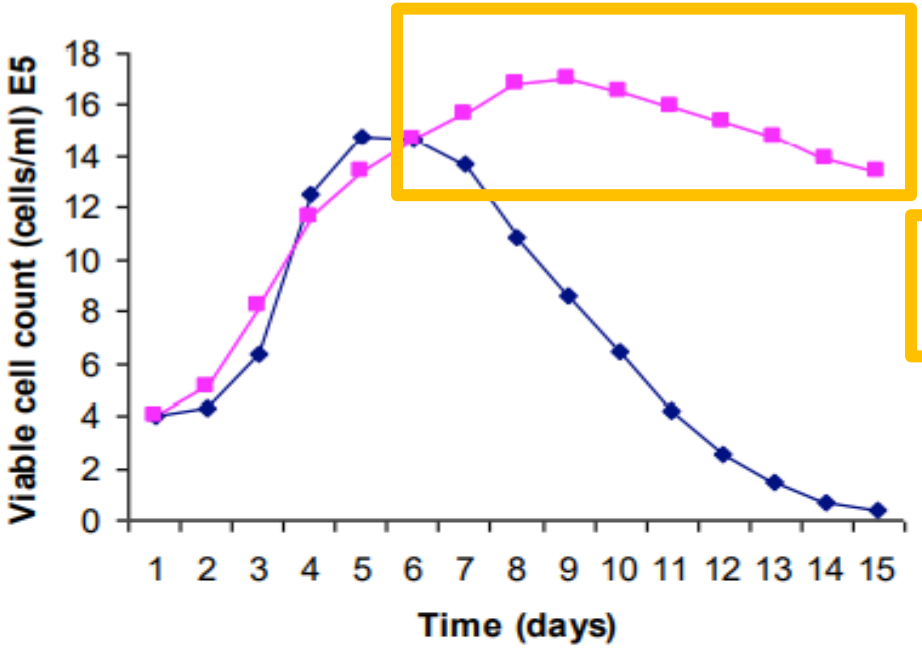


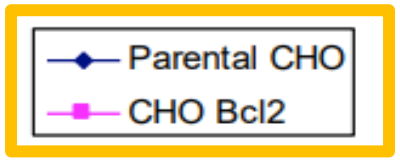
CHO-GS cell performance in optimized media

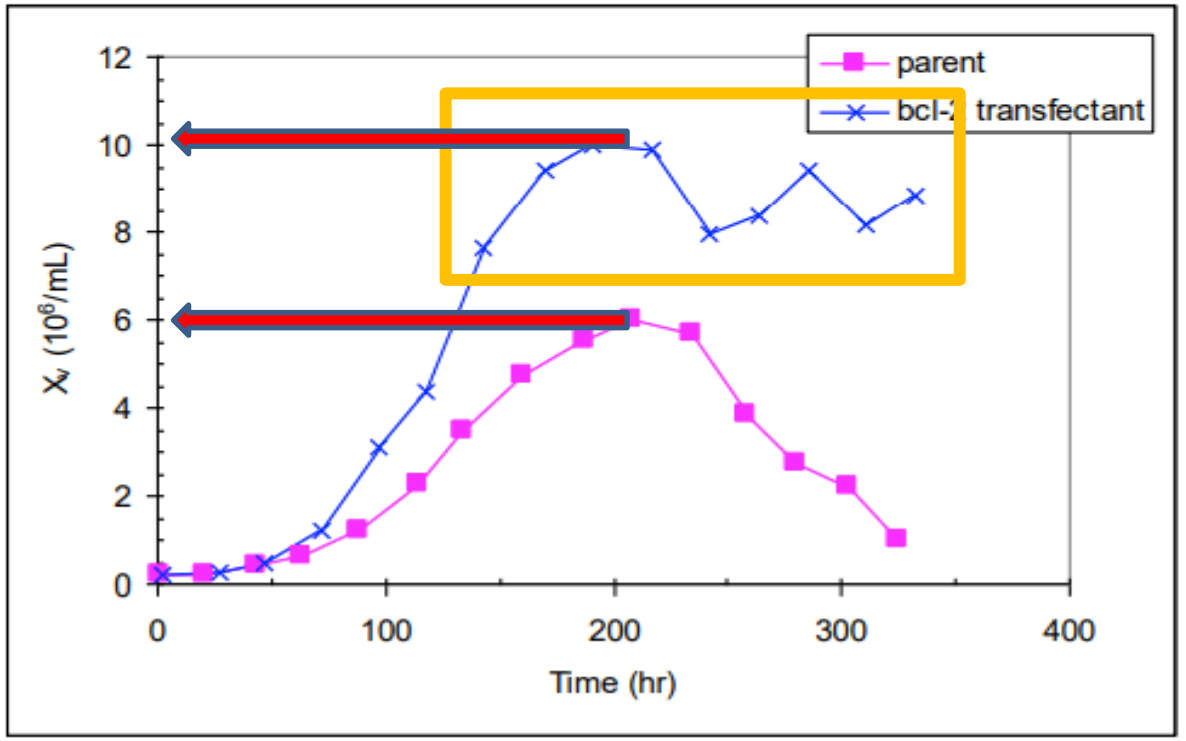
http://www.lonza.com/custom-manufacturing/development-technologies/gs-gene-expression-system/additional-gs-kits-and-services/media-and-feed.aspx



Growth of Mab-producing GS-CHO & GS-NS0 With and Without Anti-apoptosis Gene







*From: Al-Rubeai, M. Development of Cell Lines for Controlled Proliferation and Apoptosis. Presentation at 'Cell Line Development and Engineering' Meeting Zurich, May www.ucd.ie/ccerc/PowerPoint/**Cell**%20**line**%20**development**%20IBC.pps*

Summary

- Cell line development – a critical stage in biomanufacturing process development.
- Cell lines are derived from immortal cancerous cell lines.
- Relatively few suited to biomanufacturing e.g. CHO / BHK / NSO / PerC6.
- Selected lines are enhanced by genetic engineering:
  - Normal cell lines produce circa 1 – 2 pg/cell/day;
  - Engineering lines: circa 20 – 40 pcd and some up to 100 pcd.
- Transfect cells with YFG and select for high producing clones.
- Cell bank are set up for MCB and WCB in separate locations:
  - Cells must be fully characterized.

Questions?

Sample Questions

- Comment on the improvements that have taken place in the development of mammalian cell lines for biomanufacturing? Provide some specific examples of improvements in cell productivity and viable cell densities in culture.
- Write a brief note on the DHFR selection process for the identification of transfected cell lines? Why are cell lines screened through increasing concentrations of Methotrexate?
- The glutamine synthase system is widely used as an enhancing tool to develop more productive cell lines? Why and how does it work?