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DÉPARTEMENT OF BIOTECHNOLOGy

Course handout of:
Production of Biomolecules in Animal Cell Systems

Level: Master 1 (LMD)
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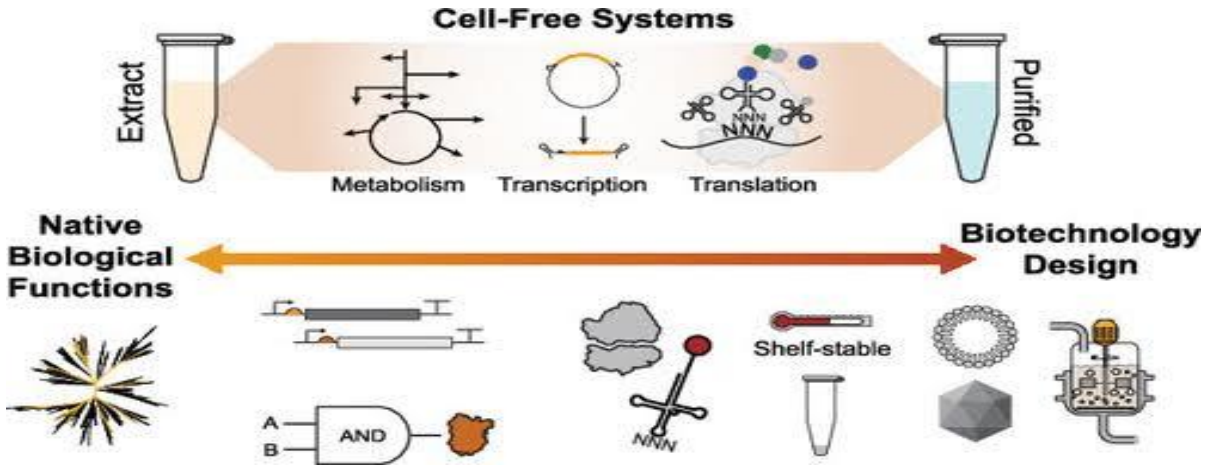

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Table of contents

List of figures

List of tables

Introduction

Biomolecular production and condensates have become a central organizing principle in cell biology, reshaping our understanding of cellular architecture. Recent research reveals that production participate in a wide range of essential processes, including gene regulation, signal transduction, metabolism, macromolecular processing, and stress responses. Beyond basic biology, condensates are linked to human diseases such as neurodegeneration and cancer, as well as to climate-relevant processes like carbon fixation underscoring their broad significance. To fully comprehend how cells function, we must understand condensates; in turn, this knowledge will allow us to target or engineer them for therapeutic and environmental applications. The concepts of phase separation and condensation provide a powerful framework for studying this membrane less organelles, offering insights into how they organize biomolecules in space and time.

- ## Cellular system

Outside of cellular systems, a new biotechnological approach has recently been developed for the production of recombinant biomolecules. This method relies on the use of a cell lysate (prokaryotic or eukaryotic) containing the transcriptional and translational machineries. This production system circumvents the experimental constraints associated with cell proliferation and allows the recombinant biomolecule to be obtained as the predominant product. This production system, called "cell-free" (or cell-free system), has enabled the mass synthesis of recombinant biological drugs on an industrial scale.

- ## Biomolecules

Biomolecules are by definition all molecules produced by a living organism, and they include proteins, polysaccharides, lipids, and nucleic acids. In the context of animal cellular systems, these biomolecules play fundamental roles: structure, signaling, enzymatic catalysis, energy storage, genetic information (**Tab 1**).

Table 1. The four major classes of biomolecules and maps them to their fundamental roles structure, signaling, enzymatic catalysis, energy storage, and genetic information specifically within animal cellular systems.

Biomolecule	Examples	Roles in Animal Cellular Systems
Proteins	• Structural (Collagen, Keratin, Actin/Myosin) • Enzymatic (Amylase, DNA polymerase, Kinases) • Signaling (Insulin, Growth factors, Receptors)	• **Structure**: Form the cytoskeleton, extracellular matrix (ECM), and connective tissues (bones, cartilage, hair). • **Signaling**: Act as hormones, neurotransmitters, and cell-surface receptors to transmit intra- and intercellular signals. • **Enzymatic Catalysis**: Act as enzymes to accelerate virtually all biochemical reactions (metabolism, DNA replication, digestion).
Polysaccharides (Carbohydrates)	• Energy storage (Glycogen) • Structural ECM (Hyaluronic acid, Chondroitin sulfate) • Cell-surface glycans	• **Structure**: Form part of the extracellular matrix (e.g., glycosaminoglycans) and provide mechanical support and hydration to tissues. • **Signaling**: Act as recognition markers on cell surfaces (glycocalyx) involved in cell-cell adhesion, immune recognition, and pathogen detection.
Lipids	• Energy storage (Triglycerides) • Structural (Phospholipids, Cholesterol) • Signaling (Steroid hormones, Prostaglandins)	• **Structure**: **Phospholipids** and **cholesterol** are the main structural components of all cellular membranes, forming the lipid bilayer and regulating membrane fluidity. • **Signaling**: Act as **steroid hormones** (e.g., estrogen, testosterone, cortisol) and local signaling molecules (e.g., prostaglandins) that regulate physiological processes. • **Enzymatic Catalysis**: Do not directly catalyze reactions (though lipid cofactors like Coenzyme Q assist in enzymatic processes in mitochondria). • **Energy Storage**: **Triglycerides** (fats) are the most concentrated, long-term energy storage molecules in animals, stored in adipocytes.
Nucleic Acids	• Deoxyribonucleic acid (DNA) • Ribonucleic acid (RNA) • Nucleotide derivatives (cAMP, ATP)	• **Structure**: **DNA** forms chromatin and chromosomes, providing the physical scaffolding for genetic material. RNA can also form complex 3D structures (e.g., ribosomes). • **Signaling**: **cAMP** (cyclic AMP) and **cGMP** act as crucial second messengers in signal transduction pathways. Regulatory non-coding RNAs (e.g., microRNAs) also modulate gene expression in response to signals. • **Enzymatic Catalysis**: Certain **ribozymes** (catalytic RNA molecules) perform enzymatic functions, such as RNA splicing and peptide bond formation in the ribosome. • **Energy Storage**: **ATP** (adenosine triphosphate) is the universal energy currency of the cell. While not a long-term storage polymer, its nucleotide structure is essential for immediate energy transfer. • **Genetic Information**: **DNA** stores the hereditary genetic blueprint. **mRNA** transmits this genetic information from the nucleus to the ribosomes for protein synthesis.

These biomolecules are used in many extremely diverse applications. Indeed, they can be found as dietary supplements or excipients in toothpastes, but also in certain textiles.

In addition, mammalian (animal) cell culture systems are the preferred platform for producing therapeutically relevant, biologically active, and glycosylated interferons. Here are some of the most prominent systems (**Tab 2**).

Table 2. Most mammalian (animal) cell culture systems

Cell Line	Type	Interferon Produced
CHO **(Chinese Hamster Ovary)**	Mammalian	IFN-β1a, IFN-γ
Murine Myeloma NSo	Mammalian	IFN-α2b
Mouse C127	Mammalian	IFN-γ
Sf9 **(Insect)**	Insect	Canine IFN-α7 (rCaIFN-α7)
Porcine Kidney (PK15)	Mammalian	Porcine IFN-α/β

I. Biotechnological Production Strategies

1. Recombinant DNA Technology & Gene Amplification

The foundation of modern interferon production is recombinant DNA technology. The gene for the desired interferon is cloned into an expression vector under a strong promoter (CMV or SV40) and introduced into the host cell line. To boost yields, a **gene amplification** strategy is often used. For example, CHO cells are co-transfected with the interferon gene and a dihydrofolate reductase (DHFR) gene. Treatment with methotrexate selectively amplifies the DHFR gene and, with it, the adjacent interferon gene, leading to a 20- to 50-fold increase in production.

2. Bioreactor Cultivation & Process Optimization

To produce interferons on an industrial scale, cells are grown in controlled bioreactors. Key strategies include:

- **Microcarrier Cultures**: For anchorage-dependent cells, microcarriers (small beads) provide a large surface area for cell growth in suspension bioreactors. This system allows for long-term (≥1 month) production of interferons like IFN-γ.
- **Fed-Batch & Perfusion Cultures**: To maximize cell density and product yield, nutrients are added (fed-batch) or fresh medium is continuously added while spent medium is removed (perfusion).
- **Process Control**: Optimizing parameters like pH, dissolved oxygen, and temperature is critical. For instance, pH control significantly improves volumetric interferon production, and applying

a temperature shift (hypothermic growth) can enhance beta-interferon titers by more than twofold.

3. Objectives of biomolecule production

In the health field, these biomolecules are used as antioxidants or antimicrobial agents, as well as for their anti-inflammatory, anticoagulant, or antitumor properties.

They are also the basis of recombinant vaccines. Their production relies on the use of homologous or heterologous living organisms whose proliferation conditions are perfectly controlled in order to achieve optimal biosynthesis of these biomolecules. Thanks to the recent rise of recombinant DNA technology and the development of certain fields such as molecular biology and cell biology, the production of biomolecules such as recombinant proteins for research or therapeutic applications has been facilitated.

A biological drug (biopharmaceutical) is a substance used to treat, prevent, or cure diseases. Biological drugs or biopharmaceuticals constitute a new class of drugs whose first uses date back to 1982, with the commercialization of the first recombinant insulin and the first growth hormone produced by genetic engineering in 1985.

Biological drugs are derived from recombinant DNA technology and fall into several categories: recombinant vaccines, therapeutic proteins (of which therapeutic monoclonal antibodies represent the majority of members), nucleic acids (of which interfering RNAs or small interfering RNA (siRNA) constitute most of the therapeutic molecules), and peptide nucleic acids (PNAs).

In essence, the objective is always to **leverage the cell's evolved machinery** (enzymes, ribosomes, chaperones, membrane transport) to perform biosynthetic tasks that are either impossible, unsafe, or uneconomical by traditional chemical synthesis or extraction.

4. The differences between chemical production and biomolecule production

Unlike traditional chemical molecules, which are obtained through chemical synthesis, the production of biopharmaceuticals relies on the development of biotechnology techniques derived from living organisms. These "biotechnological" drugs are often complex molecules that can be small (such as peptides) or very large (such as proteins), and are similar or identical to molecules found in the body. Although peptides can be obtained by chemical synthesis, protein drugs, on the other hand, are too large and too complex to be produced by chemical synthesis techniques (**Tab 3**).

Biotechnology therefore uses living "factories", namely microbial organisms, cell lines, animals, or more recently cell-free systems (in vitro) that have been genetically modified or

whose synthesis conditions have been optimized to produce the desired molecule. In general, all drugs obtained from or derived from living organisms are considered biotechnological therapies or biological drugs. Most of these biological macromolecules have been developed thanks to the rise of genetic engineering resulting from advances in molecular and cell biology and the biotechnological industry starting from the 1970s.

Understanding the molecular mechanisms governing cell cycle regulation, recombinant DNA manipulation, protein engineering, control of cell strains, and improvement of production systems (establishment of cell banks, culture media, fermenters) have made it possible to provide effective solutions for the production of these biological drugs on an industrial scale.

Table 3. A comparative table highlighting the key differences between traditional chemical production (non-biological synthesis) and biomolecule production using cellular systems (mammalian cell culture).

Feature	Chemical Production	Biomolecule Production (Cellular System)
Core process	Abiotic chemical reactions (synthesis, catalysis, purification)	Living cell metabolism (enzymatic pathways, fermentation, biotransformation)
Typical conditions	High temperature, high pressure, extreme pH, organic solvents	Mild conditions (20–40 °C, near-neutral pH, aqueous environment)
Catalysts	Metal catalysts, acids/bases, organocatalysts	Enzymes (intracellular or extracellular), whole cells
Stereochemistry	Often produces racemic mixtures; asymmetric synthesis is complex and costly	Naturally produces single enantiomers (e.g., L-amino acids, D-sugars) due to chiral enzymes
Raw materials	Petrochemicals, mineral ores, simple organic building blocks	Renewable biomass (glucose, starch, cellulose, glycerol, plant oils)
Reaction steps	Linear, often requiring protection/deprotection and multiple purifications	Multi-step pathways (10–30+ enzymatic steps) performed in one vessel (in vivo)
Product purity & complexity	Best for simple molecules (<500 Da); complex molecules require many steps, low yield	Can produce highly complex molecules (proteins, antibodies, polyketides) with correct folding and post-translational modifications
Feature	Chemical Production	Biomolecule Production (Cellular System)
By-products	Often toxic, hazardous, and need disposal (e.g., heavy metals, halogenated waste)	Mainly CO_2, water, biomass; generally biodegradable, less hazardous
Energy consumption	High (heating, cooling, pressurization)	Low (ambient conditions, but requires aeration/mixing)
Water usage	Variable, often low to moderate	High (aqueous medium), but water can be recycled
Scalability	Easy to scale (continuous flow, large reactors)	More challenging (oxygen transfer, mixing, sterility, fermentation time)
Production rate	Fast (minutes to hours)	Slow (hours to days, due to cell growth and metabolism)
Product concentration	Typically high (grams to kilograms per liter)	Lower (milligrams to tens of grams per liter; depends on host and pathway)
Downstream processing	Often simpler (distillation, crystallization, chromatography)	Usually more complex (cell disruption, removal of host cell proteins, DNA, endotoxins)

5. Methods and General Principles of Production

Developing a recombinant protein production project necessarily requires the most complete knowledge of the final use of the biological molecule and its intrinsic biochemical properties. Indeed, the choice of production method will depend on parameters based on the biochemical complexity of the molecule, the quantity to be produced, the frequency of production campaigns, and the applications (functional and/or structural studies, administration in animal models or in humans). Analysis of these data will allow selection of the purification system(s) to be implemented, and then the appropriate host/expression vector pair. Recombinant protein production can be carried out in various so-called "classical" biological organisms such as bacteria, yeasts, plants, insect cells, mammalian cells, and more recently in transgenic animals.

5.1. Vectors and Cloning Strategies

One of the challenges in obtaining recombinant proteins lies in the design of the production project. The implementation of the various steps must take several elements into account, the main ones being the final objectives for using the produced protein. Indeed, the production and purification strategy for the protein of interest depends on whether the approach is:

- a quantitative approach (production of prokaryotic or eukaryotic genes in a prokaryotic host to obtain a high level of expression);
- a qualitative approach (expression of complex proteins modified at the post-translational level in a eukaryotic system); or
- functional or structural studies.

It is also important to evaluate, from the beginning of the study, the approach that will be used for protein purification. Indeed, the purification steps can influence the choice of expression vector as well as the host organism. Finally, once the host/vector pair has been selected, one must consider whether the host organism will be able to translate the transcribed messenger RNA, whether it can correctly perform post-translational modifications, and finally whether it can support mass production of the recombinant protein without toxicity. All these elements define the production and purification strategy (**Fig 1**).

The choice of expression vector will therefore depend, on the one hand, on the final objective of protein use, but also on the host organism. These expression vectors are double-stranded circular plasmids that contain regulatory elements adapted to the host organism:

An origin of replication specific to the host organism is required for vector duplication: for example ColE1 (~40 copies/cell), pISA (~10 copies/cell), CloDF13 (~40 copies/cell), RSF1030 (100 copies/cell) (see Chapter 4, Origin of replication and metabolic load). Some plasmids contain a prokaryotic origin of replication and a eukaryotic origin of replication.

5.2. Gene expression regulatory elements

Gene expression regulatory elements are necessary for protein expression, such as a promoter and a transcription terminator, a polyadenylation site (polyA), a ribosome binding site, and enhancer sequences. Promoter and terminator regions are specific to the host cell that will produce the protein. For example, expression in the bacterium E. coli will use promoters such as T7, LacO, or araBAD, whereas mammalian cells will preferentially use SV40 or CMV promoters, yeasts will use glycolytic promoters such as ADH1 (alcohol dehydrogenase), PGK (phosphoglycerate kinase), GAP (glyceraldehyde-3-phosphate dehydrogenase), and in vitro systems will use Sp6, T3, T7 promoters.

5.3. The gene of interest

The gene of interest is cloned downstream of the promoter into a sequence containing several restriction enzyme recognition sites, called the multiple cloning site (MCS).

5.4. These plasmids

These plasmids may also contain antibiotic resistance genes: ampicillin (Ampr), chloramphenicol (Camr), kanamycin (Kanr), geneticin (G418), blasticidin, tetracycline (Tetr), zeocin, hygromycin, methotrexate, neomycin, beta-lactamase. Expression of these resistances allows selection of host organism clones that have correctly incorporated the plasmid.

5.5. Tags allowing purification or tracking of the recombinant protein

Tags allowing purification or tracking of the recombinant protein can be added at the N-terminus or C-terminus of the protein-coding sequence. They will serve, for example, in studies by western blot, immunofluorescence, immunoprecipitation, or non-invasive in vivo imaging in animals. These tags can improve the expression or solubility of the proteins of interest, such as glutathione S-transferase (GST), maltose-binding protein (MBP), but also, in some cases, the secretion of recombinant proteins. Finally, they can also be used in the case of therapeutic biological molecules for better targeting of the diseased tissue, cell, or organ (targeting peptides or homing peptides).

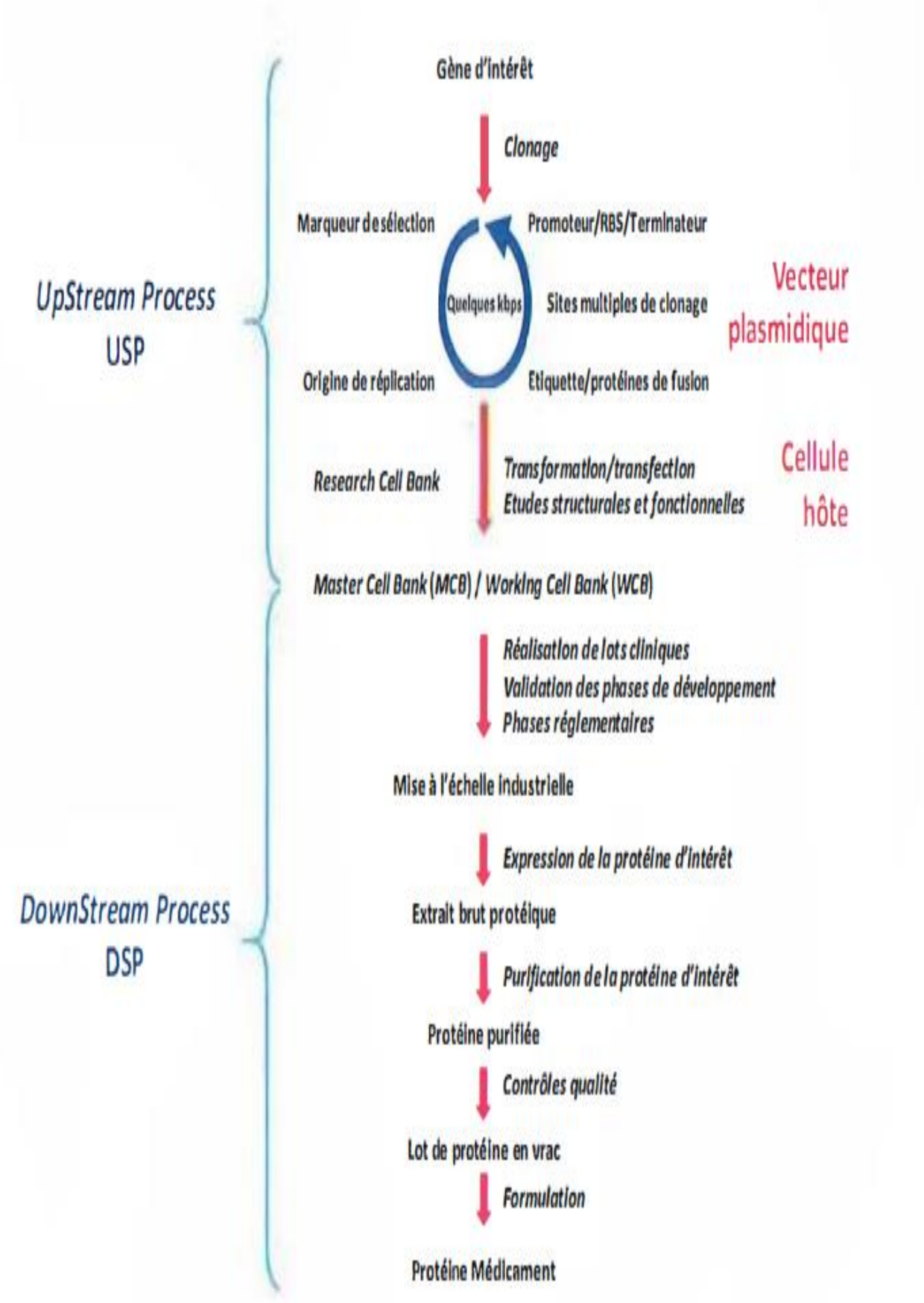

Fig 1. Schematic representation of the production strategy of a recombinant protein for research or therapeutic purposes.

5.6. Cellular Systems for Protein Expression

Many cellular systems have been developed for protein expression. Prokaryotes are the simplest, fastest, and least expensive organisms to implement. On the other hand, mammalian cells (human, monkey, hamster, etc.) are now well-controlled biological systems allowing the production of complex, high-quality proteins. In addition to mammalian eukaryotic cells, production of complex recombinant proteins is also possible in other eukaryotic expression systems such as yeasts (e.g., Pichia pastoris, Saccharomyces cerevisiae), plants (tobacco cells, maize, carrots), insect cells infected with baculovirus (Sf9, Sf21, BTI-TN5B1-4, SPCMb-92-C6, Eo4 cells), or transgenic animals (rabbit, chicken, camelids, mouse). All these systems are capable of performing more or less complex post-translational modifications, but not in an identical manner.

5.7. Protein Expression by Mammalian Eukaryotic Cells

Apart from microbial expression systems, eukaryotic expression systems are widely used to obtain complex proteins. In the context of research projects, the choice of mammalian cells as host organisms is only justifiable for certain applications. Indeed, this expression may require the prior establishment of stable cell lines, whose isolation and characterization steps are long and tedious. The development and optimization over the last 15 years of the transient gene expression (TGE) method has revolutionized recombinant protein production in mammalian systems.

These production processes are now robust and reliable enough to allow production in bioreactors with capacities exceeding 200 liters and the obtainment of several grams of protein. Optimization of these production processes has also enabled the development of inexpensive new transfection reagents for DNA transfer into host cells, as well as serum-free, protein-free, or chemically defined media.

Several mammalian cell lines are currently used routinely for recombinant protein production, including CHO (Chinese Hamster Ovary) cells, HEK293 (E1-transformed human embryonic kidney/neuronal cells) and its many derivatives, as well as PER.C6 cells of human origin (E1-transformed human embryonic retinal cells) dedicated to the production of vaccines and monoclonal antibodies.

5.8. Protein Production in Animals

Outside of cellular systems and in vitro systems, recombinant protein production can be carried out using transgenic animals. This methodology appeared in the early 1980s, following the mastery of pronuclear microinjection, and then through the development of somatic cell nuclear transfer technology. At the end of those same years, the first animal "bioreactors" for

mass production of recombinant proteins appeared. These transgenic animals, such as mice, rats, chickens, sheep, goats, pigs, or cows, are capable of producing recombinant proteins in quantity and quality suitable for research or industry (**Fig 2**).

Most of these animals produce proteins in milk, but also in blood, egg white, seminal fluid, or urine. The best yields are obtained in milk due to the volume produced each year. The advantage of using transgenic animals, compared to classical expression systems, lies in obtaining large quantities of complex proteins with correct post-translational modifications.

Moreover, the use of transgenic animals provides a less expensive alternative to the use of large-capacity fermenters. The gene of interest can be placed under the control of the promoter of the gene encoding casein or whey proteins, allowing transgene expression specific to the mammary gland tissue. By this approach, recombinant proteins have been expressed and secreted into the milk of cows, goats, or sheep.

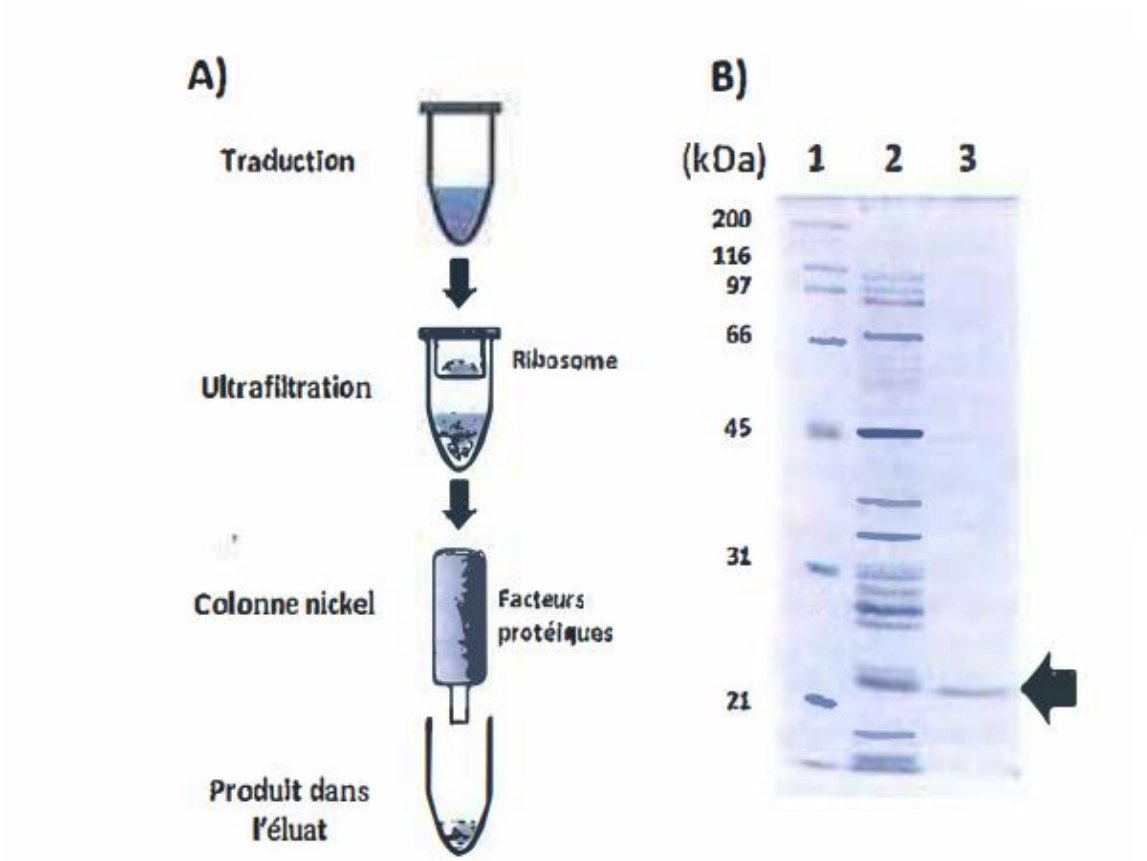

Fig 2. In vitro protein production system pure system (Protein Synthesis Using Recombinant Elements). A) Schematic representation of the production process in the PURE system. B) Purification of the protein of interest on a nickel column:1, molecular weight m

II. Important biomolecules produced

Recombinant DNA technology has enabled the large-scale production of a wide range of biomolecules that are critical for medicine, industry, and research. These molecules are produced by inserting the gene of interest into host cells (like bacteria, yeast, or mammalian cells), which then act as "factories" to synthesize the desired product (**Tab 4**).

- **The difference between biomolecules in their natural state** and **biomolecules produced via recombinant DNA technology**

While natural and recombinant biomolecules are often **chemically identical at the molecular level** for instance, recombinant human insulin possesses the exact same amino acid sequence and three-dimensional structure as the insulin produced by a healthy human pancreas their differences become starkly apparent when we consider their **source, production method, and practical attributes**.

Natural biomolecules are synthesized endogenously within an organism's own cells and must be harvested directly from animal tissues, human donors, or blood plasma, a process that is inherently limited in scale, variable in quality, and carries significant risks of contamination with pathogens like viruses, prions, or other bioactive impurities. In contrast, recombinant biomolecules are produced exogenously by genetically engineered host cells (such as E. coli, yeast, or mammalian cell lines) that have been artificially equipped with the target gene, allowing them to function as miniature "factories" in controlled bioreactors. This industrial approach enables virtually unlimited, cost-effective large-scale production under sterile, animal-free conditions, yielding exceptionally pure and highly consistent batches that are free from infectious agents.

Furthermore, recombinant technology offers a distinct advantage in flexibility, as scientists can deliberately introduce amino acid modifications to create novel analogs such as long-acting insulin variants that do not exist in nature, thereby enhancing therapeutic efficacy. Ultimately, while nature provides the original blueprint, recombinant DNA technology refines and replicates it to deliver safer, more reliable, and more plentiful biomolecules tailored for modern medicine and industry.

Table 4. The important biomolecules produced recombinantly, and by their primary function

Category	Biomolecule (Examples)	Primary Use / Application
Therapeutic Proteins & Hormones	**Insulin**	Treats diabetes (replaced animal-derived insulin).
	Human Growth Hormone (hGH) (Somatropin)	Treats growth hormone deficiency in children and adults.
	Erythropoietin (EPO)	Stimulates red blood cell production to treat anemia.
	Follicle-Stimulating Hormone (FSH)	Used in fertility treatments.
	Glucagon	Treats severe hypoglycemia (low blood sugar).
Blood Clotting Factors	**Factor VIII**	Treats hemophilia A (reduces risk vs. human-derived versions).
	Coagulation Factor XIII	Treats factor XIII deficiency and acts as an anticoagulant.
	Tissue Plasminogen Activator (tPA)	Dissolves blood clots in heart attacks and strokes.
Therapeutic Enzymes	**Taliglucerase alfa**	Enzyme replacement therapy for Gaucher's disease.
	Alpha-galactosidase A	Enzyme replacement therapy for Fabry disease.
	Alpha-L-iduronidase	Enzyme replacement therapy for Hurler/Scheie syndromes (MPS I).
	Dornase alfa	Breaks down DNA in mucus to improve lung function in cystic fibrosis.
	Asparaginase	Used in cancer therapy (depletes asparagine in leukemic cells).
Monoclonal Antibodies (mAbs)	**Adalimumab**	Anti-TNF-alpha antibody for autoimmune diseases (e.g., rheumatoid arthritis).
	Rituximab	Targets CD20 on B-cells for cancer and autoimmune therapy.
		Targeted therapy for cancers, inflammatory disorders, and infectious diseases.
Recombinant Vaccines	**Hepatitis B Vaccine**	Produces surface antigen in yeast to immunize against Hepatitis B.
	Virus-Like Particle (VLP) Vaccines	Mimics viral structure to elicit immunity without infectious material (produced in yeast/insect/plant cells).
Cytokines & Growth Factors	**Interferons (IFNs)** (e.g., IFN-α2a)	Immunostimulants used in cancer and viral therapies.

II.1. Insuline

Insulin is the master regulator of glucose, lipid, and protein metabolism. Following ingestion of an oral glucose load or mixed meal, the plasma glucose concentration rises, stimulating insulin secretion by the beta cells. The resulting hyperinsulinemia, working in concert with hyperglycemia, promotes glucose uptake into tissues, suppresses hepatic glucose production, and enhances lipid and protein synthesis.

1.1. Structure

In 1922, Canadian researchers achieved the first documented physiological response to injected animal insulin in a patient with type 1 diabetes. Insulin was the first protein to be fully sequenced. Its molecule contains 51 amino acids organized into two chains an A chain (21 amino acids) and a B chain (30 amino acids) connected by two disulfide bonds (1) . The insulin precursor, proinsulin, is transported to the beta cell's Golgi apparatus, where it undergoes processing and packaging into granules. Proinsulin is an 86-amino-acid single-chain peptide that gets cleaved into insulin and C-peptide (a connecting peptide); both are secreted from the beta cell in equimolar amounts upon stimulation by glucose and other insulin secretagogues. Although C-peptide has no known physiological function, measuring it provides an estimate of endogenous insulin secretion (**Fig 3**).

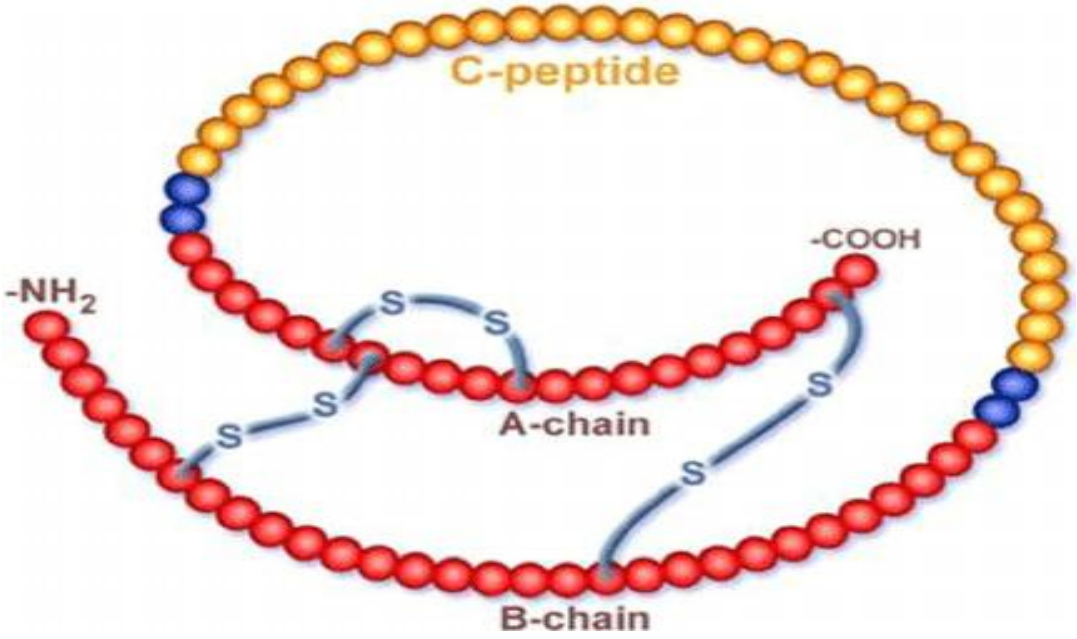

Fig 3. Structure of Proinsulin Showing the C-Peptide and the A and B Chains of Insulin

1.2. Production

Pancreatic beta cells must secrete insulin with precise timing and dosage to sustain life. Impaired beta-cell function disrupts glucose homeostasis: excessive secretion causes dangerous hypoglycemia, while insufficient secretion leads to damaging hyperglycemia. Not

surprisingly, beta cells are tightly controlled by numerous stimulatory and inhibitory factors, with glucose as the primary regulator though after more than four decades of study, the mechanisms involved are still not fully understood.

All current models of stimulus secretion coupling rightly describe beta cells as electrically excitable fuel sensors that are under hormonal and neural control. They highlight the central role of ATP-sensitive K^+ channels (KATP channels) in converting changes in glucose metabolism into alterations in membrane potential, which in turn trigger Ca^{2+} influx and a rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]c$) (**Fig 4**). Other reviews summarize the original studies that elucidated this KATP channel-dependent triggering pathway over 20 years ago, or examine the complex, multifactorial regulation of these channels, which are composed of regulatory (sulfonylurea receptor 1, SUR1) and pore-forming (Kir 6.2) subunits.

In the presence of non-stimulatory concentrations of glucose, the rate of metabolism in beta cells is relatively low and enough K_{ATP} channels are open in the plasma membrane to counteract depolarising currents and maintain the membrane potential at values more negative than the threshold for opening the voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} is minimal, $[Ca^{2+}]_c$ is low, and insulin secretion is basal. When the concentration of glucose increases, beta cell metabolism accelerates, leading to changes in the concentration of cytosolic adenine nucleotides (in particular an increase in ATP and a decrease in MgADP) which induce closure of K_{ATP} channels. The resulting decrease in K^+ efflux causes membrane depolarisation, followed by opening of Ca^{2+} channels and Ca^{2+} influx into the cell (as reflected by the electrical activity shown in the inset of (**Fig 4**).

The ensuing increase in $[Ca^{2+}]_c$ then activates an effector system that promotes exocytosis of insulin-containing granules. The triggering Ca^{2+} signal is essential. All experimental conditions that interfere with the rise in $[Ca^{2+}]_c$ impair glucose-induced insulin secretion, whereas all agents, physiological or pharmacological, that increase beta cell $[Ca^{2+}]_c$ (regardless of the mechanism) induce insulin secretion.

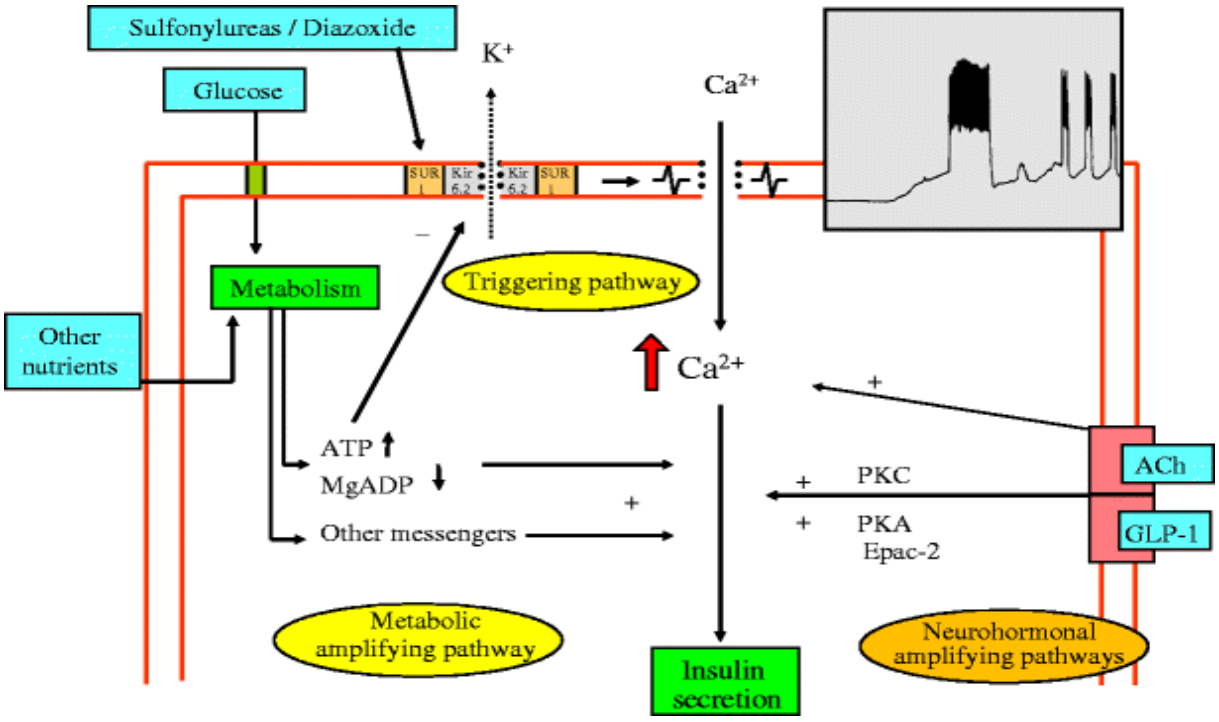

Fig 4. Production of the insulin.

Two drugs that interact with the SUR1 subunit of K_{ATP} channels have been particularly instrumental for the studies of stimulus–secretion coupling discussed below. Sulfonylureas, such as tolbutamide, mimic the action of glucose on membrane potential and Ca^{2+} influx, by closing K_{ATP} channels independently of changes in metabolism. Conversely, diazoxide reverses the effects of glucose on membrane potential and Ca^{2+} influx through the direct opening of K_{ATP} channels. Their respective stimulatory and inhibitory effects on insulin secretion are thus attributable to changes in the triggering Ca^{2+} signal (**Fig 5**). Additional mechanisms have been proposed but are unlikely to contribute to the effects that sulfonylureas and diazoxide produce at therapeutically relevant concentrations. Thus, both types of drug are inactive in beta cells lacking K_{ATP} channels because of a knockout of either Sur1 (Abcc8).

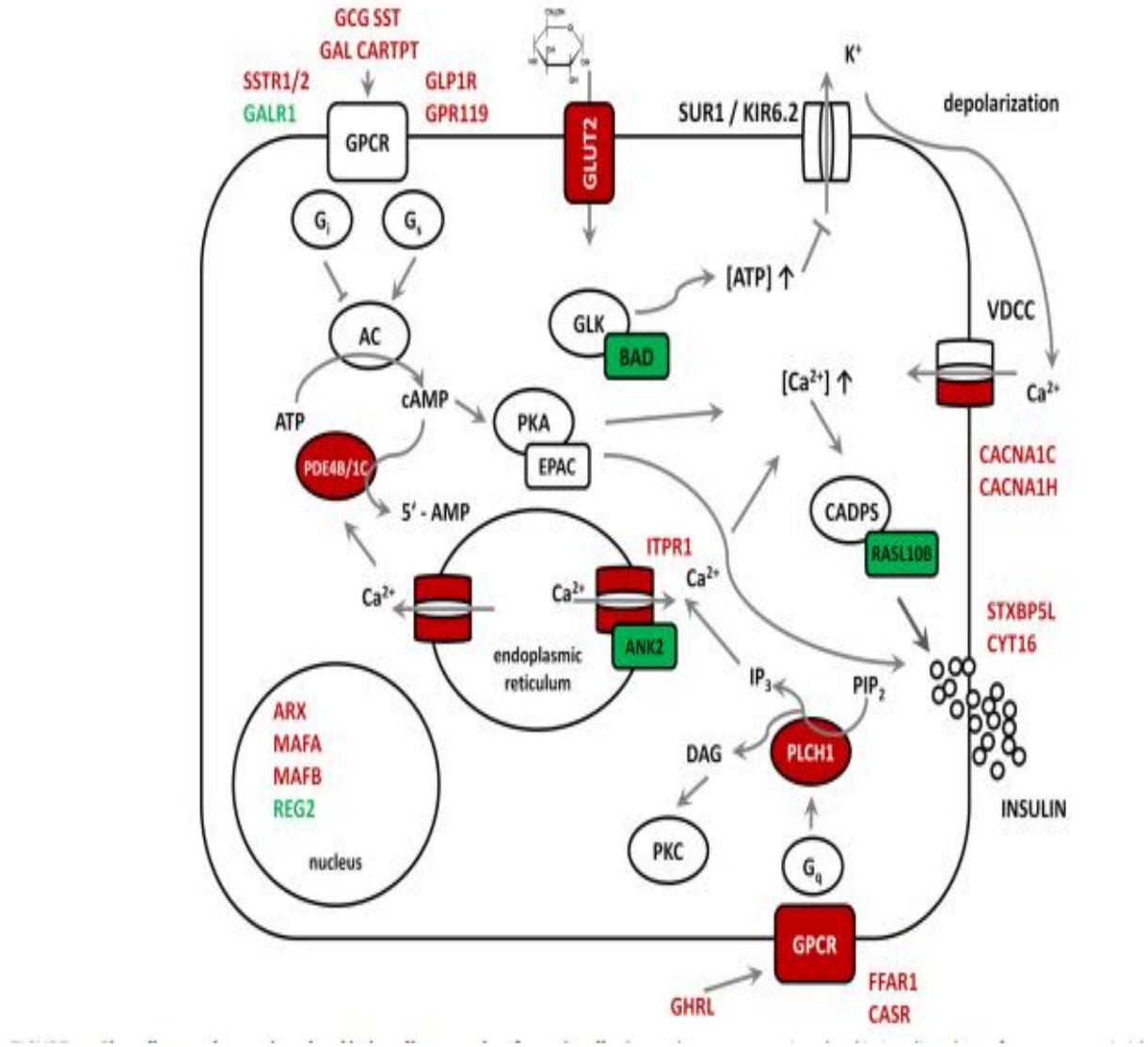

Fig 5. The triggering and metabolic amplifying pathways mediating the stimulation of insulin secretion by glucose, and of the neurohormonal amplifying pathways mediating the effects of neurotransmitters and hormones. The KATP channel, made up of SUR1 and Kir6.2 subunits, is the transducer of metabolic changes into membrane potential changes. The inset illustrates the depolarization and the electrical activity induced by glucose in one mouse beta cell within an intact islet; the recording was made with an intracellular electrode. The dotted line indicates a decreased flow. +, stimulation; −, inhibition; SUR1, sulfonylurea receptor 1; Kir, inwardly rectifying K channel; ACh, acetylcholine; Epac-2, exchange protein directly activated by cAMP 2; GLP-1, glucagon-like peptide 1; PKA, protein kinase A; PKC, protein kinase C.

1.3. Regulation

Insulin, the main hormone that reduces blood glucose levels, plays a vital role in preserving metabolic balance. This regulation depends on two closely interconnected mechanisms: the release of insulin from pancreatic β-cells and its subsequent effects on target

tissues.

Regulation of Secretion

The secretion of insulin is a highly controlled process that ensures the hormone is released precisely when blood glucose levels rise.

- Glucose-Stimulated Insulin Secretion (GSIS) Pathway

The sequence of cellular events that trigger insulin secretion begins with a rise in blood glucose levels, leading to rapid glucose uptake into pancreatic β-cells. Once inside, glucose is metabolized via glycolysis and the Krebs cycle, generating ATP. This increases the ATP/ADP ratio, which in turn causes the closure of ATP-sensitive potassium (KATP) channels. The resulting membrane depolarization opens L-type calcium channels, allowing calcium ions to enter the cell. The subsequent increase in intracellular calcium stimulates the exocytosis of insulin granules, releasing insulin into the bloodstream (**Fig 6**).

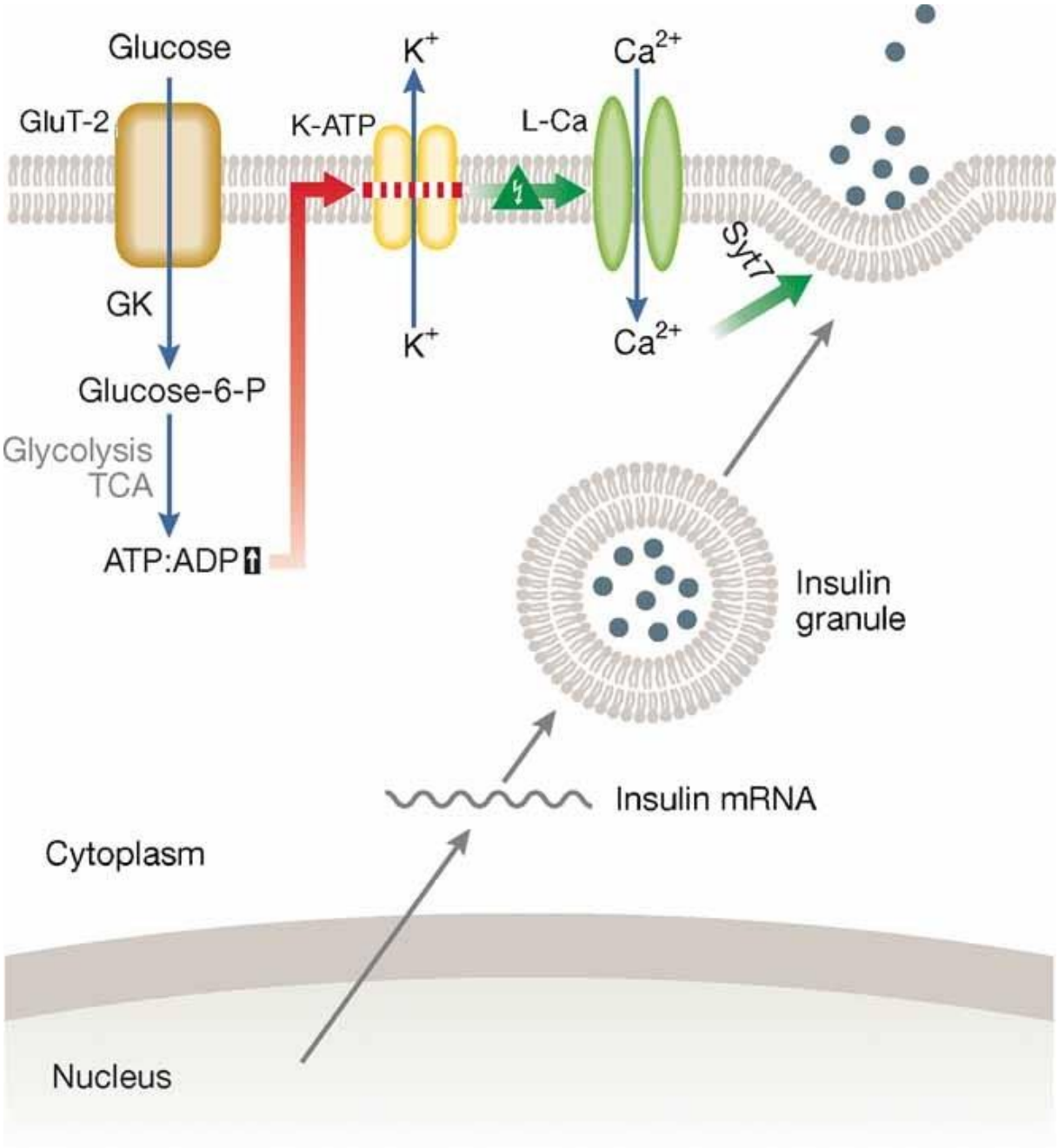

Fig 6. Cellular and molecular regulation of insulin secretion. (GluT 2 = glucose transporter 2; GK = glucokinase; TCA = tricarboxylic acid cycle; Syt7 = synaptotagmin 7; K ATP = ATP sensitive potassium channel; L Ca = L type calcium channel.)

- Dual-Phase Insulin Secretion

Glucose triggers insulin release in a characteristic two-phase pattern. The initial phase consists of a quick, short-lived burst of insulin from already stored granules, occurring within just a few minutes. This is followed by a prolonged second phase, which depends on the release of freshly synthesized insulin and requires ongoing glucose metabolism as well as calcium signaling. Emerging research has emphasized the role of pathways that boost this second phase,

including metabolic coupling factors and protein kinases such as PKA and PKC (**Fig 7**)..

- Modulation by Hormones and Neurotransmitters

Glucose enters the cell via its specific glucose transporter, after which it is phosphorylated and metabolized in the mitochondria, similarly to free fatty acids (FFA). The metabolism of glucose and FFA raises the ATP/ADP ratio, causing ATP-sensitive potassium (KATP) channels to close. This closure leads to depolarization of the plasma membrane, which in turn triggers calcium influx through voltage-gated calcium channels, thereby increasing cytoplasmic Ca^{2+} levels.

Other transmembrane channels also help regulate membrane potential: voltage-gated K^+ channels (K^+Kr), store-operated channels (SOC), the sodium background current (Na_β), and the non-selective cation channel NALCN, which can be modulated by acetylcholine via activation of M3 muscarinic receptors (M3R) (**Fig 7**)..

The rise in cytoplasmic Ca^{2+} activates several calcium-dependent enzymes, including adenylyl cyclase (AC) and phospholipase C (PLC).

- **Phosphoinositide pathway**

Phosphatidylinositol-4-phosphate (P4P) is synthesized from phosphatidylinositol (PI) by phosphatidylinositol 4-kinases; this synthesis is stimulated by PKC and Ca^{2+}. Phosphatidylinositol-4,5-bisphosphate (PIP2) is then formed mainly from P4P by phosphatidylinositol-4-phosphate 5-kinase I (**Fig 7**).

- **cAMP pathway**

Ca^{2+}/calmodulin (CaM) plays a key role. cAMP synthesis and degradation are catalyzed by adenylyl cyclase and phosphodiesterase (PDE), respectively. Soluble AC (ACc) and plasma-membrane-bound AC (ACP) exist; ACP is G-protein-regulated and can be activated by stimulatory Gαs proteins as well as by Ca^{2+}/CaM, and inhibited by inhibitory Gi/o proteins. PDE activity is enhanced by Ca^{2+}/CaM. cAMP activates protein kinase A (PKA) and the exchange protein Epac.

The incretins GLP-1 and GIP bind to their respective receptors (GLP-1R and GIPR), activating ACP and increasing intracellular cAMP. Endogenous catecholamines (adrenaline, noradrenaline) bind to α2A-adrenergic receptors (AdR) on the plasma membrane and inhibit ACP.

- **Phospholipase C (PLC) pathway**

Cytoplasmic PLC (PLCc) is activated by Ca^{2+}, while plasma-membrane-bound PLC (PLCp) can be activated by Gq-type G proteins. Acetylcholine and free fatty acids bind to their respective receptors (M3R and FFAR1/GPR40) and trigger Gαq-mediated activation of PLCp.

Both PLCc and PLCp hydrolyze membrane PIP2 to generate inositol-3-phosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) (**Fig 7**).

- **Endoplasmic reticulum (ER)**

SERCA is the ER Ca^{2+}-ATPase, and IP3R is the IP3 receptor, which can be activated by IP3 and by PKA.

Solid lines indicate fluxes, while dashed lines represent inhibitory or stimulatory effects on currents or fluxes.

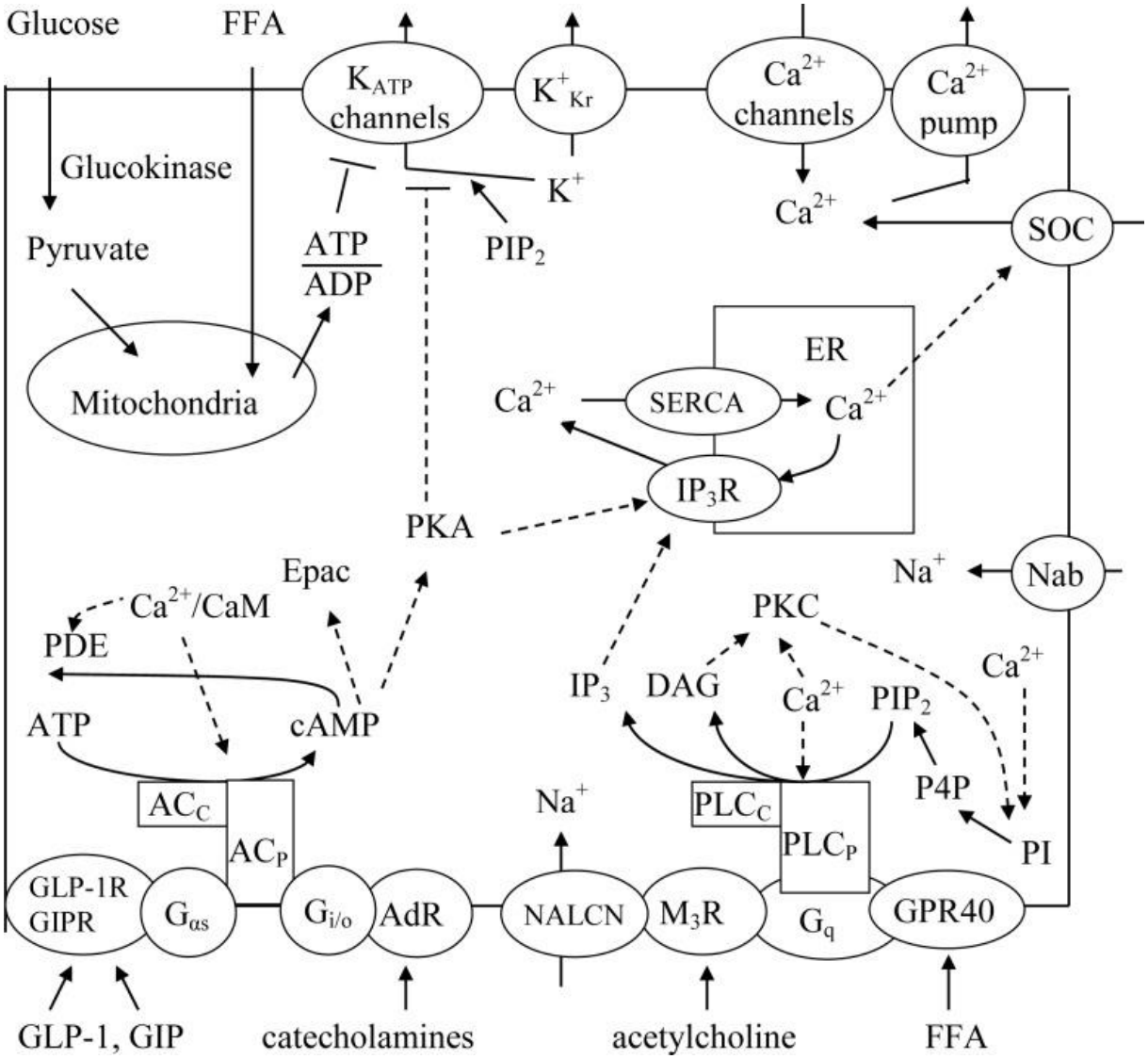

Fig 7. A schematic model of the main signaling pathways that regulate insulin secretion.

Regulation of Insulin Action

Once secreted, insulin exerts its effects by binding to insulin receptors (IR) on the surface of target cells, primarily in the liver, skeletal muscle, and adipose tissue.

- **Insulin Signaling Cascade**

The binding of insulin to its receptor (IR) triggers a complex intracellular signaling cascade. The most critical pathway for metabolic regulation is the PI3K/Akt pathway. Upon IR activation, the PI3K enzyme is recruited and generates the lipid second messenger PIP3, which then activates the protein kinase Akt (also known as PKB). Activated Akt then phosphorylates numerous downstream targets to orchestrate the metabolic effects of insulin.

- Glucose Uptake via GLUT4 Translocation

The hallmark of insulin action is the rapid increase in glucose uptake into muscle and fat cells. This is achieved through the insulin-stimulated translocation of the GLUT4 glucose transporter. In the basal state, GLUT4 is sequestered inside the cell in specialized GLUT4 storage vesicles (GSVs). Insulin signaling, primarily via Akt, leads to the phosphorylation of key regulators such as AS160, which permits the GSVs to traffic to and fuse with the plasma membrane. This fusion increases the number of GLUT4 transporters at the cell surface, allowing glucose to enter the cell efficiently.

- Metabolic Actions in Target Tissues

Beyond promoting glucose uptake, insulin orchestrates a coordinated metabolic program to lower blood glucose and promote energy storage **(Fig 8).**

- ✓ **In the Liver**: Insulin suppresses glucose production by inhibiting gluconeogenesis and glycogenolysis, while also promoting glycogen synthesis.
- ✓ **In Skeletal Muscle**: Insulin is the primary driver of glucose uptake (via GLUT4 translocation) and promotes glycogen synthesis and protein synthesis.

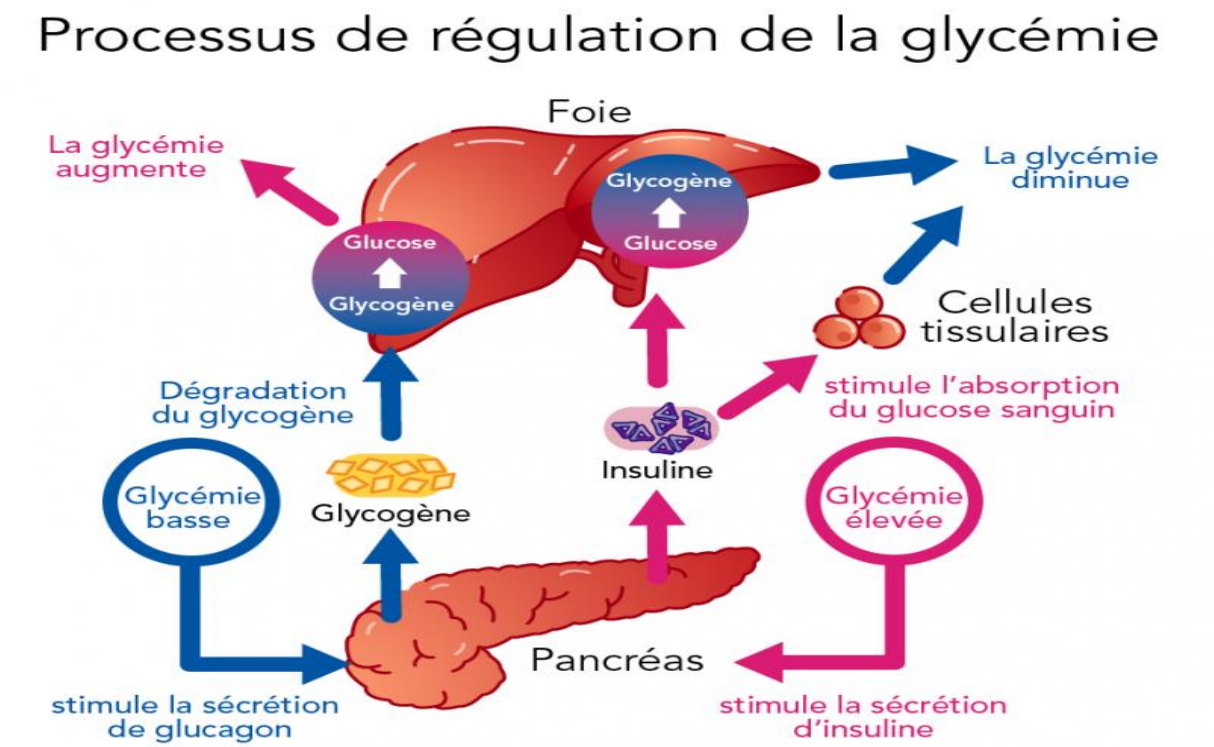

Fig 8. Metabolic Actions in Target Tissues

- ✓ **In Adipose Tissue**: Insulin inhibits lipolysis (the breakdown of fat), reducing the release of free fatty acids into the circulation, and also promotes glucose uptake for energy storage as triglycerides.

1.4. Physiological roles

Insulin is a key hormone in glucose metabolism, but its effects extend beyond simply lowering blood glucose levels. This pleiotropic hormone decreases blood glucose levels by affecting many physiological pathways.

Insulin increases glucose uptake at the level of many organs via the glucose transporter type 4 (GLUT-4), notably in the liver, skeletal muscles, and adipose tissue, which is essential in regulating glycemia. Glucagon secretion is directly affected by insulin levels, working as a feedback mechanism that keeps blood glucose levels balanced.

Insulin directly inhibits glucagon secretion during the fed state, pushing the body to use glucose as the primary energy source for ATP production. While the direct action of insulin on hepatic cellular pathways is known, particularly the activation of glycolysis, glycogenesis, and lipogenesis, new studies are targeting the indirect effects of insulin on the liver. These indirect effects include the decrease in glycerol and free fatty acids released from adipose tissues and the suppression of hepatic gluconeogenic genes (phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase).

Lastly, the action of insulin on the central nervous system is becoming a subject of

interest for many. Although the results of trials can be conflicting, some studies have shown that hypothalamic insulin action decreases hepatic glucose production and can be a target for new drugs and treatments for diabetes.

This review integrates current understanding of the physiological functions of brain insulin and its potential as a therapeutic target in AD and PD. We highlight the potential of insulin as a multifaceted therapeutic target against neurodegenerative diseases.

Insulin is mainly used to manage type 1 diabetes (T1D). The discovery of insulin in 1921 by Sir Frederick G. Banting was unquestionably one of the top scientific achievements of the 20th century. Since then, insulin has saved the lives of countless individuals suffering from T1D. However, it might also be prescribed to patients with type 2 diabetes (T2D), pregnant women developing gestational diabetes, and some people with other types of diabetes, or in certain situations to manage hyperglycemia.

Recently, there has been more attention to the significant impact of insulin on the reproductive system, particularly on women. An enormous number of studies have shown that there is a link between insulin excess and hyperandrogenism, polycystic ovary syndrome (PCOS), ovarian follicle dysfunction, and endometrial hyperplasia. Moreover, research in recent years has revealed several important connections between insulin and cognitive processes, particularly in the context of aging, metabolic disorders, and neurodegenerative diseases. This review provides a comprehensive overview of the effects of insulin actions beyond regulating glucose metabolism (**Fig 9**).

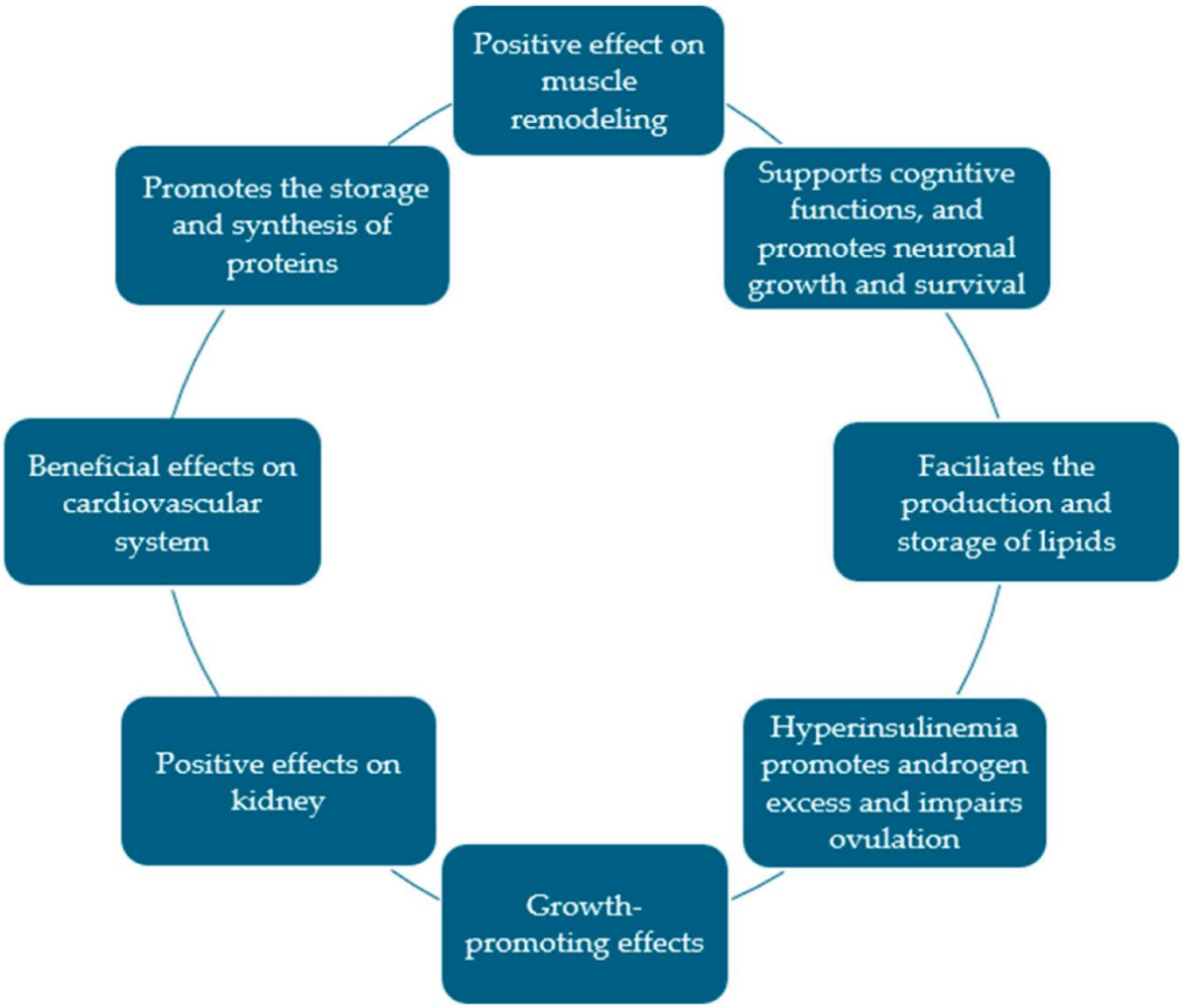

Fig 9. Illustration of the systemic actions of insulin, extending beyond glucose control.

1.5. Insulin Production in Animal Cell Systems

- Specialized Cell Lines

Continuous cell lines, such as the HIT line (hamster pancreatic beta cells transformed with SV40 virus), serve as reference tools. These cells secrete insulin in response to glucose, achieving a rate of approximately 2.4 milliunits per 10^6 cells per hour at an optimal glucose concentration of 7.5 mM. They are also responsive to regulators like somatostatin and dexamethasone.

- Primary Cultures of Pancreatic Islets

Langerhans islets isolated from various animal species (rat, mouse, pig, cow, human) retain their functionality in culture. For instance, bovine islets release significantly higher levels of insulin in the presence of 28 mM glucose compared to 2.8 mM glucose. Similarly, human, mouse, and rat islets exhibit glucose-stimulated insulin secretion even after multiple interlaboratory transports when microencapsulated.

- Stem Cell Differentiation

Mesenchymal stem cells (MSCs) derived from various animal tissues (bone marrow, adipose tissue, liver, pancreas) can be directed in vitro toward an insulin-producing cell fate. Several two- or three-step differentiation protocols have been developed, using agents such as trichostatin, mercaptoethanol, or a combination of transcription factors including PDX-1, BETA2/NeuroD, and MafA.

- Mouse bone marrow MSCs have been differentiated in vitro into cells expressing insulin and C-peptide, with glucose-stimulated insulin secretion.
- Organoids derived from canine pancreatic ducts have yielded functional beta cells that release insulin following glucose stimulation.
- Three-dimensional (3D) culture generally improves insulin secretion capacity compared to conventional 2D systems.

However, generating truly "glucose-sensitive" cells remains a challenge: in some studies, differentiated cells do produce insulin but do not always respond to elevations in glucose concentration.

1.6. In Vivo Insulin Production in Animal Cell Systems

In vivo approaches turn the animal into a bioreactor, either through transgenesis to produce recombinant human insulin or via direct genetic manipulation to induce insulin synthesis in adult animals.

Transgenic Animals as Bioreactors

Genetically modified animals express a human insulin gene under the control of a tissue-specific promoter, most often in the mammary gland.

- **Transgenic cows**:

A cow was generated by transferring a lentiviral vector containing the bovine beta-casein promoter fused to the human insulin sequence. The milk of this animal contained human insulin, with natural milk proteases ensuring the conversion of proinsulin into mature insulin. This is the first cow capable of producing human insulin in its milk, paving the way for large-scale production.

- **Transgenic pigs**:

An INS SNAP pig model was developed to study the turnover of insulin secretion granules in vivo. These pigs exhibit correct targeting of labeled insulin and normal glycemic control, enabling real-time imaging of insulin granule dynamics.

- **Transgenic mice**:

Several strategies have been tested in mice. Expression of insulin in hepatocytes (using the PEPCK promoter) demonstrated that these cells are not destroyed by autoimmune responses, although the amount of insulin produced was insufficient to prevent diabetes. Other models have targeted intestinal K cells, intestinal L cells, or gastric G cells to produce human insulin in vivo.

1.7. The Biotechnological Revolution

Resource of insulin

The advent of insulin therapy in the early 20th century, spearheaded by the landmark 1921 discovery of Banting, Best, and Collip, hinged upon a remarkably crude yet revolutionary resource: the pancreases of slaughtered pigs and cattle. For over six decades, this animal-derived insulin served as the global standard, effectively transforming diabetes mellitus from a fatal wasting disease into a survivable chronic condition and enabling the first large-scale manufacturing of the hormone.

Nonetheless, this reliance on livestock tissues posed significant logistical and clinical challenges. The production process was notoriously inefficient, requiring massive amounts of pancreatic tissue often sourced from slaughterhouse waste and complex, multi-step purification procedures to extract minute quantities of the active hormone. Moreover, the final product was a xenogeneic protein, not a perfect biochemical match for human physiology; porcine insulin varies by a single amino acid, while bovine insulin differs by three (**Tab 5**). This molecular discordance, combined with inconsistent purification standards across different batches, resulted in variable potency and purity.

Table 5: Commercial Recombinant Insulins & Analogs, their manufacturers, and production hosts.

Insulin Type	Structure	Action Profile	Host System
Human Insulin	Identical to native human insulin	Fast / short / intermediate / long-acting (depending on formulation)	E. coli, S. cerevisiae, P. pastoris
Insulin Lispro	Engineered: inversion of B28-B29 amino acids	Fast-acting	E. coli
Insulin Aspart	Engineered: B28 proline replaced by aspartic acid	Fast-acting	S. cerevisiae
Insulin Glulisine	Engineered: B3 asparagine → lysine; B29 lysine → glutamic acid	Fast-acting	E. coli
Insulin Glargine	Engineered: A21 glycine; B-chain elongated by two arginines	Long-acting	E. coli
Insulin Detemir	Engineered: B30 threonine removed; fatty acid attached to B29 lysine	Long-acting	S. cerevisiae
Insulin Degludec	Engineered: B30 threonine removed; fatty acid conjugate attached to B29 lysine	Ultra-long acting	S. cerevisiae

Consequently, patients frequently endured adverse effects, ranging from localized skin irritation to severe systemic allergic reactions. Over prolonged use, many individuals also developed anti-insulin antibodies, which could neutralize the treatment's effectiveness, leading to insulin resistance and unstable glycemic control. This precarious therapeutic landscape ultimately persisted until the dawn of genetic engineering in 1982, when the introduction of recombinant human insulin (Humulin) rendered the mass extraction from animal pancreases obsolete.

The pharmaceutical industry's transition

The pharmaceutical industry's transition from animal-derived insulin to biotechnology was not a sudden decision but a necessary evolution driven by a confluence of clinical, logistical, and scientific pressures.

The Mounting Limitations of Animal-Derived Insulin

For decades, the extraction of insulin from the pancreases of pigs and cows was a medical miracle, but it was also a process fraught with challenges that became increasingly untenable.

1. **Clinical Immunogenicity and Safety Risks**:

The fundamental issue was that animal insulin is not an exact match for human insulin; porcine insulin differs by one amino acid, and bovine insulin by three. This molecular difference meant that for many patients, the immune system recognized the animal protein as foreign. This led to the production of anti-insulin antibodies, which could neutralize the treatment's effectiveness, leading to insulin resistance. In some cases, it caused severe allergic reactions. The variable purity of early animal extracts further exacerbated these issues, leading to side effects like lipodystrophy (abnormal fat distribution at injection sites).

2. **Logistical and Supply Constraints**:

The production process was remarkably inefficient, requiring a massive and constant supply of animal pancreases from slaughterhouses. This dependency created a precarious supply chain that was vulnerable to fluctuations in the meat industry and could not easily scale to meet the rapidly growing global demand for insulin. Furthermore, emerging health concerns, such as the risk of contamination from agents like Bovine Spongiform Encephalopathy (BSE), added another layer of risk to animal-sourced products.

Recombinant Human Insulin

The solution to these problems came from the revolutionary field of molecular biology. Recombinant DNA technology, developed in the early 1970s, offered a way to produce a perfect, safe, and limitless supply of human insulin.

The breakthrough was achieved by a team at Genentech, who successfully inserted the human gene responsible for insulin production into the bacteria Escherichia coli. These genetically engineered bacteria functioned as microscopic factories, capable of synthesizing human insulin. This landmark achievement was licensed to Eli Lilly and Company, leading to the development of Humulin.

In **October 1982**, the U.S. Food and Drug Administration (FDA) approved Humulin, making it the **first recombinant DNA-based therapeutic product** approved for human use. This approval marked a pivotal moment, not just for diabetes care, but for the entire pharmaceutical industry, ushering in the era of modern biotechnology.

The Advantages of a Biotechnological Approach

The shift to recombinant insulin brought immediate and profound advantages:

1. Chemical Identity and Reduced Immunogenicity: Recombinant human insulin is chemically identical to the insulin produced by the human pancreas. This dramatically reduced the risk of allergic reactions and immune system interference, providing a safer and more reliable therapy for patients.

2. Superior Purity and Consistency: The production process in a controlled bioreactor environment is far more predictable and purer than extraction from animal tissue. This eliminated the batch-to-batch variability in potency and purity that was a major drawback of animal extracts.

3. **Unlimited Scalability**: Recombinant production is not dependent on a limited supply of animal organs. It can be scaled up almost indefinitely in industrial bioreactors, ensuring a consistent and abundant supply of insulin to meet global demand.

The Role of Engineering in the Biotech Era

The success of recombinant insulin production depends not only on the genetic engineering of bacteria but also on the sophisticated industrial infrastructure required to grow them and process the protein. This is where companies like **Boccard** play a crucial role.

Boccard has a proven track record in designing and delivering complete biotechnological production units. In one instance, they supported an Eastern European company by engineering a comprehensive solution for recombinant protein fermentation. This included the design and delivery of a bioreactor system, scaling up from a 30-liter seed reactor to a 150-liter main bioreactor. Such integrated solutions, which also encompass downstream processing equipment like centrifuges and ultrafiltration systems, are essential for translating a scientific breakthrough into a viable, large-scale, and life-saving commercial product.

Main stages of modern insulin production today

Modern insulin manufacturing usually follows one of two biotechnology routes (**Tab 6**):

- Fermentation of genetically modified E. coli or yeast producing pro-insulin: an early form of insulin produced inside pancreatic beta cells with an initial single-chain molecular structure that is later cut and folded into active insulin and its companion peptide
- Recovery, purification and conversion into human insulin or insulin analogues
- **Formulation**, the stage where purified insulin is stabilised and prepared for aseptic filling

Step 1: recombinant strain creation

Scientists identify and insert the human insulin gene into E. coli or yeast. The modified cells gain the ability to express pro-insulin or an insulin precursor. This gene insertion is validated for productivity, stability and safety before scale up.

Step 2: fermentation conducted

The recombinant microorganism is cultivated in stainless steel bioreactors. Inside these vessels, operators carefully regulate:

- Temperature
- pH
- Dissolved oxygenb
- Nutrients feeding strategy

The goal is to maximise biomass growth and pro-insulin expression while preserving product integrity.

Step 3: insulin precursor harvest

Once fermentation is complete, the culture is transferred to downstream operations. Depending on the microorganism used:

- E. coli requires **cell disruption** to release the insulin precursor
- Yeast secretes insulin precursors more readily

Harvest steps include centrifugation and filtration to remove unwanted solids.

Step 4: insulin purification

Purification may include:

- Solubilisation of inclusion bodies (in E. coli processes)
- Precipitation to remove impurities
- Multiple chromatography steps to isolate the insulin precursor
- Ultrafiltration to concentrate the product

The goal is to achieve pharmaceutical-grade purity suitable for injection.

Step 5: insulin converted into its final form

When the precursor is purified it undergoes enzymatic or chemical conversion to produce:

- Human insulin
- Rapid-acting analogues
- Long-acting analogues

Each type requires precise reaction conditions and careful monitoring.

Step 6: What happens during insulin formulation?

Purified insulin is mixed with:

- Buffers
- Preservatives
- Stabilising excipients

These allow the final medicine to remain effective and safe throughout its shelf life. This step is highly sensitive to contamination and requires flawless utilities such as **(water for injection) WFI, pure steam** and **pharmaceutical-grade compressed air.**

Step 7: the final product filled and sealed

The formulated solution is transferred through sterile piping to filling lines. The product is then:

- Filled into vials, cartridges or pens
- Inspected
- Labelled
- Packed
- for distribution

Table 6: Detaille Technical Breakdown Table

Stage	Host	Core Process Description	Critical Process Parameters (CPPs)	Intermediate	Modern Standards
1. Gene & Cell Banking	E. coli (BL21) or S. cerevisiae	**Synthesis:** Human insulin gene (or modified analog gene) is codon-optimized and inserted into a plasmid with a strong inducible promoter (e.g., T7/lac for E. coli; AOX1 for yeast). **Banking:** MCB and WCB are created, cryopreserved, and fully sequenced.	- Plasmid copy number stability. - Antibiotic selection pressure. -Cryopreservation rate (-80°C / LN_2).	**MCB & WCB** vials with confirmed genetic fidelity.	**ICH Q5D** (Cell bank characterization). **ICH Q5A** (Viral/Adventitious agent testing). Full vector sequence map required.
2. Fermentation (Upstream)	E. coli or Yeast (Stainless Steel Bioreactors, 1,000 - 15,000 L)	**Inoculation:** WCB → shake flasks → seed fermenter → main production fermenter. **Fed-Batch:** Carbon source (glucose/glycerol) fed exponentially to maximize cell density without acetate overflow (for E. coli). **Induction:** IPTG (for E. coli) or Methanol (for Pichia) added to trigger protein expression.	- **Temp:** 22-37°C (± 0.5°C). - **pH:** 6.8-7.2 (± 0.1). - **DO:** >30% saturation. - **Feed rate:** Precise to avoid metabolic byproducts.	**High-density cell paste** containing either: A) E. coli Inclusion Bodies (IBs) B) Secreted Proinsulin in supernatant.	**In-process control (IPC):** OD_{600}, off-gas mass spectrometry (O_2/CO_2), wet cell weight. **Bioburden** sampling before induction.
3. Recovery & Refolding	**A) E. coli Route:** Homogenizer + Centrifuges **B) Yeast Route:** TFF (Tangential Flow Filtration)	**A) IBs:** Cells lysed via high pressure → IBs washed with detergents → Solubilized in 6-8M urea/guanidine + reducing agent (DTT). **B) Secretion:** Cells removed via centrifugation; supernatant concentrated via ultrafiltration. **Refolding (for E. coli):** Slow dilution into redox buffer (GSH/GSSG) to allow correct disulfide bond formation.	- Homogenization pressure (800-1000 bar). - Redox ratio (GSH:GSSG, typically 1:1 to 5:1). - Dilution factor & temperature (4-10°C).	**Correctly folded proinsulin** (for E. coli) or **Concentrated proinsulin** (for yeast).	**HPLC** monitoring for refolding efficiency. **SDS-PAGE** to confirm product identity and aggregation status.
4. Enzymatic Conversion	Enzymatic Reactor (Stirred tank with precise T°C control)	The C-peptide connecting the A and B chains is cleaved using proteolytic enzymes. **Trypsin** (cleaves at Lysine/Arginine residues) and **Carboxypeptidase B** (removes terminal basic residues) are added in minute, highly controlled quantities.	- Enzyme-to-substrate ratio (critical to prevent over-digestion). - Reaction time. - Temperature (4-15°C to limit side reactions).	**Active two-chain human insulin** (or specific analog variant).	**In-process RP-HPLC** to monitor cleavage completion and identify desamido byproducts.

All steps take place in a controlled cleanroom with validated cleaning cycles and traceability.

Understanding each step makes it clear why formulation workshops and clean utilities are central to insulin quality. The formulation stage is where the bulk product becomes a stable, injectable medicine that patients depend on. Boccard's work strengthens this critical stage by ensuring sterility, utility performance and process reliability.

Industrial Insulin Production: Key Bioprocessing and Regulatory Constraints

Industrial production of recombinant insulin is far more complex than simply growing bacteria in a tank. It operates at the intersection of highly sensitive biochemistry, stringent regulatory oversight, and advanced engineering. The key constraints can be broken down into five critical pillars: **bioprocess fidelity**, **regulatory compliance**, **utility infrastructure**, **formulation stability**, and **economic scalability**.

Here is a detailed development of these constraints:

1. Bioprocess and Biochemical Fidelity (Beyond just Temperature and pH)

While temperature and pH are crucial, they are just the surface of the bioprocess constraints.

- **Critical Process Parameters (CPPs)**:

  During fermentation, parameters like dissolved oxygen (DO) and nutrient feed rates must be meticulously controlled. E. coli or yeast cells are highly sensitive; even a minor spike in temperature can induce heat-shock proteins that degrade the recombinant protein, while fluctuations in pH can alter the bacterium's metabolic pathways, reducing yield.

- **Downstream Processing (DSP)**:

  After fermentation, insulin is produced as inactive "inclusion bodies" (in E. coli) or as proinsulin (in yeast). Extracting the active hormone requires complex, multi-step chromatography (HPLC) and enzymatic conversion. These purification steps are highly constrained by buffer compositions, flow rates, and column pressures. Any deviation can compromise the removal of host-cell proteins, endotoxins, and DNA—impurities that could trigger severe immune responses in patients.

3. **Stringent Regulatory and Quality Compliance (GMP, FDA, EMA)**

   Compliance is not a one-time checkpoint but a continuous, pervasive constraint that dictates every operational decision.

- **Current Good Manufacturing Practices (cGMP)**:

Facilities must operate under cGMP, which mandates that all manufacturing processes are validated, reproducible, and meticulously documented. The "Four Ps" rule applies: **Product** (must meet release specs), **Process** (must be validated), **People** (must be rigorously trained and gowned), and **Premises** (must meet cleanroom standards).

- **Validation and Change Control**:

Any change in raw material suppliers, equipment, or even a single parameter requires formal regulatory notification and re-validation, a process that can cost millions and take months.

- **Environmental Monitoring**:

The sterile environment requires continuous, real-time monitoring of airborne particles and viable microorganisms (bacteria and fungi) in the cleanroom. A single environmental excursion can trigger a full product recall investigation.

3. Critical Utility Infrastructure (WFI, Pure Steam, and Purified Water)
The text correctly highlights WFI and pure steam, but the engineering constraints behind them are immense.

- **Water for Injection (WFI)**:

WFI is the gold standard for drug formulation. It is produced via distillation or reverse osmosis and must be stored and circulated in a **continuously heated loop (typically 65–80°C)** to prevent biofilm formation. The system must be built with sanitary, dead-leg-free piping (usually 316L stainless steel with an electro-polished internal surface) to prevent microbial attachment. Any stagnation or cooling in the loop immediately compromises sterility.

- **Pure Steam**:

Used for Sterilization-in-Place (SIP) of bioreactors, piping, and storage tanks. The pure steam must be contaminant-free (no volatile organics or pyrogens) to effectively kill all microorganisms without leaving residues that could leach into the drug product.

- **Purified Water**:

Used for cleaning equipment (Clean-in-Place, CIP) and preparing buffers. Its conductivity, total organic carbon (TOC), and microbial counts are strictly regulated and monitored online 24/7.

4. Formulation and Physical Stability (Excipients and Insulin Aggregation)
Insulin is a notoriously fragile peptide prone to physical degradation.

- **Excipient Precision**:

Excipients like zinc, phenol, meta-cresol, and glycerol are not merely inactive fillers; they are **structural stabilizers**. They work together to form stable zinc-hexamer complexes that protect the insulin from chemical degradation and physical fibrillation. The pH must be maintained within a very narrow window (typically around 7.0–7.4) because even a 0.2-unit deviation can disrupt the dissociation of hexamers into the active monomeric form upon injection, directly affecting the drug's pharmacokinetics.

- **Fibrillation Risk**:

Insulin has a strong tendency to aggregate into insoluble amyloid-like fibrils when exposed to shear stress, air-liquid interfaces, or excessive heat. Therefore, the formulation and filling processes must be executed with ultra-gentle mixing and specialized silicone-free stoppers to prevent protein denaturation.

5. Economic and Supply Chain Constraints (CAPEX/OPEX)
While not a scientific constraint, economics heavily influence production design.

- **Capital Expenditure (CAPEX)**:

Building a GMP-compliant facility with highly engineered WFI loops, HVAC systems (to maintain unidirectional airflow and pressure cascades), and stainless-steel bioreactors requires a multi-hundred-million-dollar investment.

- **Operational Expenditure (OPEX)**:

The energy required to continuously heat WFI loops and run high-volume HVAC systems makes utilities one of the largest ongoing costs. Furthermore, all raw materials entering the facility—from culture media to packaging vials—must meet stringent quality standards with full traceability, creating a complex, high-cost global supply chain.

Industrial insulin production is a holistic system where failure in one area—be it a leaking valve in the WFI loop, a non-validated cleaning protocol, or a 0.5°C drift in the fermentation tank—immediately jeopardizes the batch. To overcome these constraints, modern manufacturers adopt **Quality by Design (QbD)** principles, building robust process control strategies and resilient utility networks that ensure every final vial is pure, potent, and perfectly sterile.

II.2. Interféron

Interferons (IFNs) are glycoproteins belonging to the cytokine family, or rather signaling molecules that regulate the immune system. They were discovered in 1957 by Isaacs and Lindenmann, who observed that chicken cells infected with the influenza virus produced a factor that enabled other cells to become resistant to that virus.

This factor was named interferon, because it allows viral interference that is, the acquisition of resistance to a virus by a cell. It was later demonstrated that this factor was actually composed of several different proteins from the same family, which were found to play various roles in vertebrates.

2.1. The diversity of interferons

IFNs are categorised into three types: types I and III are involved in antiviral innate immunity in most cells of the body, whereas type II plays a predominant role as a communication molecule between specialized cells of the immune system.

Type I interferons are themselves divided into several subtypes: IFN-α and β, which are the most common and most studied, but also IFN-ω, ε, and κ, which are less studied and whose action is limited to certain organs, unlike IFN-α and β, which are almost ubiquitous throughout the body.

The type II interferon family consists exclusively of IFNγ, which functions similarly to other cytokines by primarily facilitating intercellular signaling within the immune system. For this reason, its specific properties will not be discussed further in this article.

Type III encompasses the IFNλs a recently identified group whose biological roles remain a subject of ongoing debate. Nevertheless, their functions appear to closely parallel those of type I IFNs: they trigger a non-specific antiviral state and utilize the same signal transduction cascade, with the sole distinction being their receptor.

In general, humans possess 21 genes encoding interferons: 13 for IFN-α, 3 for IFN-λ, and one each for the β, ω, ε, κ, and γ classes. The grouping of several genes within a single

subtype is due to the very high homology of their sequences; these genes are said to constitute a multigenic family.

2.2. Structure and biosynthesis

All interferons (IFNs) share an α-helical architecture forming an antiparallel four-helix bundle (A, C, D, and F). However, each family adopts a distinct conformation: type I IFNs are monomers with long, straight, and parallel helices (16 members with 35% to 95% sequence identity), whereas type III IFNs (IFNλ) possess shorter and kinked helices, forming a more compact bundle that structurally brings them closer to IL-22 than to type I IFNs

In contrast to the monomeric types I and III, IFNγ (type II) adopts an intercalated dimeric structure, where helices E and F are swapped between subunits, thereby bringing it closer to IL-10. This structural diversity reflects distinct regulatory mechanisms: IFNγ, which is highly conserved and unique, acts through homodimerization of its receptor, whereas type I and type III IFNs exploit their variable sequences (28% to 96% identity) to modulate their biological activities via varied contacts with their respective receptors (**Fig10**).

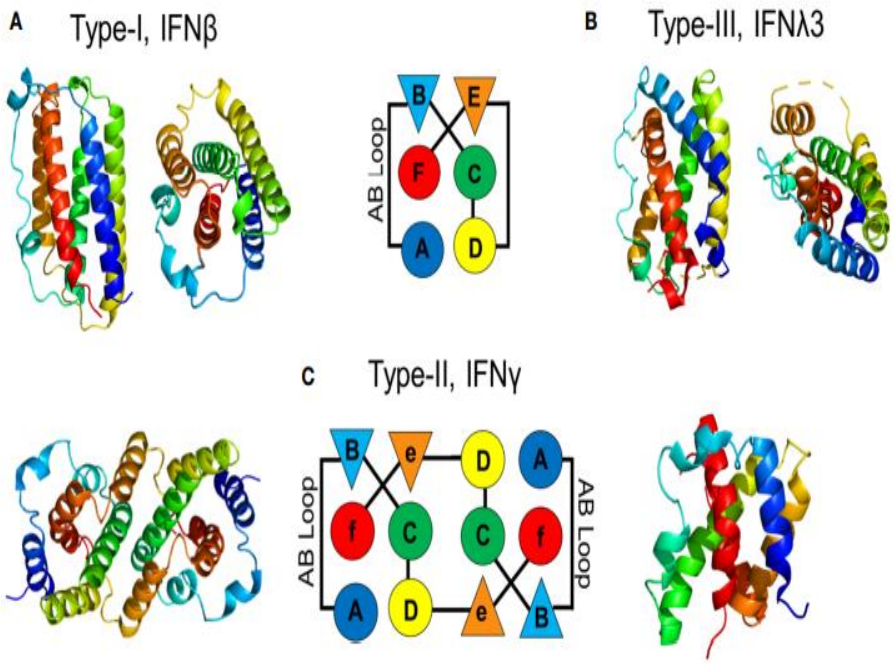

Fig 10. This representation of the three-dimensional structure of interferon α highlights the numerous alpha helices that compose it (indicated by the gray cylinders), organized into domains, each shown in a different color.s. Schematic, and ribbon diagrams, s. Schematic, and ribbon diagrams, show the six secondary structural elements of the type-I (A), pdbid = 1AU, type-III (B), pdbid = 3HHC, and type-II (C), pdbid = 6E3K IFNs. IFN structures are rainbow colored from the N-terminus helix A (blue) to the C-terminal helix F (red).

2.3. Interferon production

In vertebrates, type I interferons can be produced by virtually all cell types. Type III IFNs, while similarly capable of inducing an antiviral state across a wide range of cells, appear to be more specifically tailored to tissues that are highly exposed to viral invasion—particularly mucosal surfaces. Notably, a specialized subset of immune cells known as plasmacytoid dendritic cells (pDCs) has been identified for its remarkable ability to generate massive amounts of interferons in response to viral infection. These circulating cells secrete up to 10,000 times more IFN-α than ordinary cells, a trait that has earned them the designation of IPCs (interferon-producing cells).

Interferon production is triggered when host pattern-recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) signaling viral infection. This innate immune response is rapidly deployed upon first exposure to a pathogen, without requiring the prolonged maturation period characteristic of adaptive immunity, which relies on the generation of highly specific antibodies and lymphocytes.

PAMPs can be nucleic acids located in unusual cellular compartments. For instance, the presence of cytosolic double-stranded RNA or DNA signals infection, since vertebrate cells normally confine DNA to the nucleus or mitochondria and do not produce free dsRNA. Many viruses either release these nucleic acids into the cytosol upon entry or generate them during replication, directly stimulating innate immunity and, notably, interferon production. PAMPs, however, also encompass a variety of other molecular structures (**Fig 11**).

Viral DNA and RNA act as PAMPs that can engage multiple types of PRRs, including Toll-like receptors (TLRs), RIG-I (a member of the RLR family), and cGAS. Through adaptor proteins, these PRRs activate the transcription factors IRF3 and IRF7, which subsequently drive the production of interferons that are secreted into the extracellular environment (**Fig 11)**.

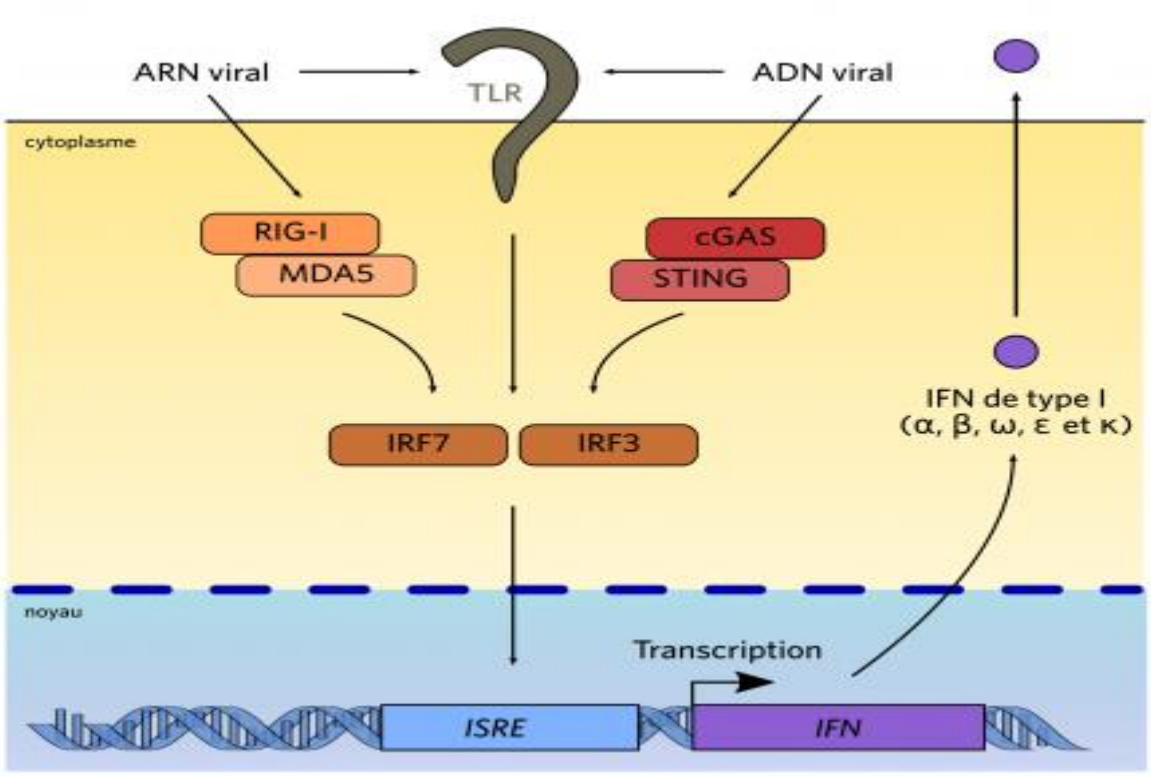

Fig 11. Mechanisms of type I interferon production

Upon IFN binding, receptor-associated JAK kinases become activated and phosphorylate both the receptor subunits and themselves (autophosphorylation). These active JAKs then phosphorylate STAT proteins, prompting them to dimerize and associate with the auxiliary protein IRF9 forming the ISGF3 transcriptional complex. This complex subsequently translocates to the nucleus, where it drives the expression of interferon-stimulated genes (ISGs).

Upon IFN binding, a membrane receptor in its monomeric state undergoes dimerization with a second subunit. This association induces conformational changes in both receptor chains, which in turn activates receptor-associated Janus kinases (JAKs) specifically JAK1 on one subunit and TYK2 on the other. These two kinases trans-phosphorylate and activate each other following receptor dimerization, and subsequently phosphorylate free cytosolic STAT proteins. This phosphorylation promotes STAT dimerization, causing a conformational rearrangement that exposes nuclear localization signals.

The resulting STAT1/STAT2 heterodimer can then bind to its consensus DNA sequence, known as the interferon-stimulated response element (ISRE), which is present in the promoters of numerous genes. Binding of the STAT dimer to these ISREs drives target gene transcription. This signaling cascade is referred to as the JAK/STAT pathway **(Fig 12**).

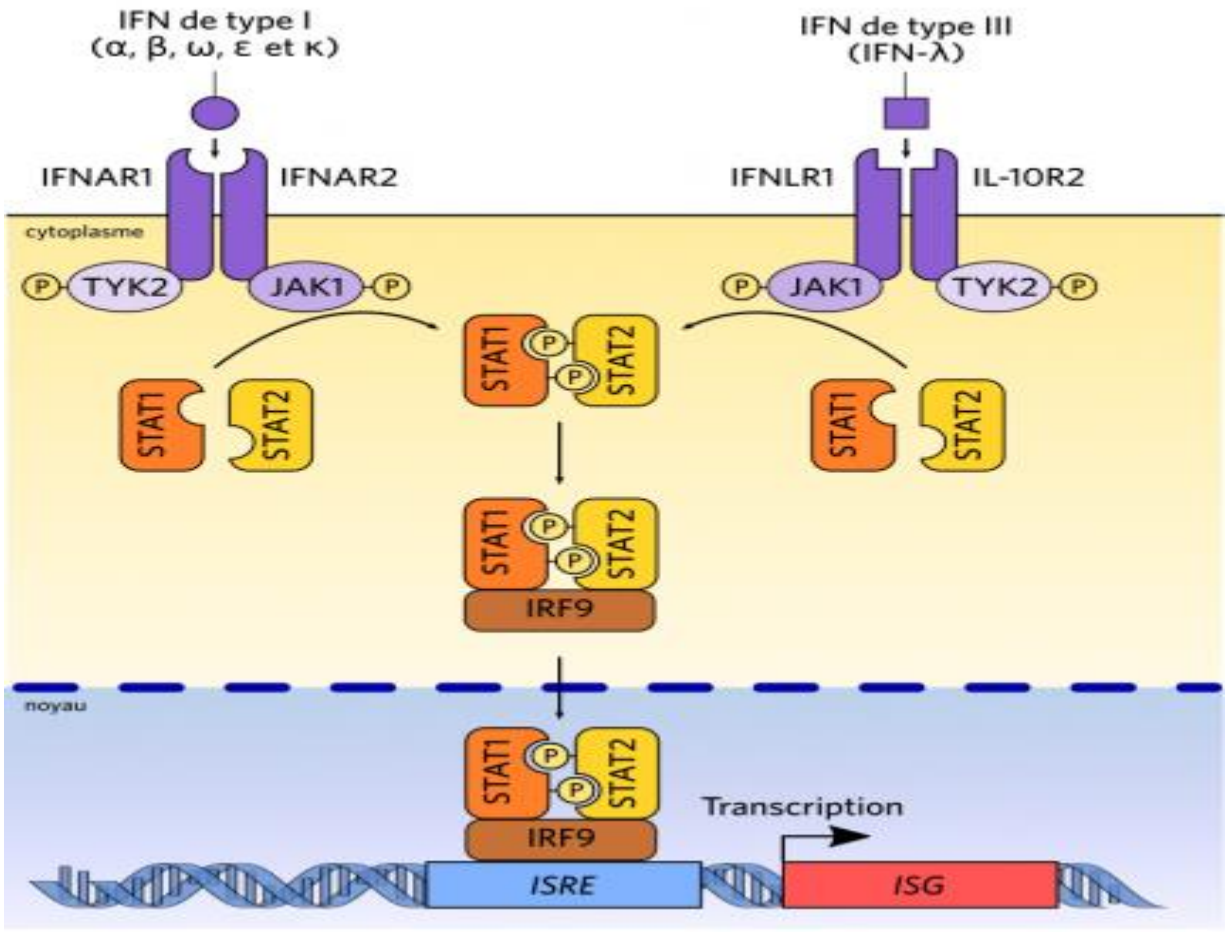

Fig 1. JAK/STAT signal transduction pathways activated by type I and type III interferons

2.4. Regulatory Factors and Mechanisms of Interferons

The intracellular signaling cascades that control interferon production are intricate and subject to stringent regulation. Unlike type II IFN (IFN-γ), which is produced exclusively by Th1 cells, $CD8^+$ T lymphocytes, and natural killer (NK) cells upon stimulation by bacterial, viral, or other pathogenic agents, the induction of type I and type III IFNs is largely dependent on PRR signaling pathways triggered by pathogen-associated molecular patterns (PAMPs) or endogenous danger signals (DAMPs). These PRRs fall into four main families: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), cytosolic DNA sensors, and NOD-like receptors (NLRs). Of these, TLRs, RLRs, and cytosolic DNA sensors are the principal drivers of type I and type III IFN production (**Fig 13**).

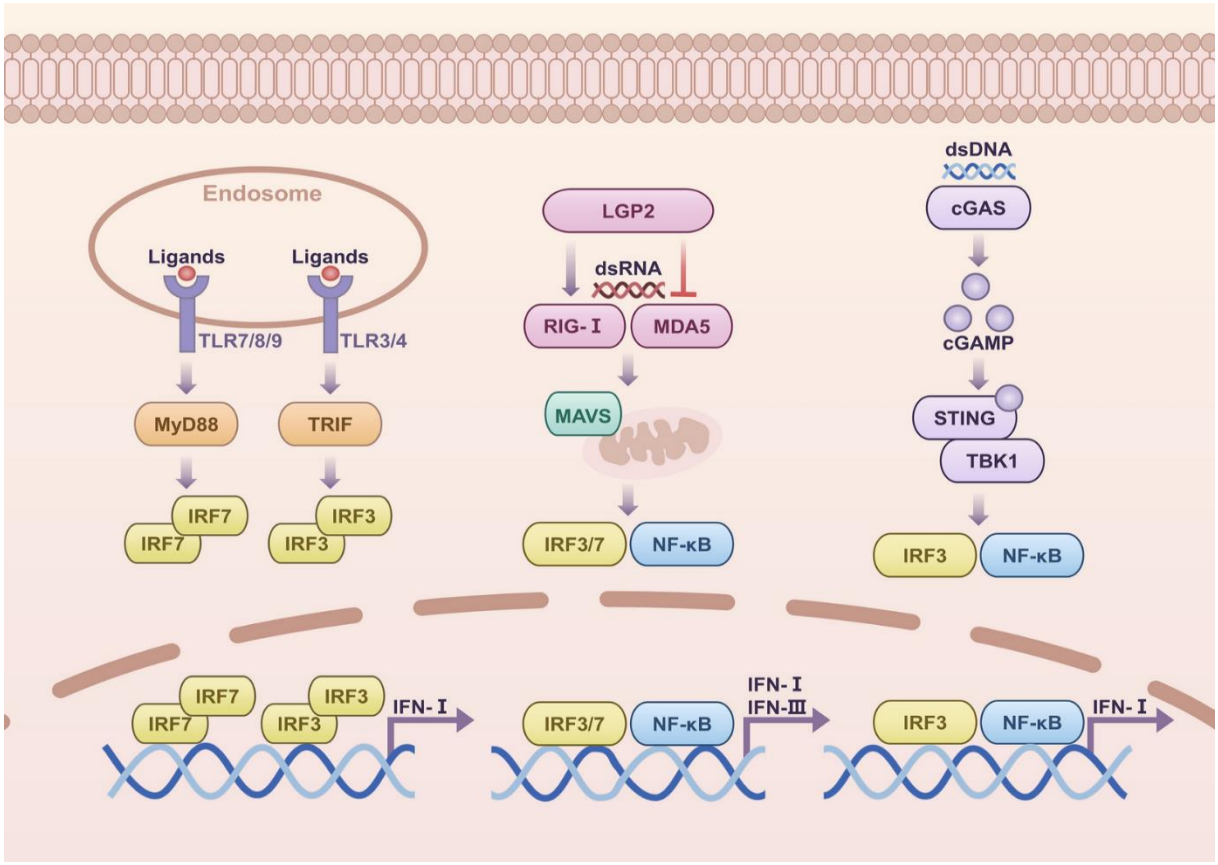

Fig 2. Regulatory Factors and Mechanisms of Interferons

The transcriptional regulation of interferon (IFN) production is orchestrated by cellular pattern recognition receptors (PRRs). These sensors detect either exogenous pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs) such as double-stranded RNA (dsRNA) and double-stranded DNA (dsDNA) derived from pathogens. This recognition event triggers downstream signaling cascades involving the TLR, RLR, and cGAS-STING pathways, which ultimately converge to activate IRF3, IRF7, and the NF-κB axis. The coordinated activation of these transcription factors then drives the expression of type I and type III interferons (IFN-I and IFN-III).

2.5. Clinical use of interferons

The discovery of the effectiveness of interferons in combating viral infections has paved the way for their therapeutic use. Recombinant interferon molecules are produced by bacteria, yeasts, or cultured human cells into which one of the human interferon genes has been cloned; these are known as recombinant IFNs. This method enables the production of large quantities of pure interferons.

These molecules are primarily used to treat hepatitis B and C, as they induce an antiviral state, and are used in combination with other direct-acting antiviral agents. However,

they can cause side effects, such as fevers or migraines, though they remain well tolerated by the majority of patients

2.6. Recombinant interferon

Recombinant interferon produced from animal cells refers to interferon proteins created by introducing the interferon gene into animal cell hosts, which then manufacture the protein. This is a key method for producing interferons for therapeutic use in both veterinary and human medicine.

3.1. Animal Cell Production Systems

A primary system for this is the **baculovirus-insect cell system**. This involves using a genetically engineered baculovirus to carry the interferon gene into insect cells (like Sf9 cells) or even live insect larvae, which then produce the recombinant interferon.

- **Canine Interferon (rCaIFN-α7)**

The development of a biotechnological process for producing recombinant canine interferon-alpha 7 (rCaIFN-α7) using the baculovirus-insect cell and larvae system was undertaken to evaluate its antiviral and antitumor activities for potential veterinary applications against viral and oncological diseases. To achieve this, the rCaIFN-α7 gene was expressed using a baculovirus vector in two distinct platforms: Sf9 insect cell culture, where the recombinant protein was purified from the supernatant via single-step immobilized metal ion chromatography, and a scalable alternative utilizing 200 Rachiplusia nu larvae infected with the same recombinant virus. The production in Sf9 cells proved highly successful, yielding 4.43 mg/L of rCaIFN-α7 at an impressive 95% purity; this purified protein exhibited significant antiviral activity against Vesicular Stomatitis Virus in MDCK canine kidney cells, with a recovery of 5.34×10^7 AU/mL, and also demonstrated antitumor efficacy against canine mucosal melanoma cell lines.

In parallel, the larval scale-up also generated a biologically active product with a potent antiviral titer of 4.5×10^7 AU/mL and a yield of 5.36×10^6 AU/g of larvae, yet this approach was hindered by a low purification yield of only 20% from the larval extract, largely attributed to protein aggregation or degradation. Consequently, while the baculovirus-insect cell system is highly effective for producing high-purity rCaIFN-α7 with potent biological activity, the larval system, although offering a scalable alternative, requires further optimization to overcome the challenges posed by protein instability.

- **Feline Interferon (FeIFN-α7)**

The primary objective of this study was to express and purify recombinant feline interferon-alpha 7 (FeIFN-α7) alongside an arginine-tagged variant (FeIFN-α7xArg) using the baculovirus-insect cell and larvae systems, while also comparing the expression efficiency across different insect hosts. To accomplish this, both FeIFN-α7 and its eight-arginine-tagged counterpart were expressed in Sf9 insect cells maintained in suspension culture, as well as in the larvae of two distinct insect species: Rachiplusia nu and Spodoptera frugiperda. The results obtained from Sf9 cell cultures at 4 days post-infection demonstrated robust expression levels, reaching $(1.28 \pm 0.15) \times 10^6$ U mL^{-1} for the native FeIFN-α7 and $(1.3 \pm 0.2) \times 10^6$ U mL^{-1} for the tagged version, indicating that the arginine tag did not hinder production in this system.

When comparing the two larval hosts, Rachiplusia nu clearly emerged as the superior expression platform, achieving maximum expression at just 2 days post-infection with values of $(3.7 \pm 0.2) \times 10^6$ U mL^{-1} for FeIFN-α7 and $(3.5 \pm 0.4) \times 10^6$ U mL^{-1} for FeIFN-α7xArg, whereas Spodoptera frugiperda larvae exhibited considerably lower peak expression at 5 days post-infection, with titers of only $(1.1 \pm 0.2) \times 10^6$ U mL^{-1} and $(1.0 \pm 0.15) \times 10^6$ U mL^{-1}, respectively.

Importantly, the presence of the 8xArg tag was found to have no detrimental effect on the biological activity of the interferon, preserving its full functional potential. In conclusion, the baculovirus-insect larvae system proves to be highly effective for FeIFN-α7 production, with Rachiplusia nu demonstrating significantly superior expression capabilities compared to Spodoptera frugiperda, while the arginine tag offers a valuable purification tool that does not compromise biological function.

- **Equine Interferon (eIFN-γ)**

The aim of this study was to express biologically active recombinant equine interferon-gamma (reIFN-γ) using two distinct baculovirus gene expression platforms: one based on insect cell culture and the other utilizing silkworm larvae.

To achieve this, the full-length equine IFN-γ cDNA, which included its native secretion signal peptide, was first cloned into the baculovirus transfer vector pAcYM1, and this construct was subsequently used to generate two recombinant viruses—AcEIFN-γ, derived from Autographa californica nuclear polyhedrosis virus, and HyEIFN-γ, based on a hybrid nuclear polyhedrosis virus. These viruses were then employed to infect Trichoplusia ni-derived BTI TN 5B1-4 insect cells and Bombyx mori silkworm larvae, respectively, with the latter receiving the HyEIFN-γ construct.

The results demonstrated that recombinant eIFN-γ was successfully accumulated both in the culture fluid of infected insect cells and in the hemolymph of infected silkworm larvae, and the protein was produced as four distinct glycoforms with molecular masses of 14, 16, 18, and 20 kDa, as confirmed by SDS-PAGE analysis and tunicamycin treatment which verified proper glycosylation. Importantly, the recombinant protein exhibited high-level biological activity, including potent antiviral effects against Vesicular Stomatitis Virus and the capacity to induce MHC class II antigen expression on equine fetal kidney cells, confirming its functional integrity.

In conclusion, both the insect cell and silkworm larvae baculovirus expression systems are fully capable of producing biologically active, glycosylated recombinant equine IFN-γ, thereby providing a viable and versatile approach for generating this cytokine for research purposes and potential therapeutic applications in equine medicine.

- **Porcine Interferon (spI Interferon)**

The primary objective of this investigation was to biochemically and biologically characterize a novel, atypical short porcine type I interferon, designated spI interferon, which was expressed in insect cells using a recombinant baculovirus system.

To accomplish this, a recombinant baculovirus was engineered to express the spI interferon gene a gene naturally transcribed in pig trophoblast during implantation and this virus was subsequently used to infect Sf9 insect cells, with the recombinant protein being secreted into the culture medium and purified through ion-exchange and reverse-phase high-performance liquid chromatography.

The results revealed that the protein was secreted into the culture medium at 3 days post-infection, reaching a titer of 60,000 IU/mL, and N-terminal sequencing confirmed the correct cleavage of the signal peptide, yielding a mature protein of 149 amino acids, which represents the shortest reported type I interferon to date. The purified spI interferon was characterized as an N-glycosylated monomer of 19 kDa, exhibiting a specific antiviral activity of 3.7×10^7 IU/mg on Madin-Darby bovine kidney cells, yet remarkably, it demonstrated no activity on human cells in either antiviral or antiproliferative assays, highlighting its species-specific nature with a preference for pig cells over bovine cells.

Furthermore, serological tests confirmed that spI interferon is not antigenically related to other interferon families, suggesting that it may represent an entirely new family of type I

interferon, and interestingly, the carbohydrate chain was found to be non-essential for its bioactivity.

In conclusion, the baculovirus-insect cell system successfully produced a fully functional, atypical porcine type I interferon, and its unique biochemical properties and species-specific activity distinguish it from conventional type I interferons, marking it as a potential new member of the interferon family with specialized biological functions.

- **Chicken Interferon (chIFN-α)**

The primary objective of this study was to enhance the production of recombinant chicken interferon-alpha (chIFN-α), which has traditionally been limited by low yields, through the use of an improved baculovirus expression system, while simultaneously evaluating its antiviral efficacy both in vitro and in vivo. To accomplish this, an enhanced baculovirus expression system was employed, wherein a recombinant bacmid was constructed carrying two copies of the chIFN-α gene specifically designed to boost expression levels, and the resulting protein was subsequently produced and subjected to rigorous antiviral testing.

The results demonstrated that this improved system successfully produced chIFN-α at high yields, overcoming the previous limitations associated with low productivity, and the purified protein exhibited potent anti-VSV activity reaching up to 5.0×10^6 IU/mL in vitro, confirming its strong antiviral potential at the cellular level.

Furthermore, when administered to specific-pathogen-free (SPF) chickens in vivo, the recombinant chIFN-α effectively inhibited the proliferation of the H9N2 subtype low pathogenicity avian influenza virus and significantly reduced the viral-shedding rate, thereby demonstrating its therapeutic efficacy in a live animal model.

In conclusion, the improved baculovirus expression system featuring a dual-gene construct successfully addressed the longstanding challenge of low yields for chIFN-α production, and the resulting protein exhibited robust antiviral activity both in cell culture and in live chickens, presenting a highly promising approach for producing therapeutic chIFN-α for applications in the poultry industry.

Other Production Methods

While animal cells are a key system, recombinant interferons are also produced using other methods:

- **Bacterial Fermentation (E. coli, Bacillus megaterium)**

Bacterial systems represent the most well-established and economical approach for recombinant protein production. Their ease of cultivation, rapid division rates, and straightforward, scalable fermentation processes make them ideal for large-scale manufacturing. Bacillus megaterium, for instance, is recognized as a safe host capable of achieving high protein yields.

However, as prokaryotes, bacteria lack the complex post-translational modification (PTM) machinery required for the full biological function of many eukaryotic proteins. Glycosylation the attachment of sugar moieties is particularly critical, as it directly influences protein stability, bioactivity, and circulatory half-life.

In the absence of these enzymatic systems, recombinant proteins frequently misfold and amass into insoluble inclusion bodies. While recovering functional protein necessitates rigorous extraction, denaturation, and refolding, this aggregation can advantageously serve as a crude preliminary purification step.

This prokaryotic limitation is notably leveraged in the production of interferon beta-1b, a non-glycosylated variant clinically approved for treating multiple sclerosis. Additionally, researchers have successfully generated biologically active recombinant porcine IFN-α and IFN-β using B. megaterium.

- **Yeast Systems (Pichia pastoris)**

Yeasts, as eukaryotes, represent an interesting compromise between the simplicity of bacterial systems and the complexity of mammalian cells. They have the advantage of carrying out certain essential post-translational modifications (PTMs), including glycosylation, which is often crucial for protein functionality.

The Pichia pastoris system is particularly prized for its ability to reach high cell densities and to secrete large quantities of recombinant proteins directly into the culture medium, which considerably simplifies downstream purification steps. A yield of 136 mg/L of porcine interferon-alpha has thus been obtained with this system, and operating costs remain significantly lower than those for mammalian cell cultures.

However, the glycosylation profile in yeasts differs from that of human cells, which can alter the properties of the produced protein. To overcome this drawback, so-called "glycoengineered" strains are currently being developed to humanize this process. Furthermore, under very high expression levels, protein misfolding can still occur, leading to their

accumulation in the endoplasmic reticulum (ER) and requiring additional purification steps. Despite these challenges, research has successfully optimized the intracellular expression of human interferon beta-1a in P. pastoris, achieving a yield of 25 mg per liter of culture.

- **Mammalian Cell Culture (CHO Cells)**

Mammalian cells represent the absolute gold standard for producing complex therapeutic proteins that require authentic post-translational modifications (PTMs), i.e., those closely resembling those observed in humans. Indeed, they possess the full cellular machinery required to ensure correct folding, precise assembly, and sophisticated PTMs, including appropriate glycosylation. Interferon produced in this system is thus practically identical to the native human protein.

This reliability explains why nearly 70% of all recombinant therapeutic proteins currently on the market are produced in CHO (Chinese Hamster Ovary) cells.

Furthermore, these cell lines demonstrate a strong productivity potential after engineering: for example, modified CHO cells have been able to constitutively produce up to 16 mg of purified IFN-β1 per liter. They can also be adapted for long-term, stable production in large-scale cultures.

However, this system has major drawbacks, related to its cost and technical complexity. It is indeed the most expensive and skill-intensive method: cultures require complex, serum-free media, exhibit slower growth than microorganisms, and remain particularly sensitive to contamination.

In terms of applications, this system is used to produce interferon beta-1a, a glycosylated form also approved for the treatment of multiple sclerosis. A concrete example: human interferon-gamma (IFN-γ) produced in CHO cells has a higher molecular weight than its counterpart derived from E. coli, confirming the successful occurrence of the glycosylation process (**Tab 7**).

Table 7. Comparison Production Methods

Feature	Bacterial Fermentation	Yeast Systems	Mammalian Cell Culture
Cost	Low	Medium	High
Speed	Fast	Moderate	Slow
Glycosylation	None	Yes (but may differ from human)	Yes (authentic human-like)
Protein Folding	Often misfolds (inclusion bodies)	Can misfold under high expression	Proper folding
Example Product	IFN-β1b (non-glycosylated)	IFN-α, IFN-β	IFN-β1a (glycosylated)

Each system represents a trade-off between cost, speed, and the biological complexity required for the final product. The choice depends on the specific interferon and its intended therapeutic application.

Applications

In veterinary medicine, recombinant animal interferons are primarily valued for their antiviral, antitumor, and immunomodulatory properties, which has driven their ongoing development against a wide spectrum of viral diseases across various species. Canine applications are specifically aimed at viral and oncological conditions, while bovine treatments are directed toward general viral infections.

In porcine medicine, these interferons are being formulated to tackle notable pathogens such as PRRSV, Seneca Valley virus, PEDV, and swine influenza, and for felines, they are being investigated to address a range of common viral diseases encountered in clinical practice.

II.3 Erythropoietin (EPO)

Erythropoietin (EPO) is a hormone naturally produced in our bodies, primarily by the kidneys. Its role is to stimulate red blood cells production by the bone marrow when the body lacks oxygen. This anti-anemic agent is produced on an industrial scale for use in human therapy in the treatment of anemia, chronic kidney failure, and cancer anemia.

3.1. Structure and biosynthesis

3.1.1. Structure

Erythropoietin (EPO) is a glycoprotein whose gene is located on human chromosome 7. The EPO gene encodes a precursor protein of 193 amino acids. During secretion, a hydrophobic signal peptide of 27 amino acids is cleaved. In addition, for a reason that is still unknown, there is a loss of a carboxy-terminal arginine by intracellular enzymes, giving the mature form of the hormone, composed of 165 amino acids. The theoretical molecular mass of this protein is approximately 18.4 kDa; however, due to its extensive glycosylation, its apparent molecular mass varies between 34 and 38 kDa (**Fig 14**)

EPO is a highly glycosylated glycoprotein, and this glycosylation is essential for its stability and biological activity. The molecule has three N-glycosylation sites located on asparagine residues 24, 38, and 83, as well as an O-glycosylation site at serine 126. Each of these four carbohydrate chains contains between two and four sialic acid molecules. The carbohydrate chains play a crucial role in the hormone's function: the removal of sialic acid residues leads to a loss of biological activity in vivo, while complete deglycosylation results in the disappearance of activity both in vitro and in vivo.

The three-dimensional structure of EPO is also stabilized by four cysteine residues that form two disulfide bridges (cys 7-161 and cys 29-33) (**Fig 14**). These are essential for maintaining the correct conformation of the protein and its biological activity.

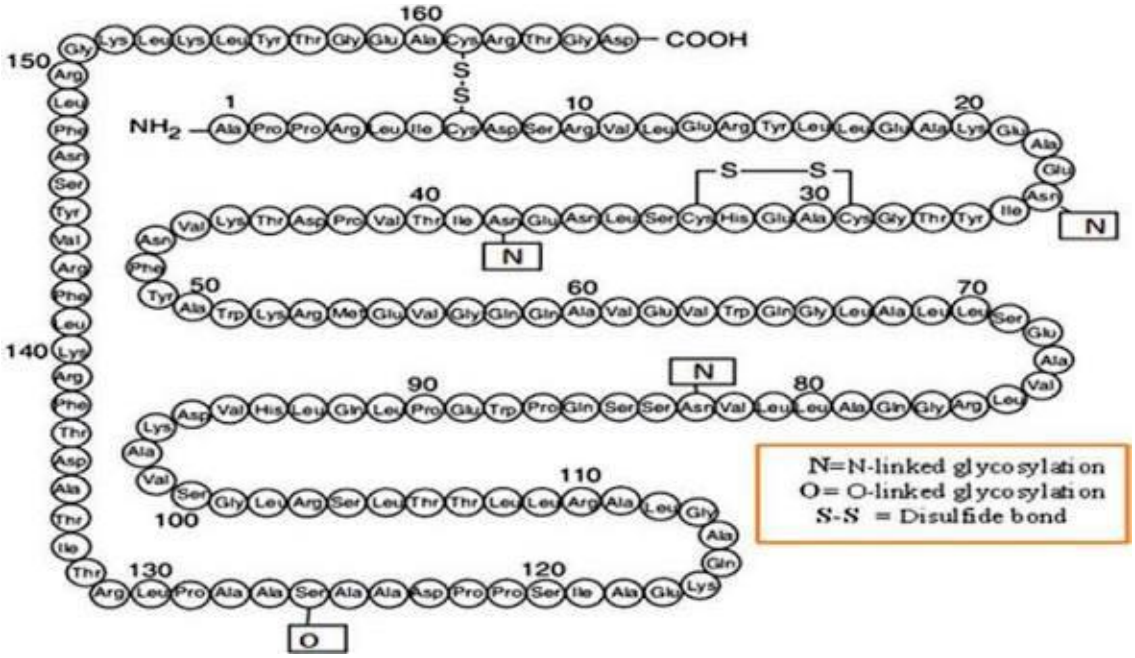

Fig 14. Primary structure of EPO hormone

3.1.2. Biosynthesis

Erythropoietin (EPO) is a glycoprotein primarily synthesized by the peritubular interstitial cells of the renal cortex (<10% in the liver) in adults. However, during fetal development, the liver is the main site of production of this hormone.

EPO biosynthesis is linked to oxygen availability; under hypoxic conditions, the transcription factor HIF-1 (Hypoxia-Induced Factor 1) is activated and binds to a specific regulatory sequence on the EPO gene, thus stimulating its transcription. The resulting messenger RNA is then exported to the cytoplasm where it is translated into a polypeptide chain. This chain is then targeted to the rough endoplasmic reticulum and subsequently to the Golgi apparatus, where it undergoes several post-translational modifications, including glycosylation, which is essential for its stability, solubility, and biological activity.

Once mature, erythropoietin is immediately secreted into the bloodstream, without prior intracellular storage.Then it acts on erythroid precursors in the bone marrow, stimulating their proliferation, differentiation, and survival, which leads to an increase in red blood cell production.

3.2. Physiological role of erythropoietin

Erythropoietin (EPO) is a glycoprotein cytokine that plays a crucial role in regulating erythropoiesis. It acts primarily on erythroid progenitors in the bone marrow, particularly CFU-E (Colony Forming Unit-Erythroid) cells and proerythroblasts. It stimulates their proliferation, promotes their differentiation, and increases their survival by inhibiting apoptosis. This action leads to an increase in the number of circulating erythrocytes, hemoglobin concentration, and hematocrit.

So, EPO is the main hormonal factor enabling erythropoiesis to adapt to the body's metabolic needs. It plays a particularly important role in hypoxic situations such as anemia, hemorrhage, respiratory illnesses (**Fig 15**).

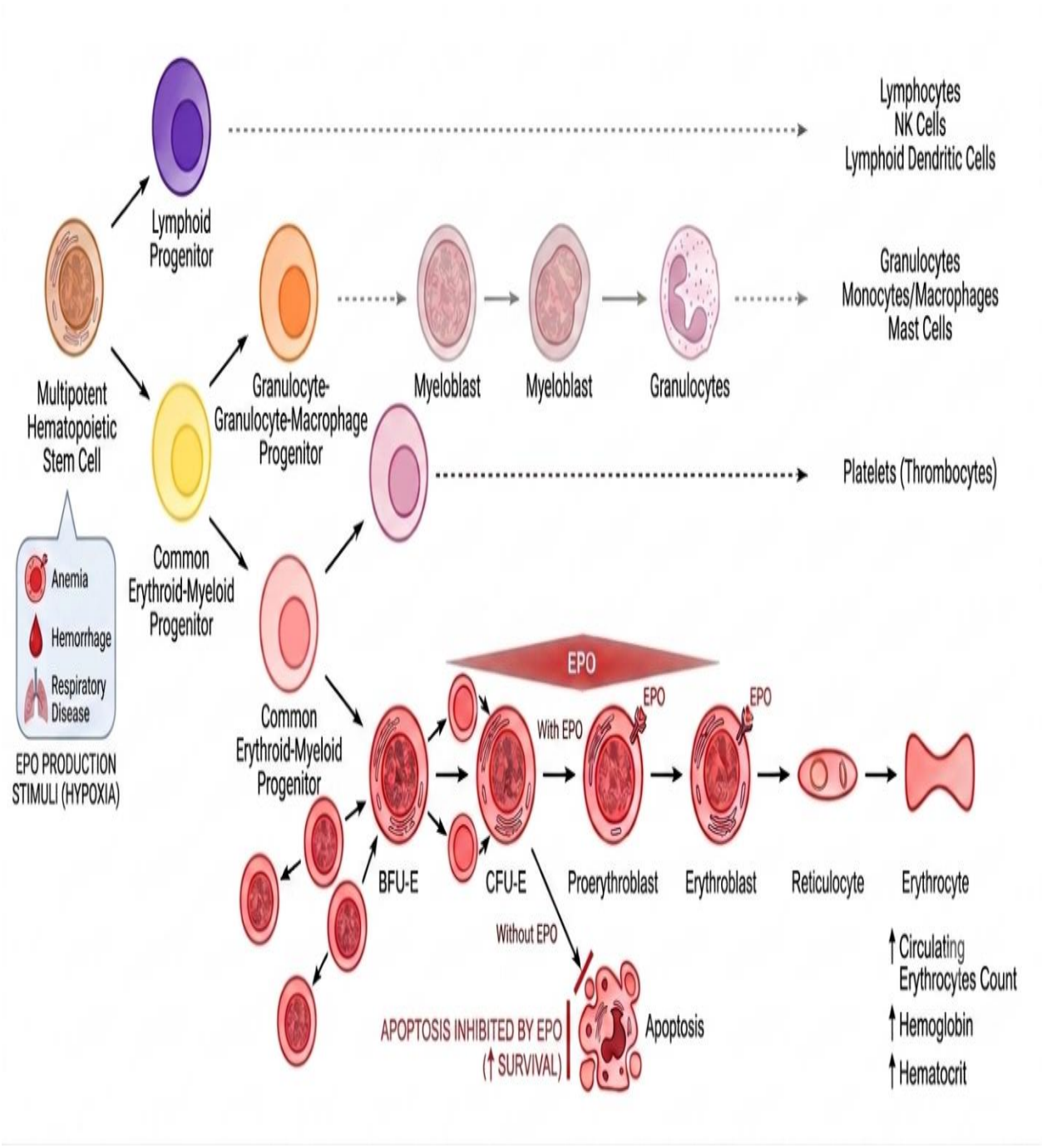

Fig 15. Erythropoiesis and the Physiological Role of Erythropoietin (EPO)

3.3. Erythropoietin receptors and mechanism of action

The biological action of EPO is mediated by a specific membrane receptor called EPOR (Erythropoietin Receptor), belonging to the superfamily of type I cytokine receptors. This receptor is mainly expressed on the surface of precursor cells of the erythroid lineage.

The binding of EPO to its receptor induces dimerization of the receptor and activates the tyrosine kinase JAK2 (Janus Kinase 2) associated with the receptor's intracellular domain. JAK2 phosphorylates several tyrosine residues of the receptor, thereby creating binding sites

for various signaling proteins.

This activation triggers several intracellular pathways:

a. JAK2/STAT5 pathway

Phosphorylation of STAT5 leads to its dimerization and then its translocation into the nucleus where it activates the transcription of genes involved in the proliferation, differentiation and survival of erythroid precursors (**Fig 16**).

b. PI3K/Akt pathway

Activation of phosphatidylinositol-3-kinase (PI3K) leads to the activation of the Akt protein, which exerts an anti-apoptotic effect by promoting the survival of erythroid cells.

c. Ras/MAPK pathway

This pathway contributes to cell proliferation and differentiation by activating several transcription factors involved in erythropoiesis.

All of these mechanisms result in increased red blood cell production and the maintenance of oxygen homeostasis.

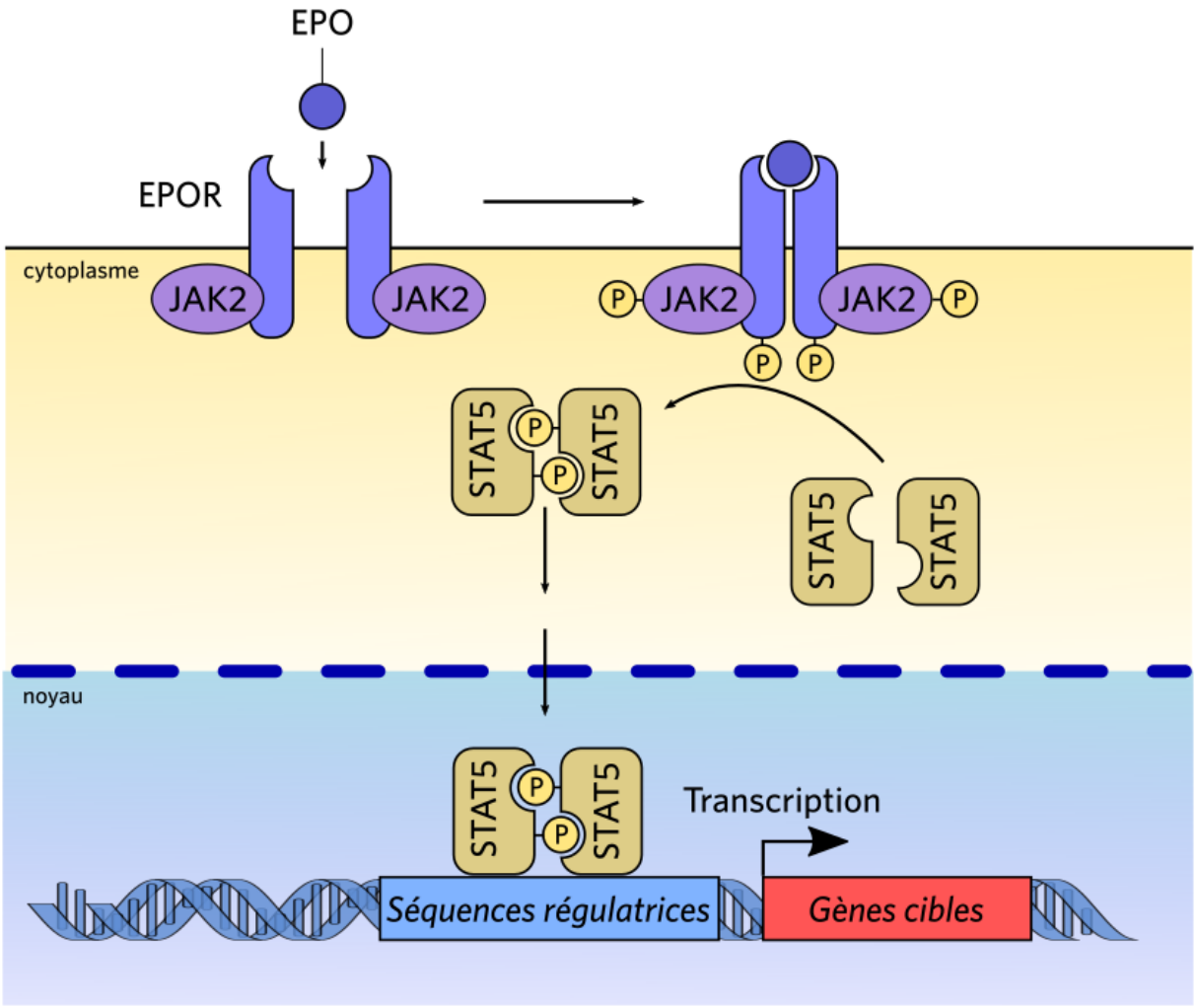

Fig 16. Simplified diagram of signal transduction induced by EPO binding to its receptor explaining JAK2/STAT5 pathway.

3.4. Regulation of erythropoietin production

EPO synthesis is primarily regulated by the availability of oxygen at the tissue level; hypoxia is the main stimulus for its expression.

Under normoxic conditions, the HIF-1α (Hypoxia Inducible Factor-1α) subunit is hydroxylated and then rapidly degraded by the proteasome. Under these conditions, EPO gene expression remains low.

However, Under hypoxic conditions, HIF-1α is stabilized and accumulates in the cytoplasm. Then it associates with the ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) protein, or HIF-1β, to form the HIF-1 complex. This complex migrates to the nucleus and binds to hypoxia response elements (HREs) present in the regulatory regions of the EPO gene, thereby activating its transcription.

Increased EPO synthesis stimulates bone marrow erythropoiesis, leading to an increase

in erythrocyte numbers and improved tissue oxygenation. When oxygen supply returns to normal, HIF-1α is degraded again and EPO production decreases via a negative feedback mechanism.

Furthermore, the regulation of erythropoiesis also involves the Fas/Fas-Ligand system, which exerts negative control over erythroid precursors in order to limit their excessive proliferation and maintain the balance of blood cell production (**Fig 16**).

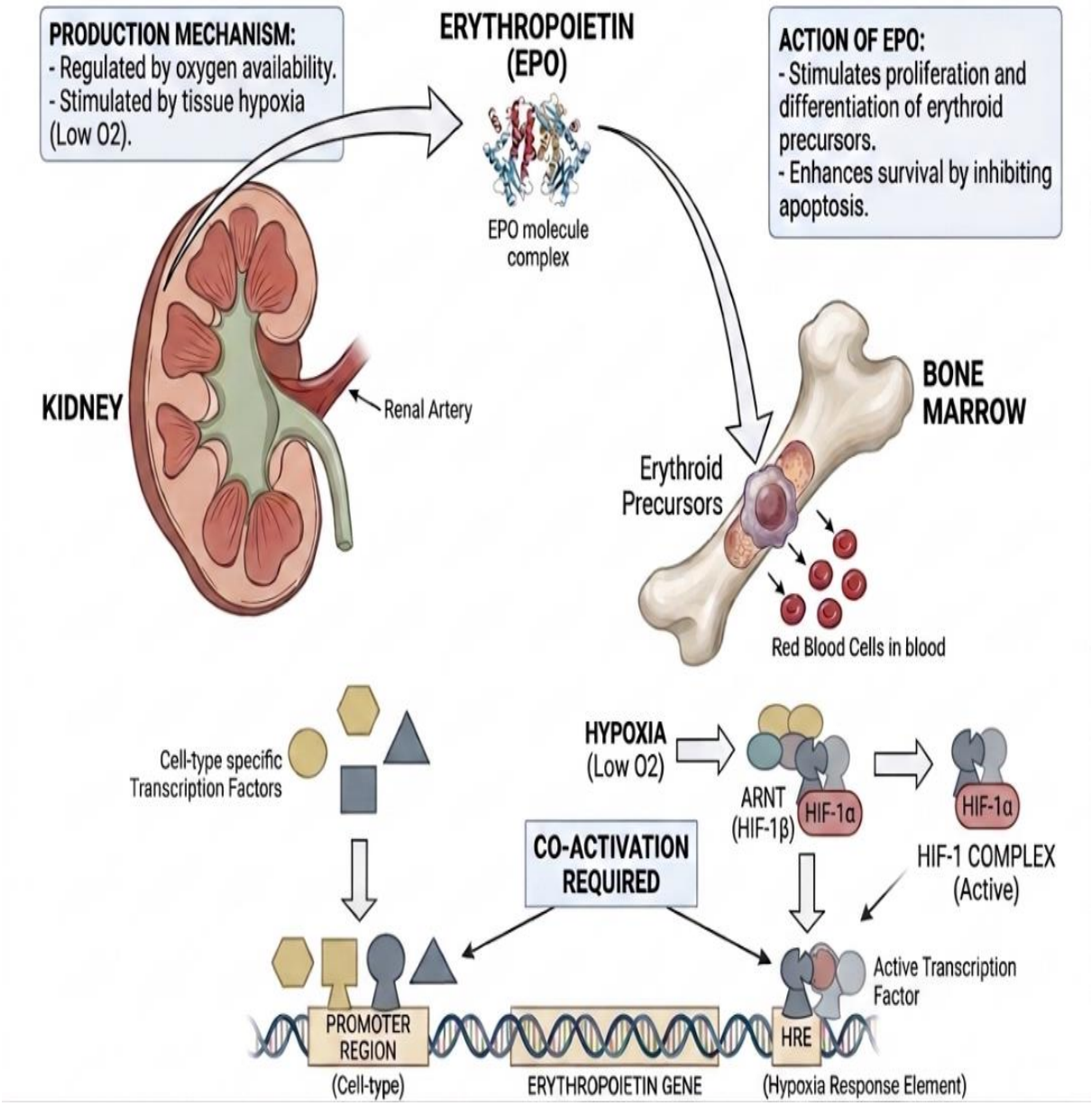

Fig 17. Overview of Erythropoietin (EPO) Production Regulation and Its Mechanism of Action

3.5. EPO Production

As previously explained, erythropoietin (EPO) is an essential glycoprotein hormone that regulates red blood cell production in the body. Due to its structural complexity and therapeutic importance, its industrial production has necessitated the development of new manufacturing technologies.

Production history

Before 100 years, French scientists P. Bert and D. Jourdan first described the direct relationship between the stimulation of red blood cell production and the decrease in atmospheric oxygen pressure. This intuition was supported in 1906 by Carnot and Deflandre, who suggested that this mechanism regulating erythropoiesis was hormonal in nature.

In 1950 Reissmann experimentally demonstrated it through cross-circulation experiments in animals, then in 1957 Jacobson and his collaborators formally identified the kidney as the main endocrine organ responsible for its synthesis.

In 1977, E. Goldwasser and T. Miyake succeeded in completely isolating and purifying natural human EPO. However, this initial production remained extremely limited, as it depended exclusively on extraction from massive volumes of urine from patients with severe aplastic anemia.

The real industrial and therapeutic turning point came in December 1983, when the team at the biotechnology company Amgen, under the leadership of Fu-Kuen Lin, successfully cloned the human EPO gene. This technological success paved the way, as early as 1985, for the large-scale expression of the gene in mammalian cells, leading to the first marketing authorizations for recombinant EPO in 1988 and 1989.

3.6. Production of Human Recombinant EPO

EPO is a highly complex glycoprotein. This structural complexity makes its pure chemical synthesis impossible; therefore, recombinant DNA technology has been used for its production

The industrial production of recombinant EPO begins with the cloning and construction of the expression vector, where the gene encoding human EPO is isolated and then inserted into a plasmid. This vector is then introduced into a selected cell expression system (Chinese hamster ovary (CHO) cells). At this stage, the bacteria such as E. coli is not used because they are unable to perform the glycosylation essential for the hormone's activity in vivo. In contrast, mammalian cells, primarily Chinese hamster ovary (CHO) cells, possess the enzymatic machinery required to add carbohydrate chains similar to those of natural human EPO.

Once the cell line is validated, the culture (or fermentation) step is initiated on a large scale within industrial bioreactors, where environmental parameters such as pH, temperature and oxygenation are optimized to maximize the secretion of the glycoprotein into the medium

Finally, the process concludes with the purification phase, during which the culture supernatant is harvested and subjected to chromatography techniques to isolate EPO from any debris or host protein and achieve a degree of pharmaceutical purity greater than 99%.

3.7. Types of human recombinant EPO

a. epoetins-α: (Ex: Eprex, Erythropoietin α). This is the historically most widely produced and used form. It is generally administered several times a week due to its short half-life.

b. epoetins-β: (Ex: NeoRecormon). Also produced on CHO cells, its carbohydrate structure varies only slightly compared to the alpha form, offering very similar pharmacokinetic properties.

c. darbepoetin -α

In this form, biotechnology engineers modified the DNA sequence of EPO by replacing 5 amino acids: alanine at 30 and tryptophan at 88 were replaced by asparagine; histidine at 32 and proline at 90 were replaced by threonine and proline at 87 by valine, thus creating two new N-glycosylation sites (5 sites in total instead of 3).

3.8. Recombinant Erythropoietin

Recombinant human erythropoietin (rHuEPO) is a man-made version of a natural hormone called erythropoietin (EPO), which is primarily produced by the kidneys. It is a **glycoprotein** (a protein with sugar molecules attached) that is manufactured using **recombinant DNA technology**. This involves inserting the human gene for erythropoietin into mammalian cells, which then produce the protein. The resulting medication, often known by the generic name **epoetin alfa**, is biologically identical to the natural EPO produced by the body. It is a 165-amino acid protein with a molecular weight of about 30,400 daltons.

Erythropoietin is the principal regulator of red blood cell production (erythropoiesis). Recombinant EPO works by binding to specific receptors on the surface of red blood cell precursors in the bone marrow.

This binding triggers a series of intracellular signals that lead to:

- **Proliferation** and **differentiation** of these precursor cells into mature red blood cells.
- **Protection** of these cells from apoptosis (programmed cell death).

Essentially, it stimulates the bone marrow to produce more red blood cells, thereby increasing the oxygen-carrying capacity of the blood.

Recombinant erythropoietin is primarily used to treat **anemia** (a low red blood cell count). Its main indications include:

- **Anemia due to chronic kidney disease** (in patients on dialysis and those not on dialysis).
- **Anemia caused by chemotherapy** in cancer patients.
- **Reducing the need for red blood cell transfusions** in certain surgical patients.

Like all medications, recombinant EPO can cause side effects. Common side effects may include:

- Nausea, vomiting, and diarrhea
- Headache and fever
- Joint, bone, or muscle pain
- High blood pressure (hypertension)
- Rash or allergic reactions

Recombinant EPO carries several **serious black box warnings** from the FDA due to significant risks:

- **Increased Risk of Death, Heart Attack, Stroke, and Blood Clots:** Especially when used to target high hemoglobin levels (e.g., above 13 g/dL).
- **Increased Risk of Tumor Progression or Recurrence:** In some cancer patients, it may speed up tumor growth or shorten survival time.
- **Pure Red Cell Aplasia (PRCA):** A rare but serious condition where the body stops producing red blood cells, which can occur after using EPO.

3.9. Recombinant Erythropoietin steps process.

1. Gene Cloning & Cell Line Engineering: The Foundation

This initial stage involves creating the "factory" that will produce the protein.

- **Gene Isolation and Vector Construction**:

The first step is to isolate the specific human gene that codes for erythropoietin. This gene is then inserted into a circular DNA molecule called a **plasmid**, which acts as a delivery vehicle (or "vector"). The vector also contains a **selectable marker gene** (e.g., dihydrofolate reductase or DHFR), which allows scientists to identify which cells have successfully taken up the new DNA.

- **Transfection**:

The engineered plasmid is introduced into the host cells. For EPO, the gold-standard host is the **Chinese Hamster Ovary (CHO)** cell line. CHO cells are favored because they are robust, grow well in large quantities, and can perform the complex, human-like glycosylation that is essential for EPO's biological activity.

- **Gene Amplification**:

To achieve high production yields, the number of EPO gene copies within the CHO cells must be increased. This is done using a selective agent like **methotrexate (MTX)**. Cells with more copies of the DHFR gene can survive in higher concentrations of MTX. Because the EPO gene is located next to the DHFR gene on the plasmid, it gets "amplified" along with it, leading to cells with many copies of the EPO gene and, consequently, much higher protein production.

- **Stable Cell Line Generation**:

The transfection and amplification process results in a mixed population of cells. The most productive and stable cells are selected, isolated, and grown to create a **stable clonal cell line**. This ensures a consistent and reliable "master cell bank" for all future production batches.

2. Large-Scale Bioreactor Cultivation: The Production Phase

Once a stable cell line is established, it must be grown on a massive scale to produce the protein.

- **Culture Medium**:

The cells are cultivated in a precisely defined, nutrient-rich liquid medium. To ensure product safety and consistency, modern processes often use **serum-free media** that lack any components derived from animals. The media are carefully formulated with amino acids, carbohydrates (like glucose), vitamins, trace elements, and other supplements to support cell growth and protein production.

- **Fed-Batch Bioreactor**:

The production typically takes place in large **stirred-tank bioreactors**, which can hold thousands of liters. The most common strategy is **fed-batch culture**. In this process:

1. Cells are inoculated into the bioreactor with an initial batch of medium.

2. As the cells grow and consume nutrients, concentrated feeds (containing amino acids, carbohydrates, and other nutrients) are added periodically to extend the production phase and maximize the final yield of EPO.

3. The process is "discontinuous," meaning it is run in batches.

- **Critical Process Parameters (CPPs)**:

The environment inside the bioreactor is tightly controlled to optimize cell health and protein production. Key parameters include:

- **Temperature**: Typically maintained at 37°C.
- **pH**: Often controlled via a **pH-shift strategy**, starting at 7.1 and then reducing to 6.9 later in the process to boost EPO concentration.
- **Dissolved Oxygen (pO_2)**: Maintained at a specific level (e.g., 50% air saturation) to ensure the cells have enough oxygen for respiration.
- **Glucose Concentration**: Monitored and kept within a target range (e.g., 3-4 g/L) to prevent nutrient starvation.

3. Downstream Purification: Isolating and Refining the Product

After the fermentation is complete, the EPO must be separated from the billions of cells and thousands of other proteins in the broth. This is a complex, multi-step process that can account for a significant portion of the production cost.

- **Harvest and Clarification**:

The first step is to remove the cells and cellular debris from the liquid containing the EPO. This is done through a series of physical separation methods:

- **Centrifugation**: The harvest broth is spun in a **disc stack separator** to sediment the larger cells and particles.
- **Depth Filtration**: The centrifuged liquid is then passed through a series of filters (e.g., depth filters) to remove any remaining fine particles and clarify the solution.

- **Chromatographic Purification**:

This is the core of the purification process, using a series of different chromatography columns to isolate the EPO based on its various chemical properties. A typical purification train can involve anywhere from **3 to 7 chromatographic steps** and is organized into three stages:

- **Capture**: The first step concentrates the EPO and removes the bulk of the impurities. An **anion-exchange chromatography** step is often used here because EPO is an acidic protein that binds well to positively charged resins. This step can significantly increase the purity of the product.

- **Intermediate Purification**: This stage removes remaining major impurities like other proteins and DNA. Techniques like **hydrophobic interaction chromatography (HIC)** or **multimodal chromatography** are commonly employed.

- **Polishing**: The final chromatography steps are designed to remove the last trace amounts of impurities and ensure the product is of the highest purity. This may involve another **ion-exchange** step, **size-exclusion chromatography**, or **hydroxyapatite chromatography**. The goal is to achieve a purity of over **98-99%**.

4. Analytical Techniques for Quality Control

Because EPO is a heterogeneous mixture of different **glycoforms** (isoforms with different sugar structures), rigorous quality control is essential to ensure each batch is safe, potent, and consistent. This is achieved through a suite of advanced analytical techniques.

- **Glycoprofiling (Analyzing the Sugar Structures)**: The pattern of glycosylation is critical for EPO's function and half-life. To analyze this, scientists use high-resolution separation methods:

  - **High-Performance Liquid Chromatography (HPLC)**: This technique can separate different EPO glycoforms based on their interactions with a column, providing a "fingerprint" of the glycan profile.

  - **Capillary Electrophoresis (CE)**: This method separates molecules based on their charge and size in a fine capillary tube. It is highly effective at resolving the different sialylated isoforms (sialoforms) of EPO. Often, CE is coupled with **Mass Spectrometry (MS)** to get precise molecular weight and structural information.

- **Bioassay (Testing Biological Activity)**: Chemical analysis alone is not enough. The final product must be proven to be biologically active. This is done using a **cell-based bioassay**. The purified EPO is added to a cell line that is dependent on EPO for growth (e.g., the TF-1 cell line). The amount of cell proliferation directly correlates with the potency of the EPO, confirming that the protein is functional.

III. Challenges and Future Directions

Despite their advantages, animal cell systems have drawbacks, including higher production costs, slower growth rates, and more complex culture media compared to bacterial systems. Research continues to focus on:

- **Improving Yields**: Through cell line engineering, media optimization, and novel bioreactor designs.
- **Alternative Animal Systems**: Exploring insect cell-baculovirus systems and even insect larvae for potentially more cost-effective production.
- **Process Intensification**: Developing continuous perfusion processes for longer, more stable production runs.

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