Protein Drug Discovery & Development

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What is a biological?

What different types of biologicals?

Examples of protein drugs

- Protein design (improvement)

Antibodies as drugs

- Biology
- Technologies
- (Production and purification)

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Biologic = biological drug = biopharmaceutical =

A drug that is produced by/from living organisms or contains components of living organisms

Is this a good definition?

Think about the difference between *synthetic* and *natural* vitamins!

Although biologics are the most rapidly growing drug class, they are not new, but are among the oldest, truly efficacious medical interventions.

1st vaccination performed by Edward Jenner (1796)

Vaccines

- Live virus
- Killed virus
- Recombinant vaccines
- DNA/RNA vaccines
- Tolerogens

Gene therapy

- Viruses
- DNA
- RNA

Cell therapy

- Stemm cells
- CAR-T

Proteins

- Antibodies: polyclonal (“antisera”) & monoclonal
- Protein hormones, growth factors & cytokines
- Enzymes
- Protein toxins

Transplants

- cell, organ & tissue transplants (bone marrow, blood, blood products
- fecal transplant
- xenotransplants
- biomaterials

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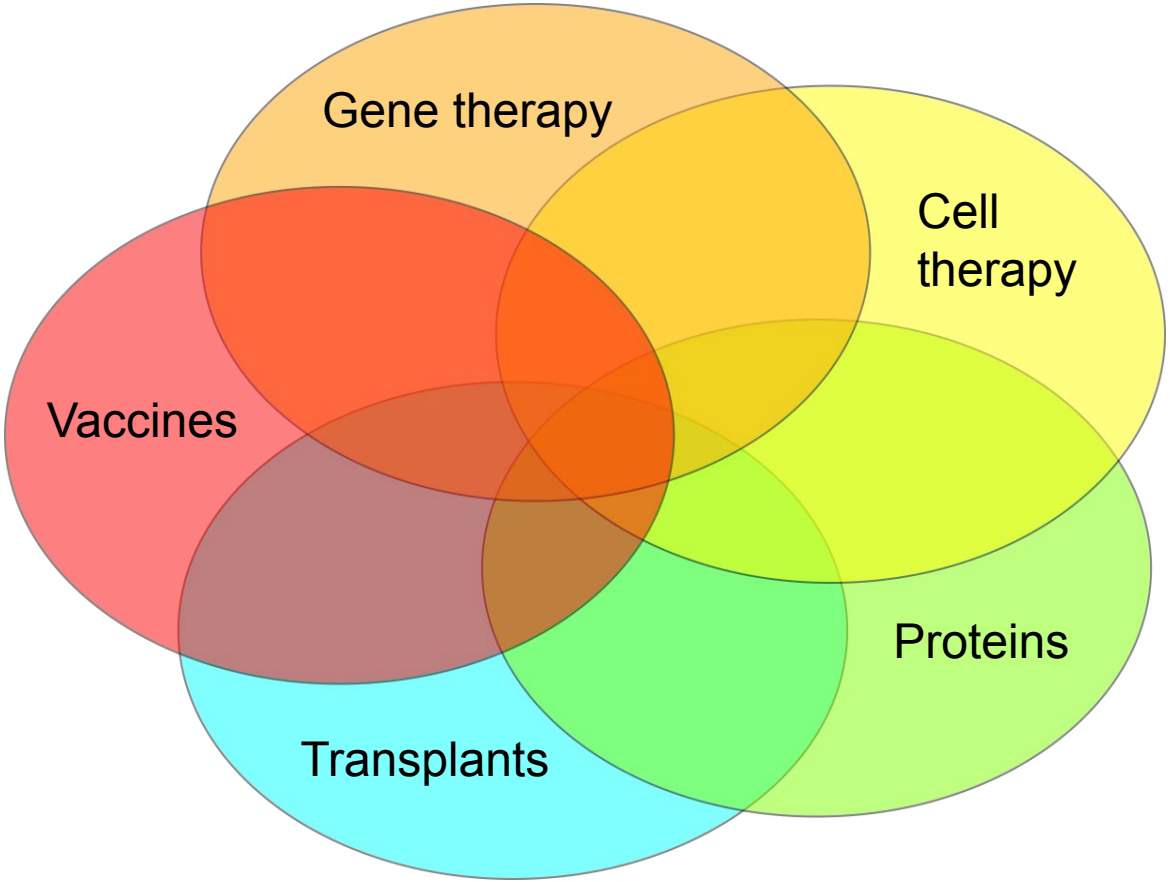
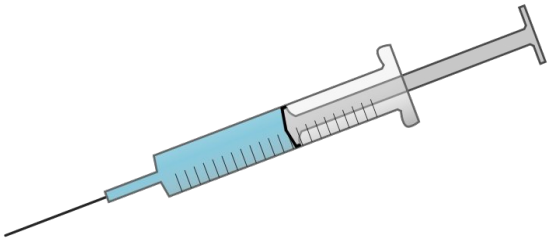
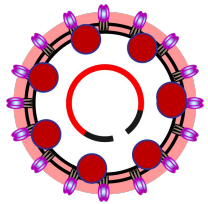




Any unifying theme?
Almost all biologics need to be “injected” with the exception of viruses.



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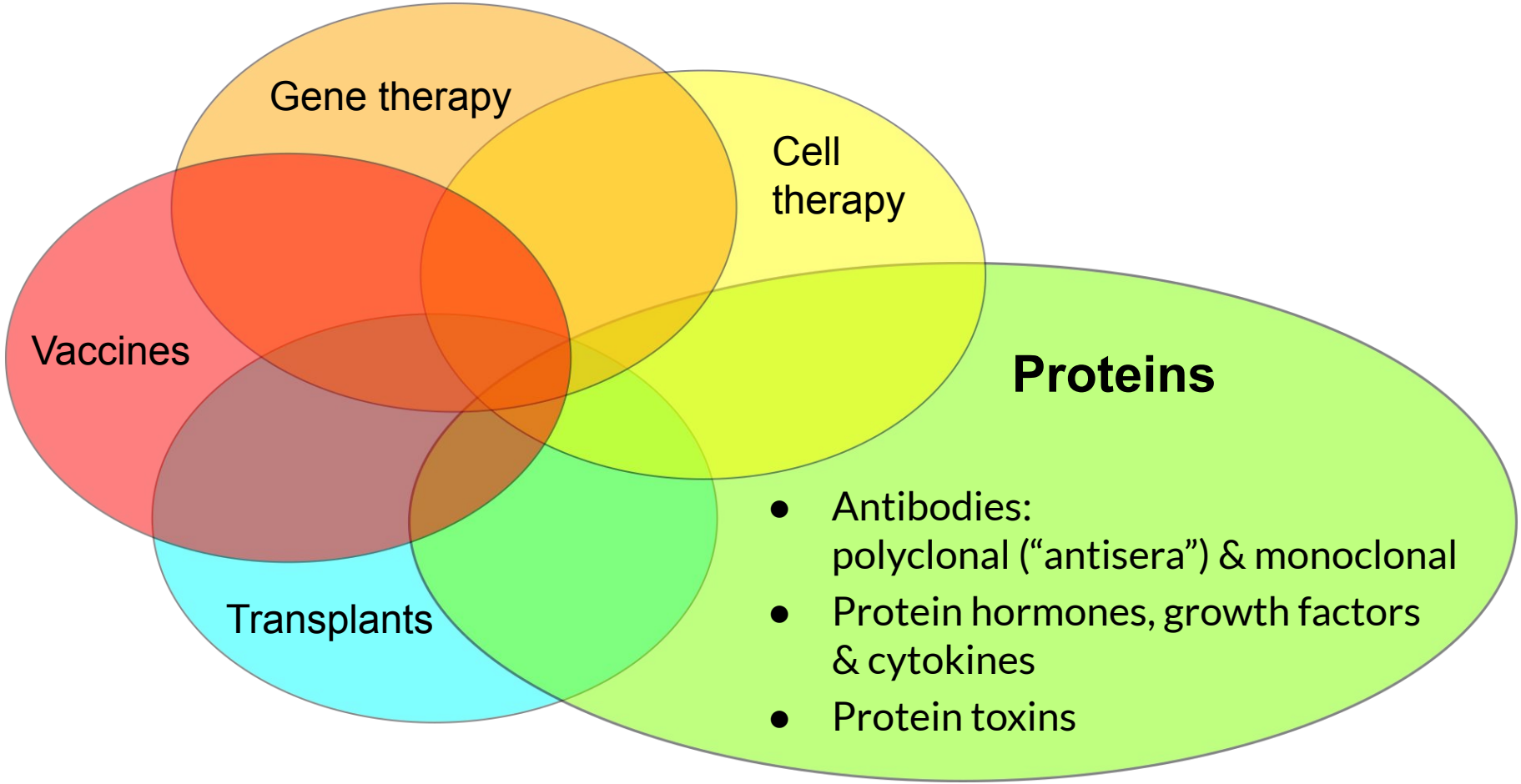

The three major protein drug classes

- **Growth factors/cytokines/protein hormones (mostly human proteins)**
  - *Darbepoetin alfa*, *erythropoietin/"epo"*, *Aranesp®*: stimulate red blood cell production in the bone marrow
  - *Insulin (Hypurin®*, *Humulin®*, *Novolin®*, *Fiasp®*, *etc.)*: hormone replacement therapy for diabetics
- **Toxins (bacterial, animal, plant, etc. proteins)**
  - *Ziconotide*, *ω-MVIIA*, *Prialt®*: pain killer
  - *Botulinum toxin*, *OnabotulinumtoxinA*, *BOTOX®*: muscle relaxant

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- **Antibodies (Abs)**
  - *Tetanus antitoxin (anti-tetanus toxin polyclonal Ab, "antiserum")*: passive immunization/post exposure vaccination
  - *Avastin™*, *bevacizumab (anti-VEGF-A monoclonal Ab)*: cancer drug

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- Purification
- Modification (limited)

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- Generation
- Screening

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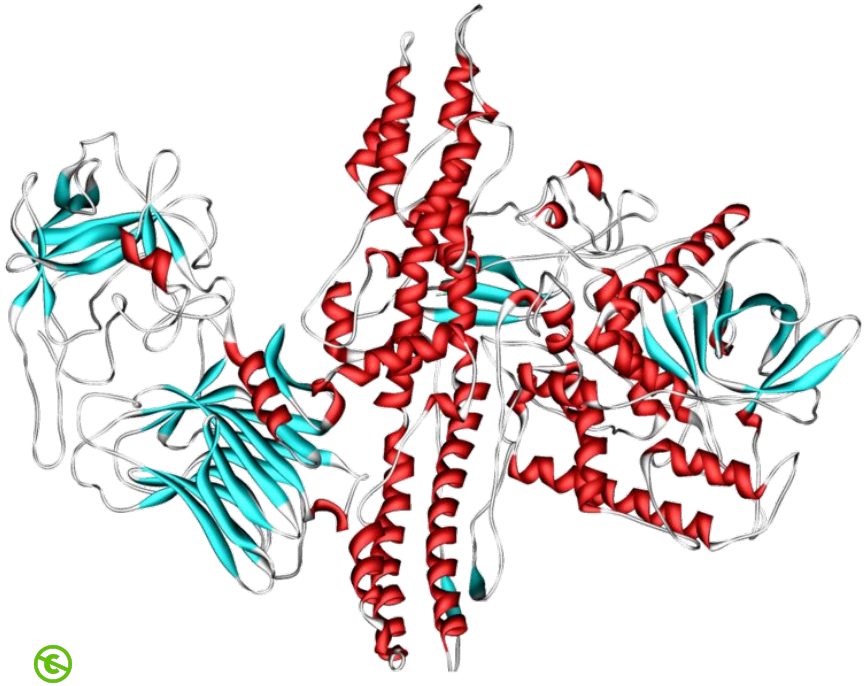
- (Most) early protein drugs:
  1. Drug discovery → 2. Mechanistic understanding
- (Most) current protein drugs:
  1. Mechanistic understanding → 2. Drug discovery
- Target identification and validation for protein drugs does not really differ much from other drug types
- drug library => look for effects
  drug target => look for drug
- For most protein drugs, the drug target is either:
  - itself the drug (= replacement therapy, e.g. insulin) or
  - the antigen of an antibody (e.g. many recent cancer drugs)

- *Justinus Kerner*, "inventor of BOTOX"
- After self experimentation, the German poet was in 1820 the first to suggest that botulinum toxin ("sausage poison") could be used therapeutically to block the "sympathetic nervous system"
- This proposal became reality 150 years later, when various muscle spasms were successfully treated with local botulinum toxin injections.

https://doi.org/10.1212/WNL.53.8.1850

Botox® (OnabotulinumtoxinA)



Botulinum toxins (BTs)

- A group of neurotoxic proteins produced by some species in the bacterial genus *Clostridium*.
- Size: ~150 kDa
- Botulinum toxin type A is the most lethal, naturally occurring toxin known to man.

https://doi.org/10.1016%2Fj.coph.2004.12.006

- *Indications:* different spasms, chronic migraine, strabismus (“crossed eyes”)
- *Available in Finland:* yes
- *Company:* Allergan, Inc (US) →
- *Interesting:* Used as a bioweapon
- *Market introduction:* late 1970s

Judah Folkman (1933 – 2008)

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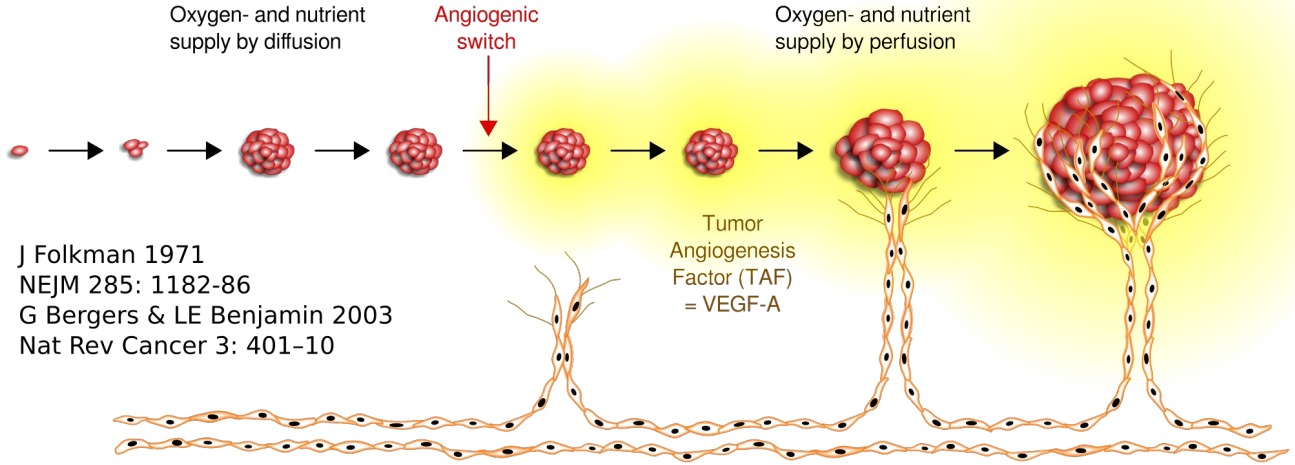
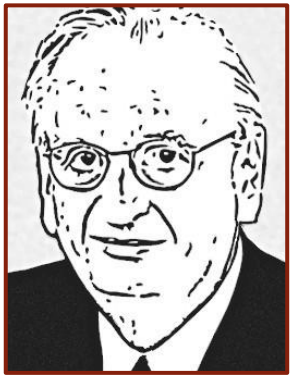
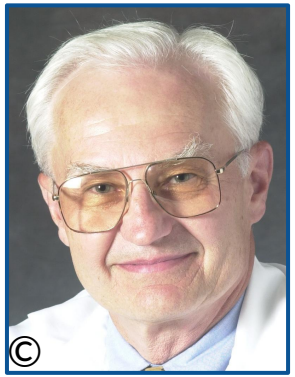


J Folkman 1971
NEJM 285: 1182-86
G Bergers & LE Benjamin 2003
Nat Rev Cancer 3: 401–10

1971 Judah Folkman proposes the concept of antiangiogenic tumor therapy



1983 Harold Dvorak isolates Vascular Endothelial Growth Factor (VEGF)

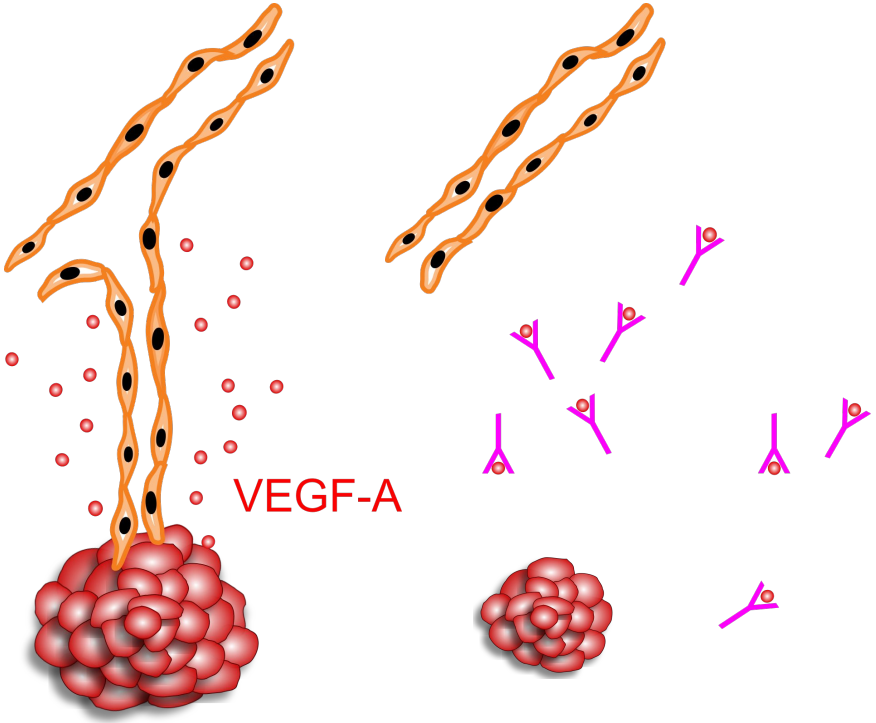


1992 Napoleone Ferrara generates neutralizing mouse antibodies against VEGF

1997 Clinical trials start with the humanized anti-VEGF antibody (“bevacizumab”)

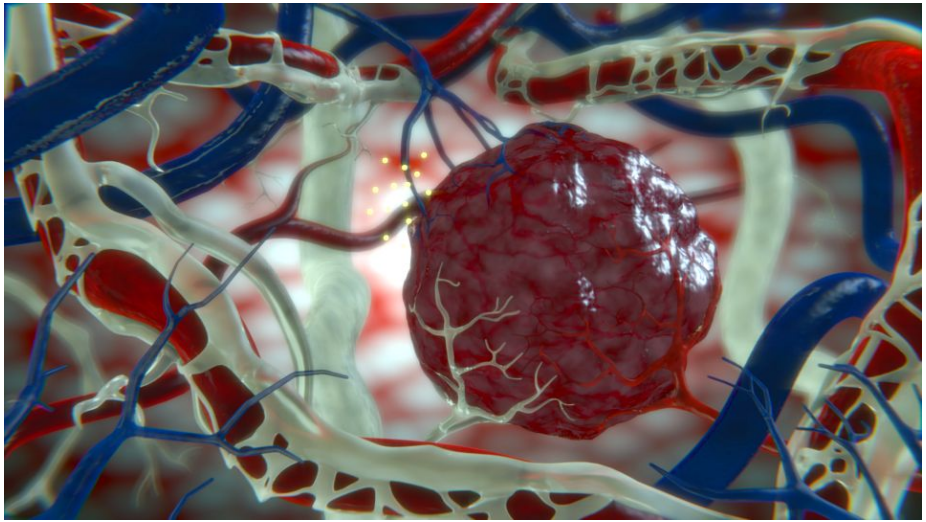
2004 Bevacizumab receives FDA approval for treatment of colon cancer

Avastin® (Bevacizumab)



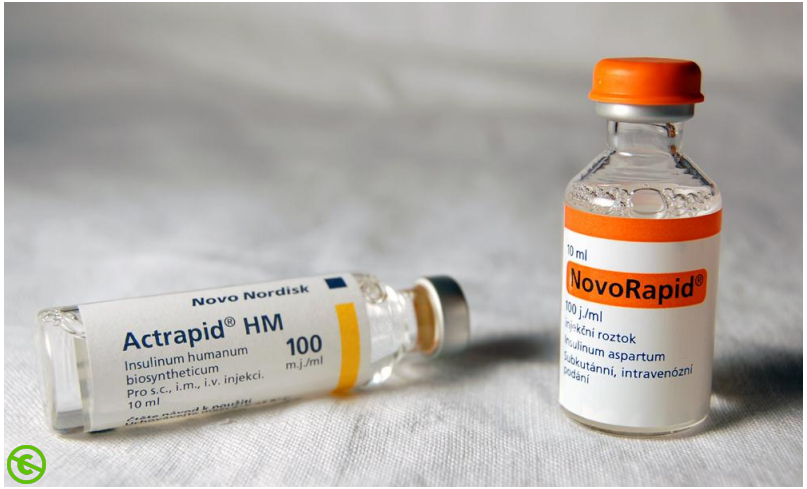
- Humanized mouse monoclonal antibody
- Suppresses the growth of blood vessels (“anti-angiogenic”)
- Hypothesis: Tumors need blood vessels to grow big

https://doi.org/10.1016/j.bbrc.2005.05.132



- *Indications:* different cancers (colorectal, lung)
- *Available in Finland:* yes
- *Company:* Genentech (US) →
- *Interesting:* This drug was predicted in 1971 by Judah Folkman
- Market introduction: 2004

Insulin



- First human successful treatment in 1922 with bovine (= cow) insulin (team Banting, Best & Collip)*
- Size: ~5.8 kDa
- Since 1982: human insulin (recombinant human insulin produced in *E. coli* bacteria)

https://doi.org/10.3389/fendo.2018.00613

*The fact that pancreatic insulin-containing extracts were able to treat diabetes had been discovered three times independently before (by George Ludwig Zuelzer in 1906, Israel Kleiner in 1915, Nicolae Paulescu in 1916).

- Protein purification from “natural” sources: “pig/cow insulin”, BOTOX
- Recombinant protein production, requires recombinant DNA technology (“genetic engineering”)

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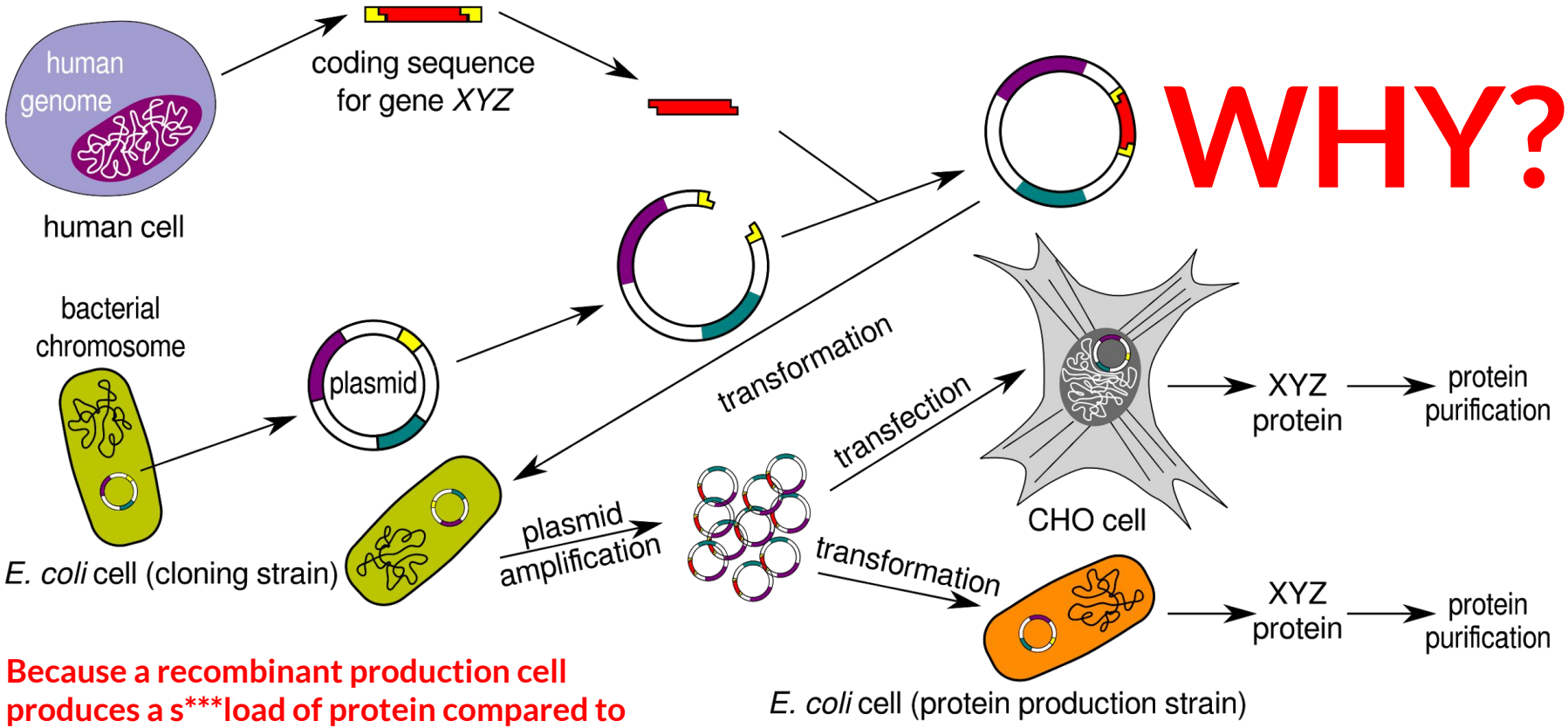

Because a recombinant production cell produces a s*load of protein compared to what can be found in natural sources**

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Growth factors/cytokines/protein hormones/toxins

- Protein purification from “natural” sources (“pig/cow insulin”, BOTOX)
- Improvements by limited modification

Antibodies (Abs)

- Generation
- Screening

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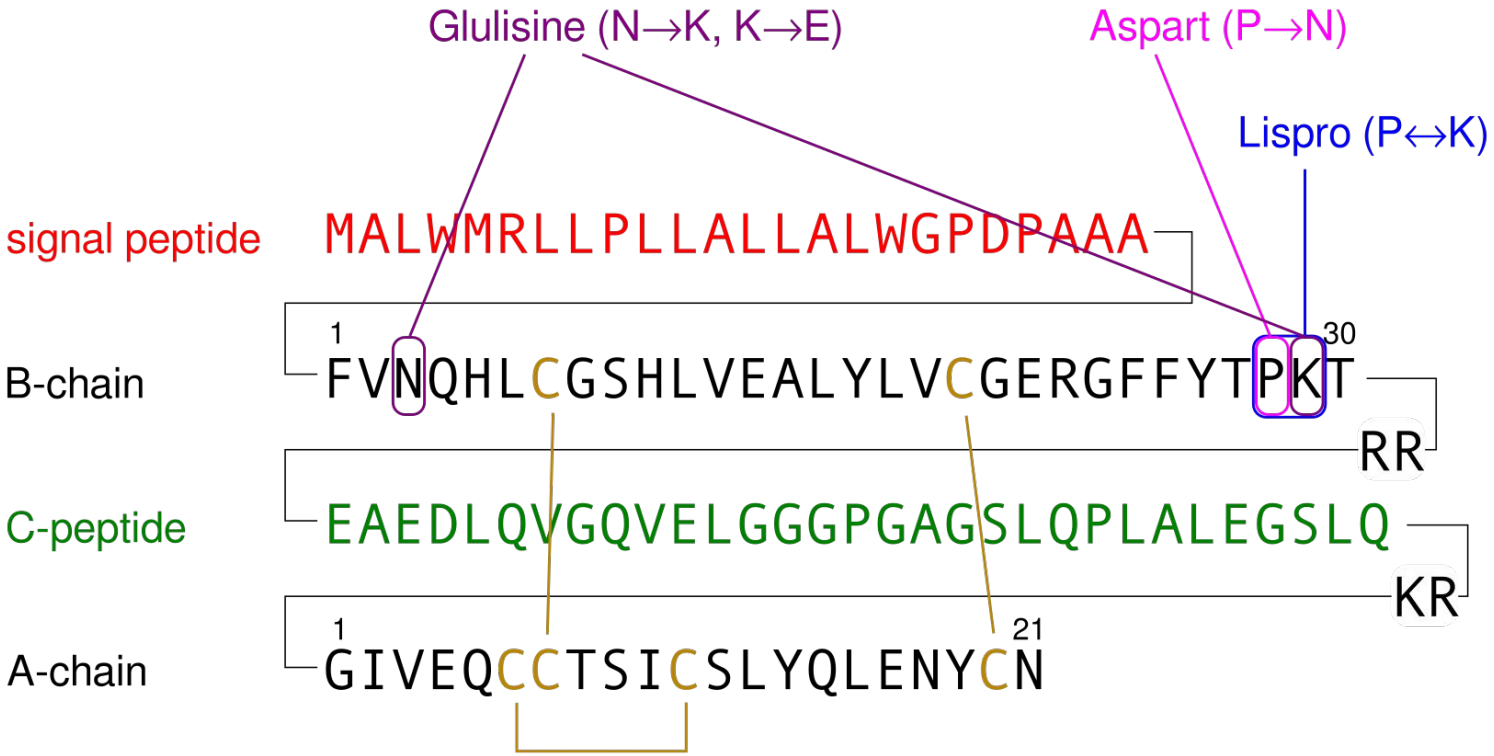

How are intermediate & long-acting insulins made?

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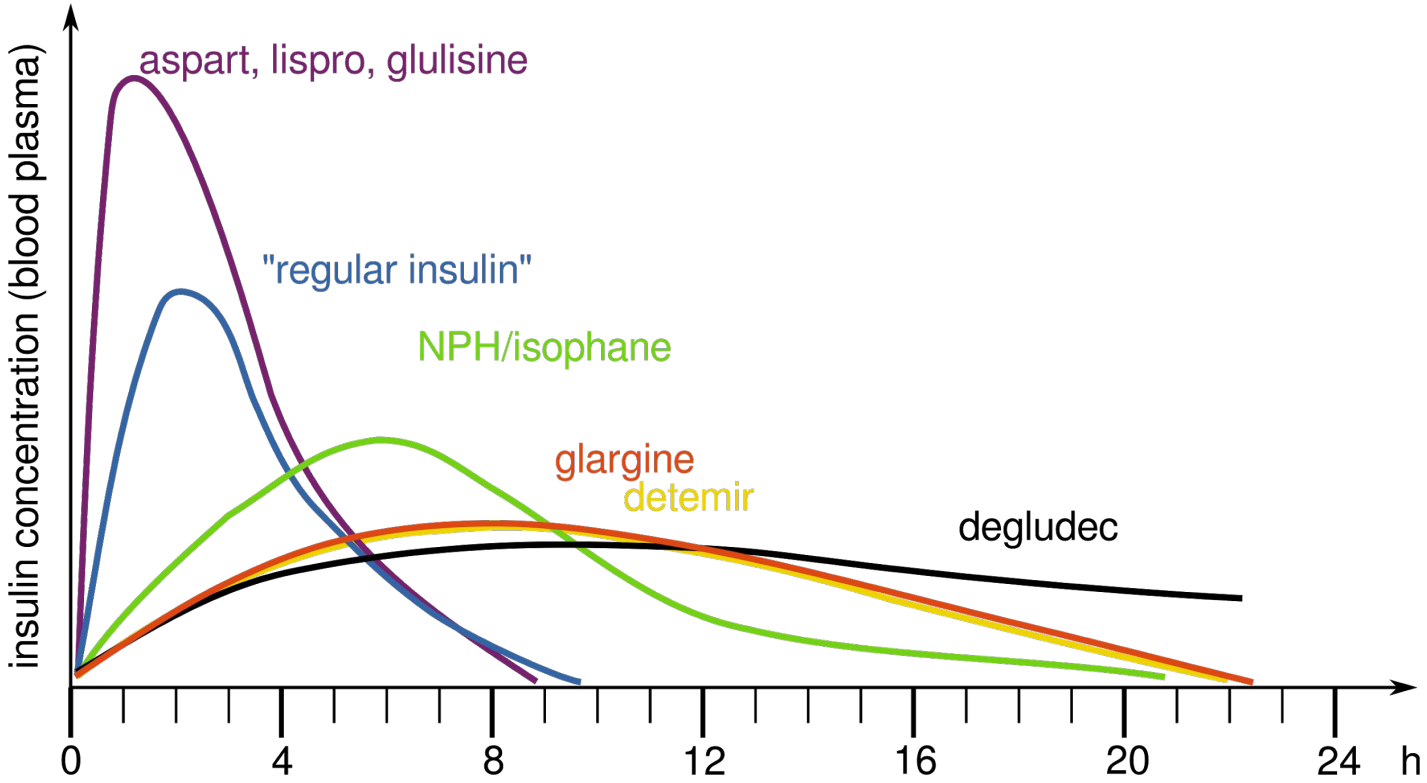

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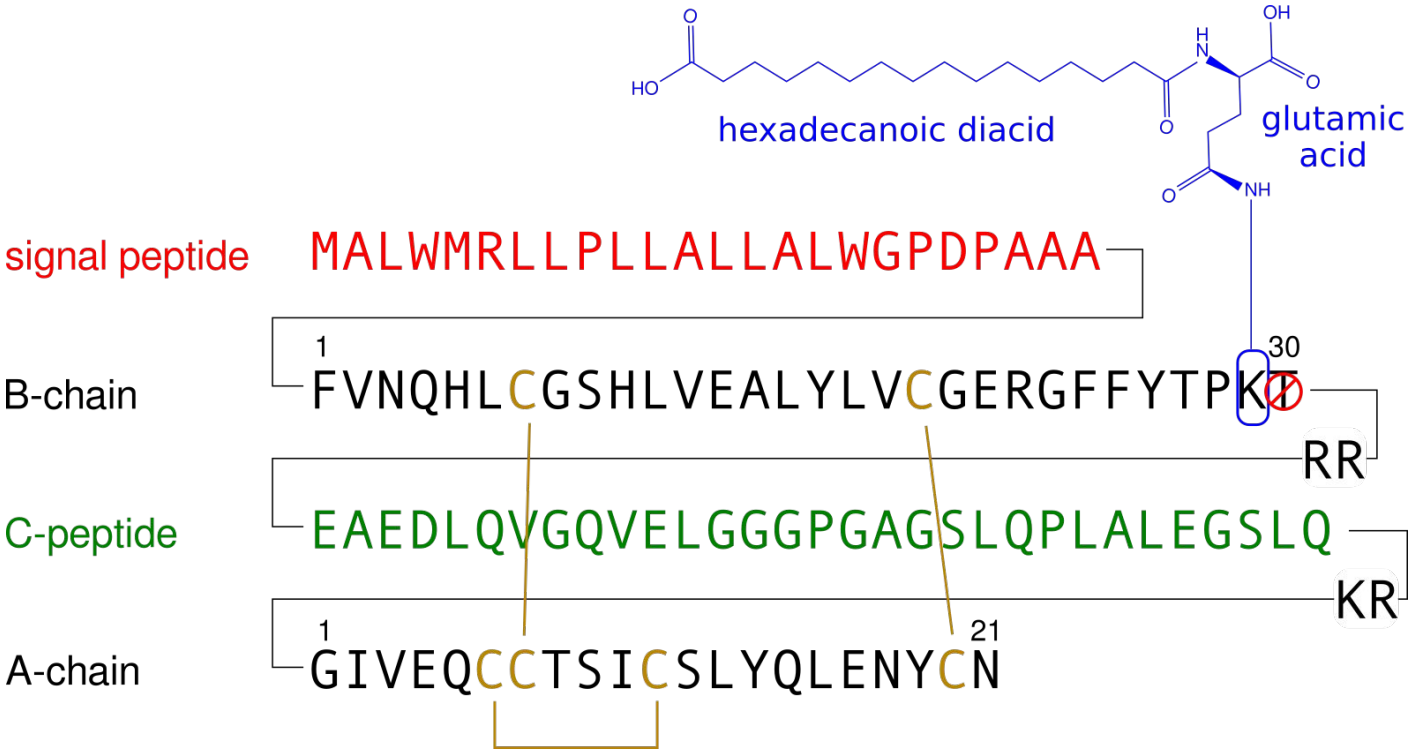

How to “optimize” a protein: In-vitro/directed evolution

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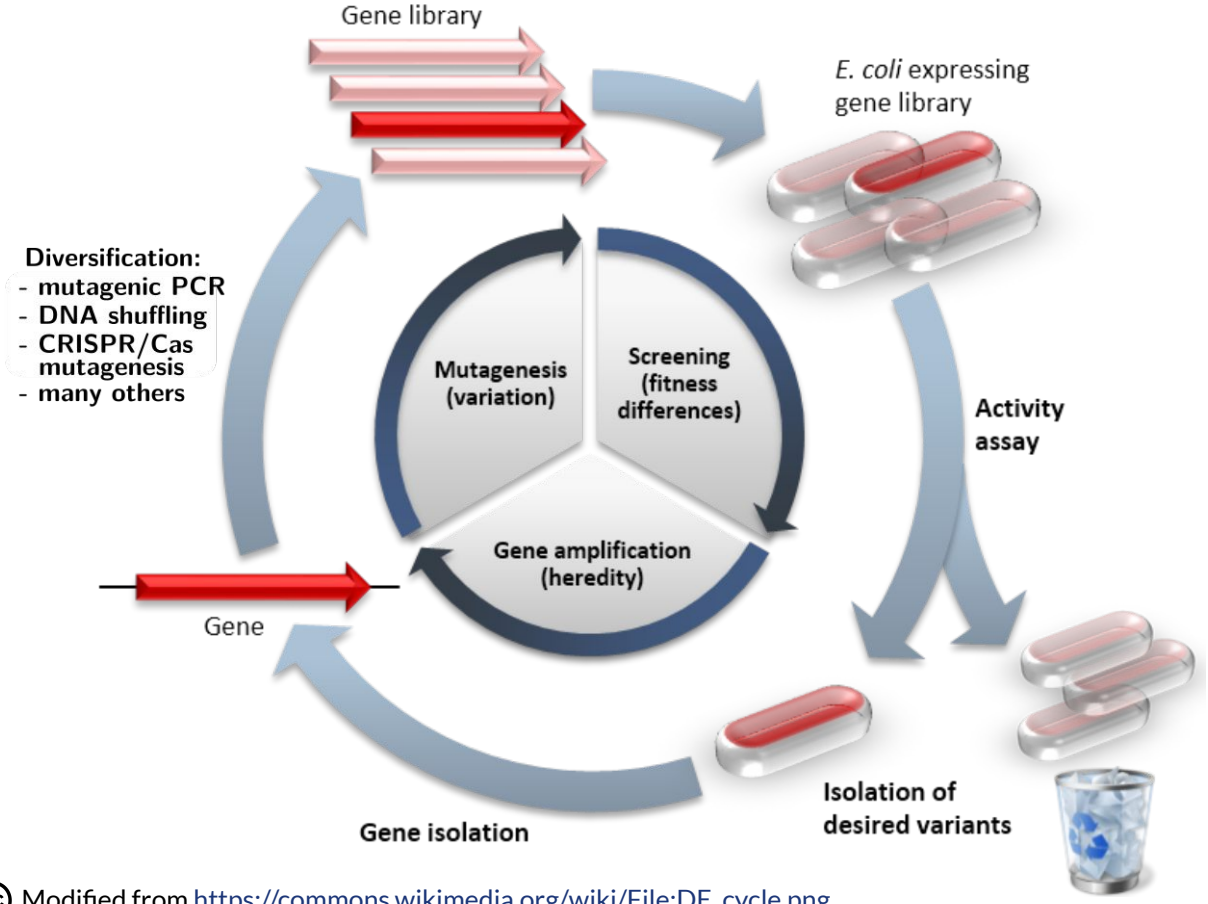

Modified from https://commons.wikimedia.org/wiki/File:DE_cycle.png

Example: Making a super vascular endothelial growth factor (super-VEGF)

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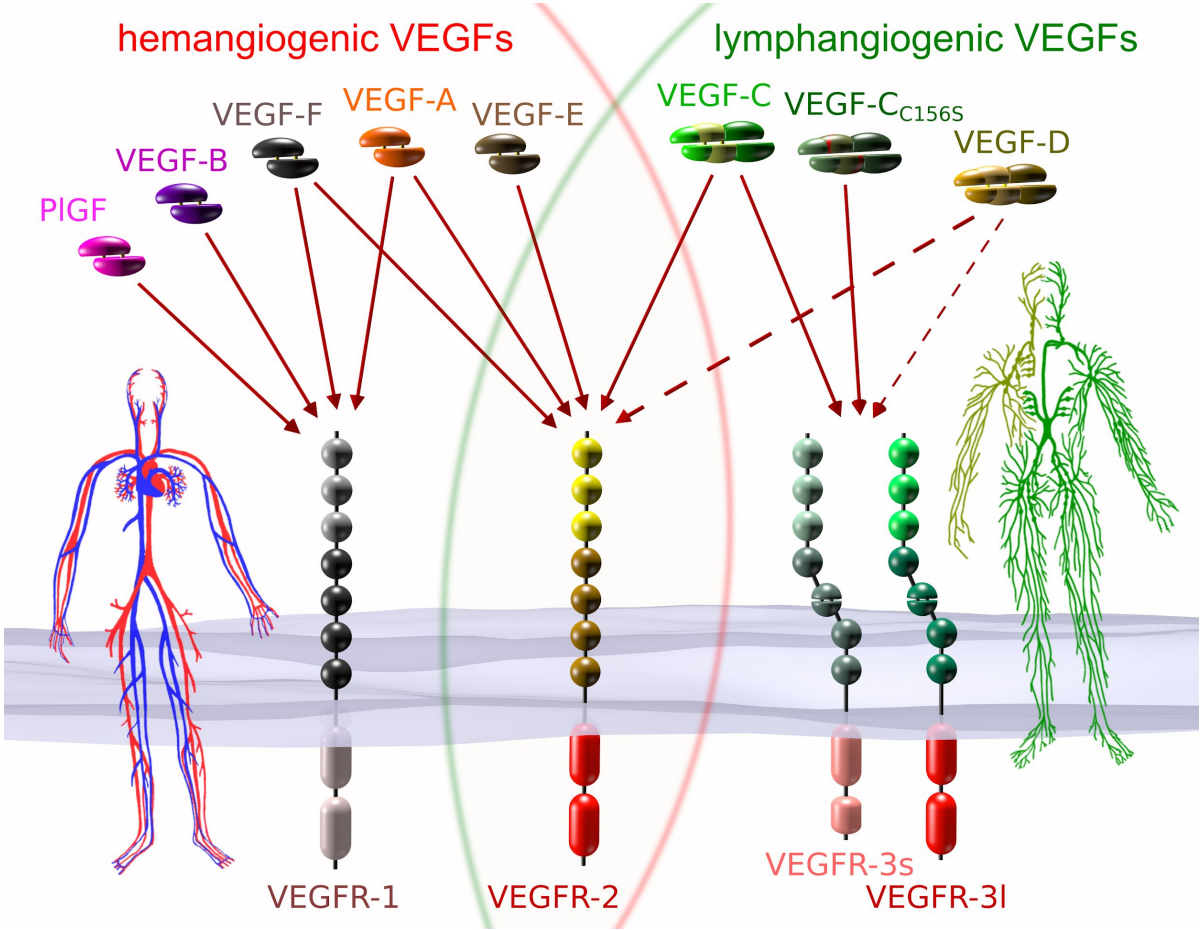

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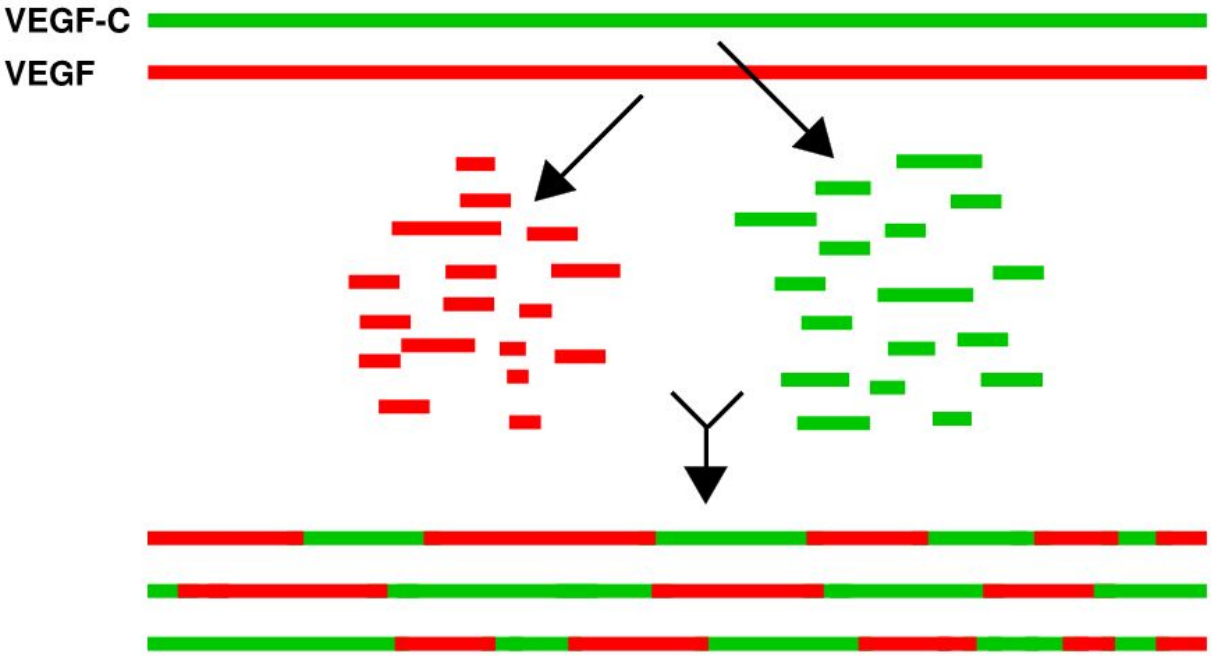


https://doi.org/10.1074/jbc.M511593200

Synthetic, man-made, human insulin: unclear usage, has historically been used to designate recombinant insulin as opposed to insulin from animal sources

Biosynthetic insulin: recombinant insulin

Fully synthetic insulin: by chemical synthesis in-vitro (“test tube”)

Insulin analogs (*lispro*, *aspart*, *glulisine*): recombinant insulin with modifications

- 1963 first fully synthetic insulin (Meienhofer et al. 1963, *Z Naturforsch* 18b: 1120f)
- 1978 fully synthetic insulin (Giba-Geigy) used for therapy (Teuscher 1979, *Schweiz Med Wochenschr* 109: 743ff)
- 1978 first recombinant insulin from *E. coli* (Genentech, Goeddel et al. 1979, *PNAS* 76: 106ff)

Human Insulin as Good as Costly Synthetic Versions

https://www.embibe.com/study/production-of-genetically-engineered-human-insulin-concept

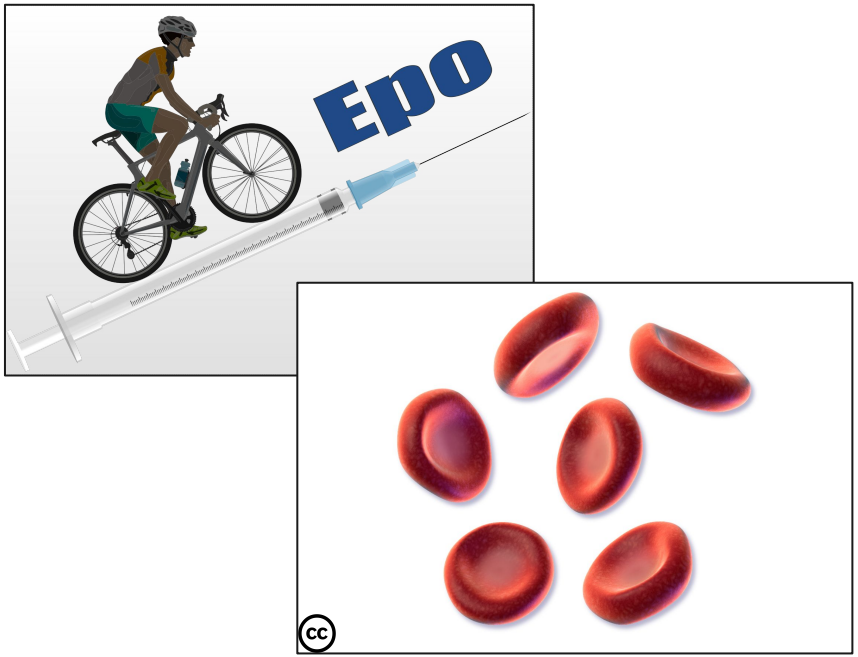
- Modify (reduce or increase) biological half-life: adding glycosylation (“glycoengineering”, most therapeutic proteins are naturally glycosylated as they are produced in eukaryotic cells), e.g. the erythropoietin homolog darbepoetin alfa

Aranesp® (Darbepoetin alfa)

```
>sp|P01588|EPO_HUMAN
Erythropoietin OS=Homo sapiens
OX=9606 GN=EPO PE=1 SV=1
MGVHECPAWLWLLLSLLSLPLGLPVLGAPP
                          N  T
RLICDSRVLERYLLEAKEAENITTGCAEHC
SLNENITVPDTKVNFYAWKRMEVGQQAVEV
                         VN  T
WQGLALLSEAVLRGQALLVNSSQPWEPLQL
HVDKAVSGLRSLTTLLRALGAQKEAISPPD
AASAAPLRTITADTFRKLFRVYSNFLRGKL
KLYTGEACRTGDR
```

- Homolog of the endogenous protein hormone erythropoietin (“Epo”)
- Five point mutations to create NXT sites to increase the biological half life
- Stimulates red blood cell production

https://doi.org/10.1002/dta.1341



- *Indications*: several types of anemias
- *Available in Finland*: yes
- *Company*: Amgen (US)
- *Interesting*: The name erythropoietin was coined in Finland by Eeva Jalavisto & Eva Bonsdorf
- Market introduction: 2001

- Modify (reduce or increase) biological half-life: adding glycosylation (“glycoengineering”, most therapeutic proteins are naturally glycosylated as they are produced in eukaryotic cells), e.g. the erythropoietin homolog darbepoetin alfa
- Improve biological activity (e.g. receptor binding affinities): e.g. consensus interferon is many times more effective than any single specific α-interferon (for Hepatitis C virus infection)

Consensus interferon

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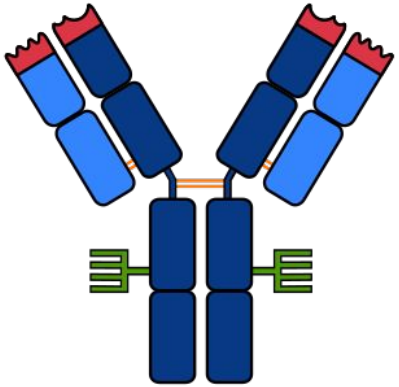
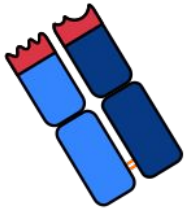
- Modify (reduce or increase) biological half-life: adding glycosylation (“glycoengineering”, most therapeutic proteins are naturally glycosylated as they are produced in eukaryotic cells), e.g. the erythropoietin homolog darbepoetin alfa
- Improve biological activity (e.g. receptor binding affinities): e.g. consensus interferon is many times more effective than any single specific α-interferon (for Hepatitis C virus infection)
- Reduce size (proteins: high solubility, low permeability): reducing the size of antibodies (~140 kDa to ~25 kDa) for better tumor penetration



IgG_1
full antibody

~140 kDa



Fab
fragment
antigen binding

~50 kDa

scFv
single chain
variable fragment

~27 kDa

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Immune system

Cell-mediated immunity ⟷ **Humoral immunity**

(proteins in body fluids):

- *Antibodies*
- *Complement System*
- *Antimicrobial peptides*

Innate immunity ⟷ **Adaptive/Acquired immunity**

- *Antibodies (in humans)*
- *CRISPR/Cas systems (in bacteria)*

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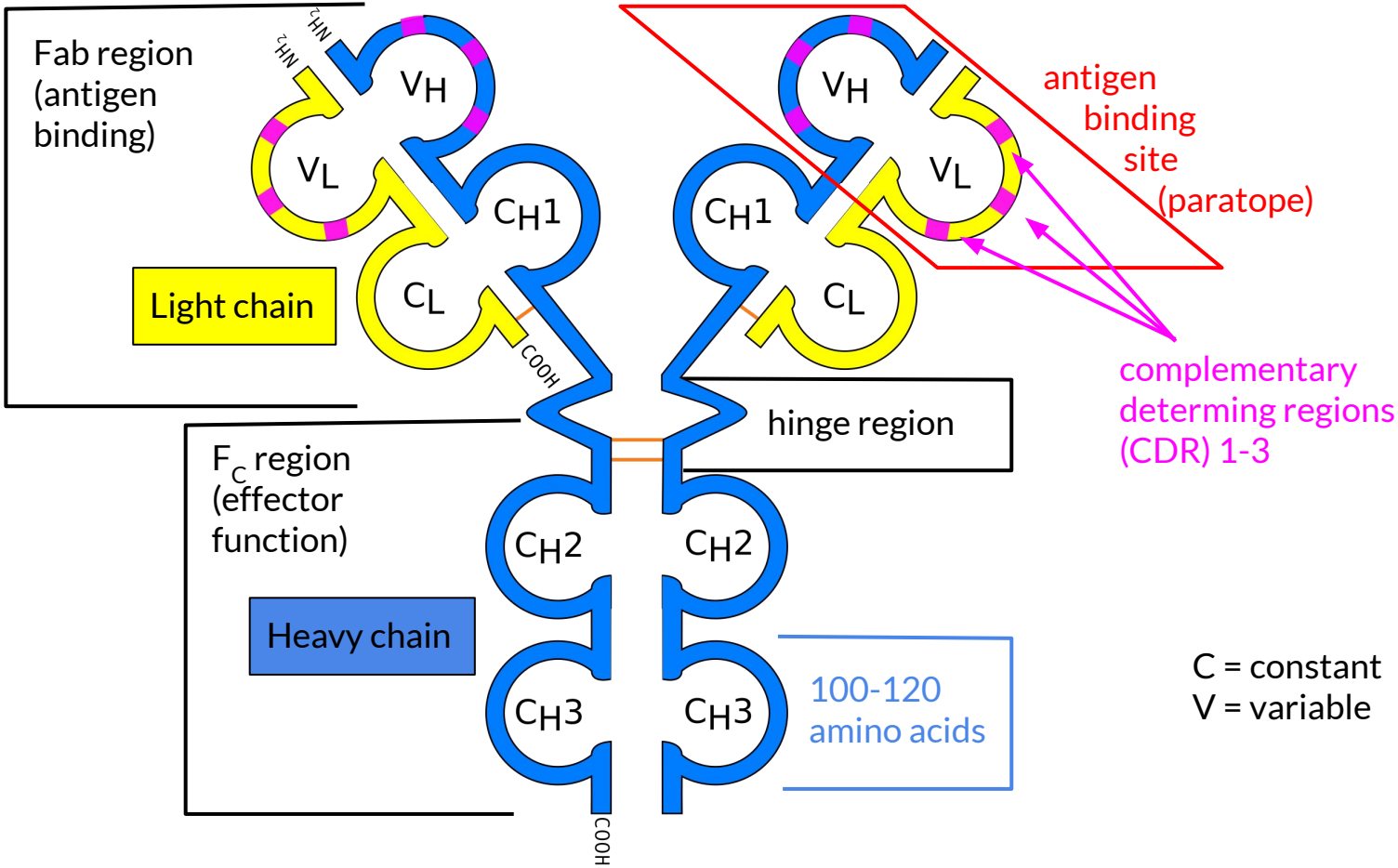
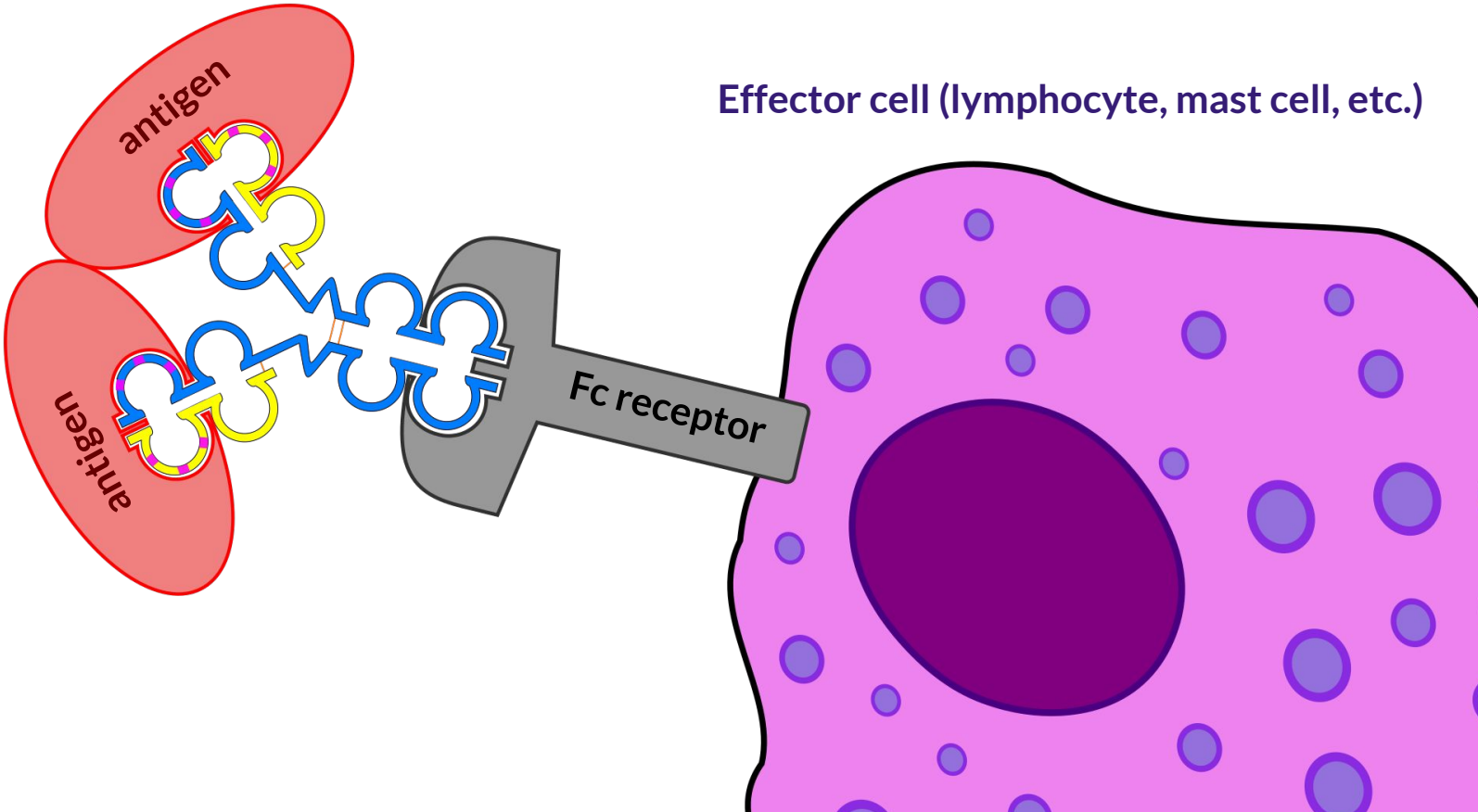
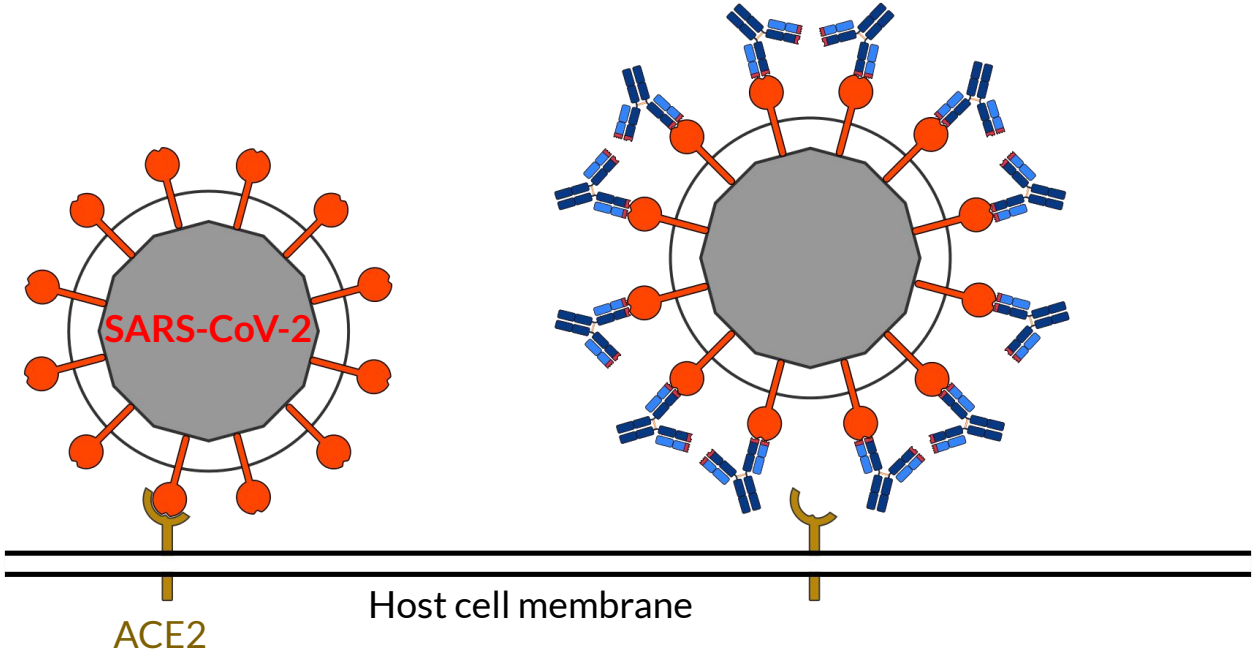


C = constant
V = variable


antigen
antigen
Fc receptor
Effector cell (lymphocyte, mast cell, etc.)

Primary function of antibodies

Neutralization of toxins and pathogens (“neutralizing/blocking” antibody)



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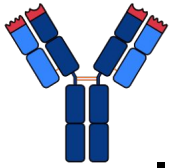
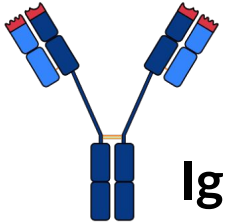
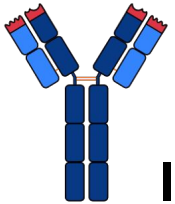


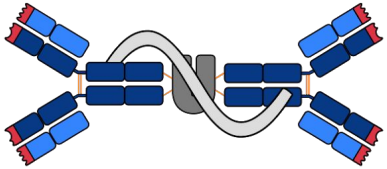
IgG



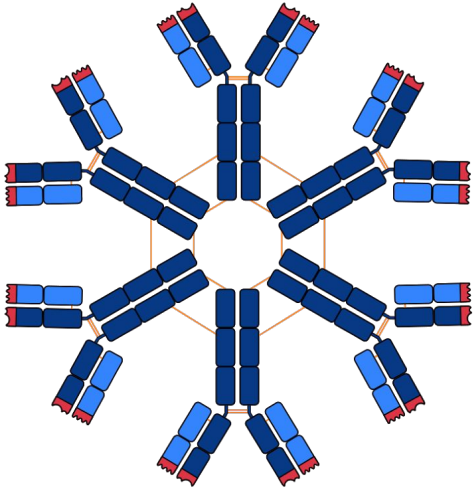
IgD

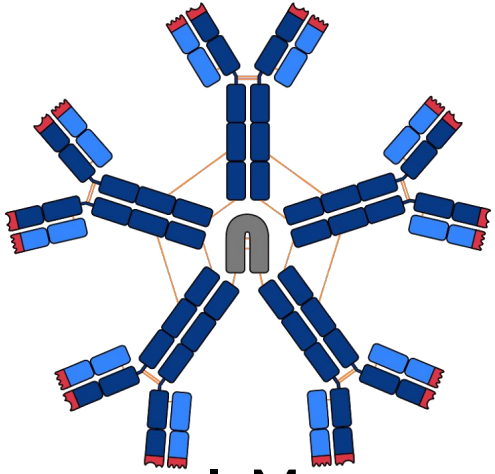


IgE

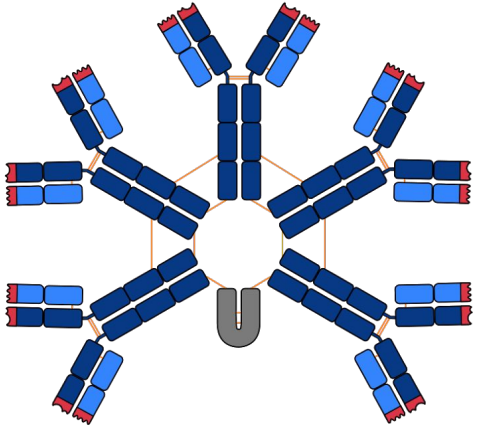


secretory IgA





IgM



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- Millions of different antigens, but only 4 immunoglobulin genes: *IGH* (Ig heavy chain), *IGK*, *IGL* (light chains Ig Kappa and Ig Lambda) and IGJ (joining chain)
- Each of us has $<4x10^8$ different antibodies, roughly the same magnitude as B cells in the blood (but most B cells are not in the blood)
    - How does the body generate so many different antibodies?

V-D-J recombination (heavy chain, simplified)

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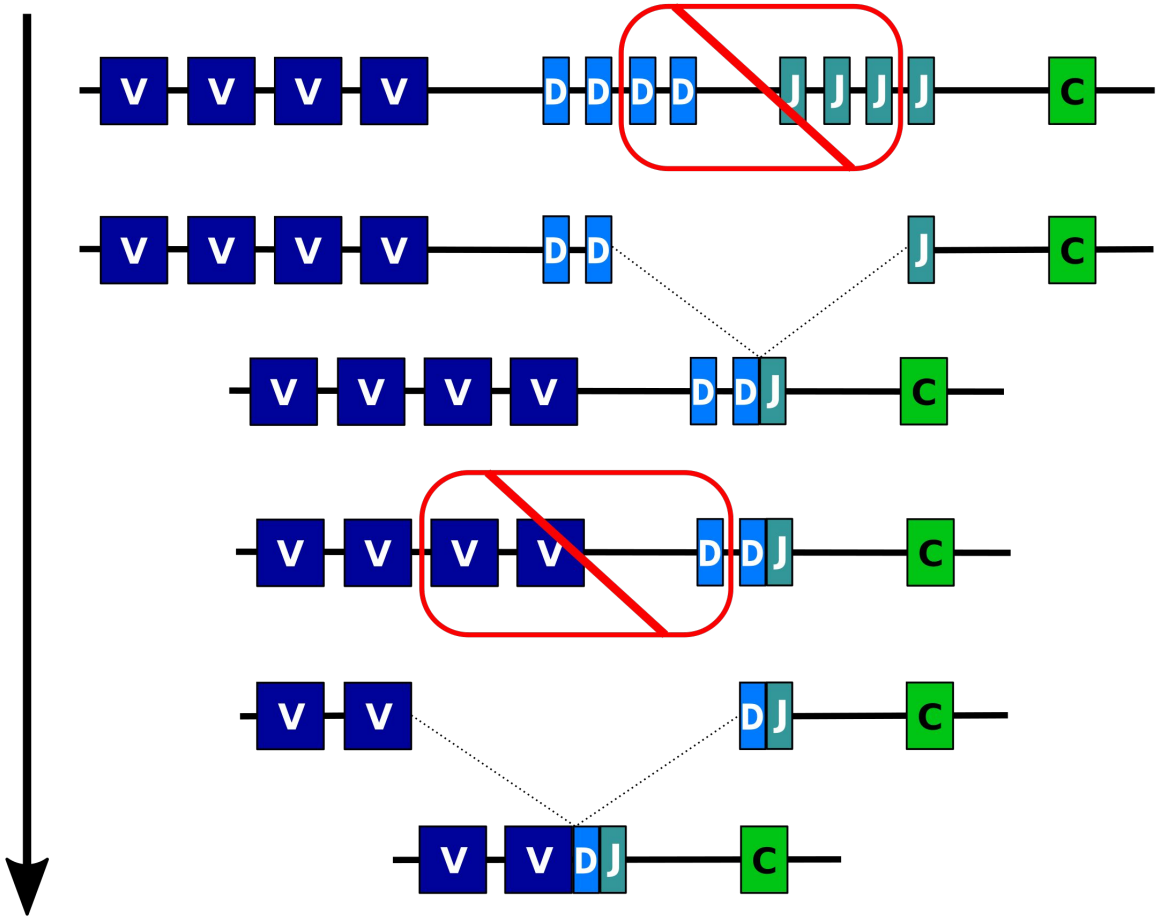

V-D-J recombination & class switching (heavy chain)

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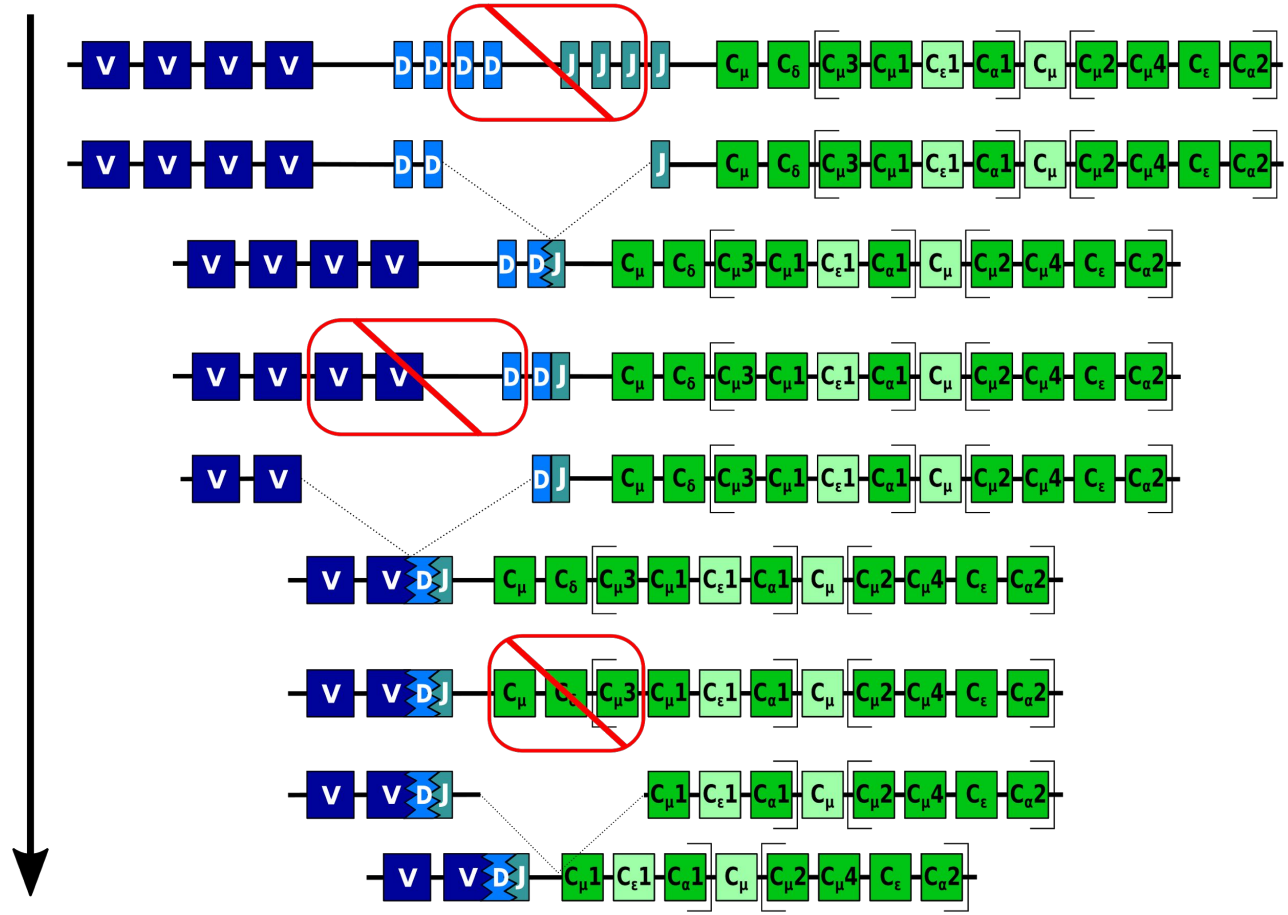

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1. Assembly of the heavy chain by recombination from V (+D) + J + C genes
2. Assembly of the light chain by recombination from V + J (two different sets: kappa & lambda)
3. Heavy and light chain combinations
4. Addition and deletion of nucleotides during recombination (“junctional diversity”)
5. Somatic hypermutation upon B cell activation by AID (activation-induces cytidine amidase) enzyme

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Polyclonal antibody ("antiserum") production

- **Ingredients for immunization (more or less unchanged for the last 100 years)**
  1. Antigen: (highly) purified protein, synthetic peptides (up to ~100 aa)
  2. Host: Rabbit, Mouse, Goat, Horse, Human
  3. Adjuvants (Freund's complete adjuvant (FCA)*, aluminium salts): to be mixed (mostly emulgated) with the antigen to boost the immune response
  4. Injection syringe for subq (intradermal, intraperitoneal, footpad, intramuscular) injection

- Pre-immune serum sample
- Repeat injection ("booster"): e.g. up to 5 times in rabbits in 3-week-intervals, many different protocols
- "Test bleeds" (e.g. starting from 2 weeks after 2nd booster) for analysis
- For small animals usually "final bleed", for larger animals (incl. humans): repeated blood donation/plasmapheresis

*Inactivated mycobacteria in mineral oil. Not for human use and only limited for animal immunization due to intensive inflammatory reaction

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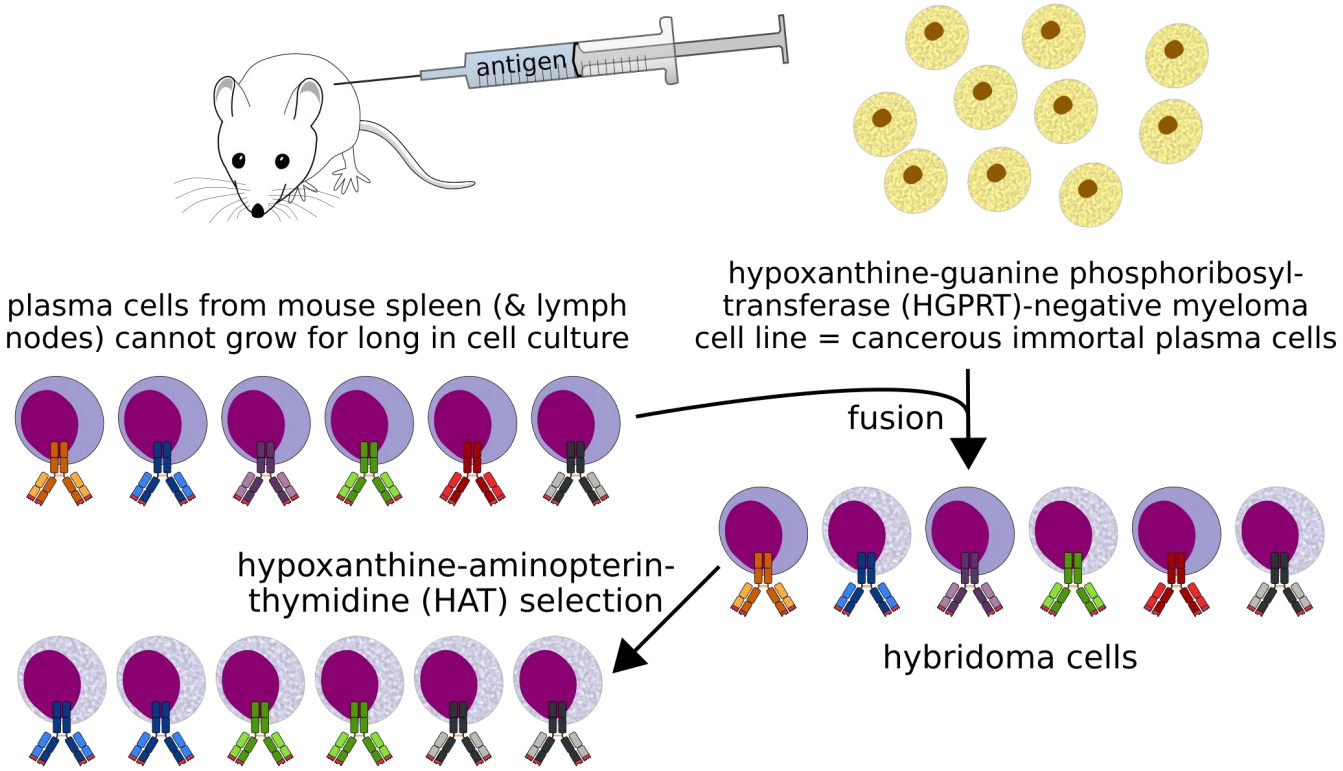
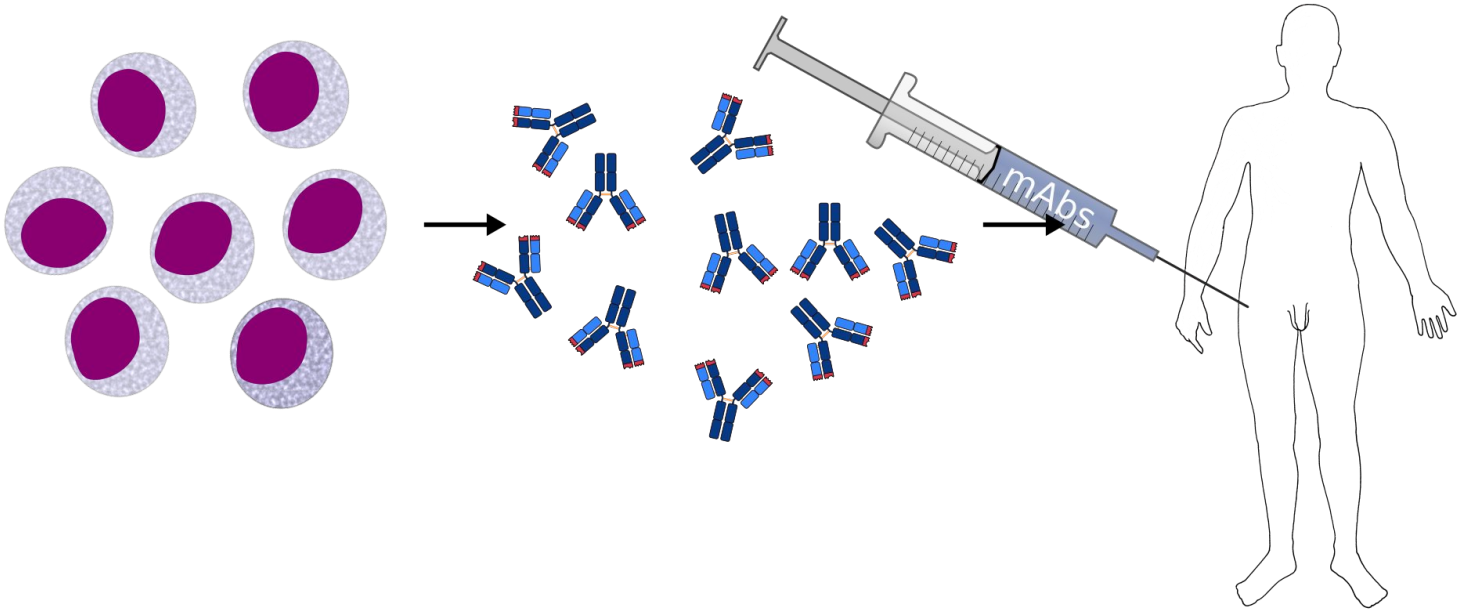


Köhler & Milstein 1975 Nature.



What happens if you inject mouse monoclonal antibodies (mAbs) into humans?

They are eliminated by an immune response!

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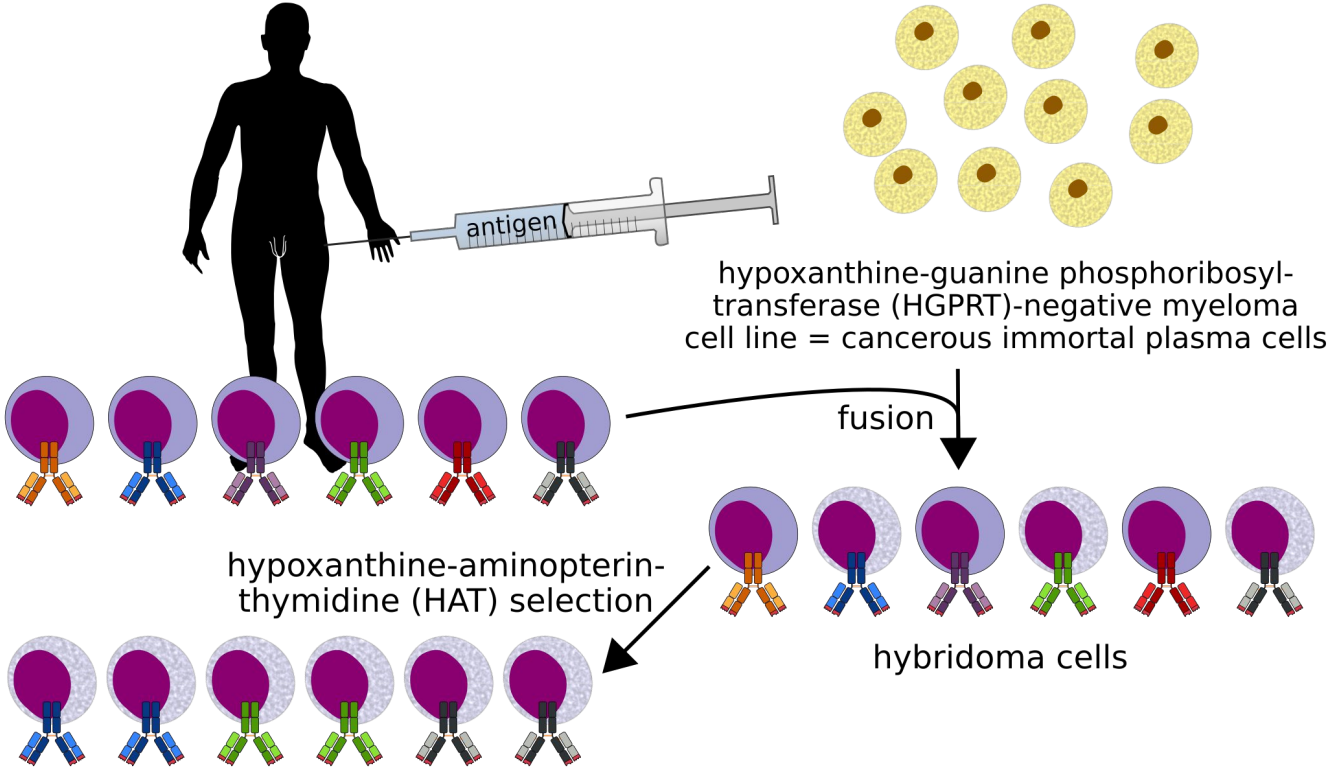
& UH



How to make ***human*** monoclonals?

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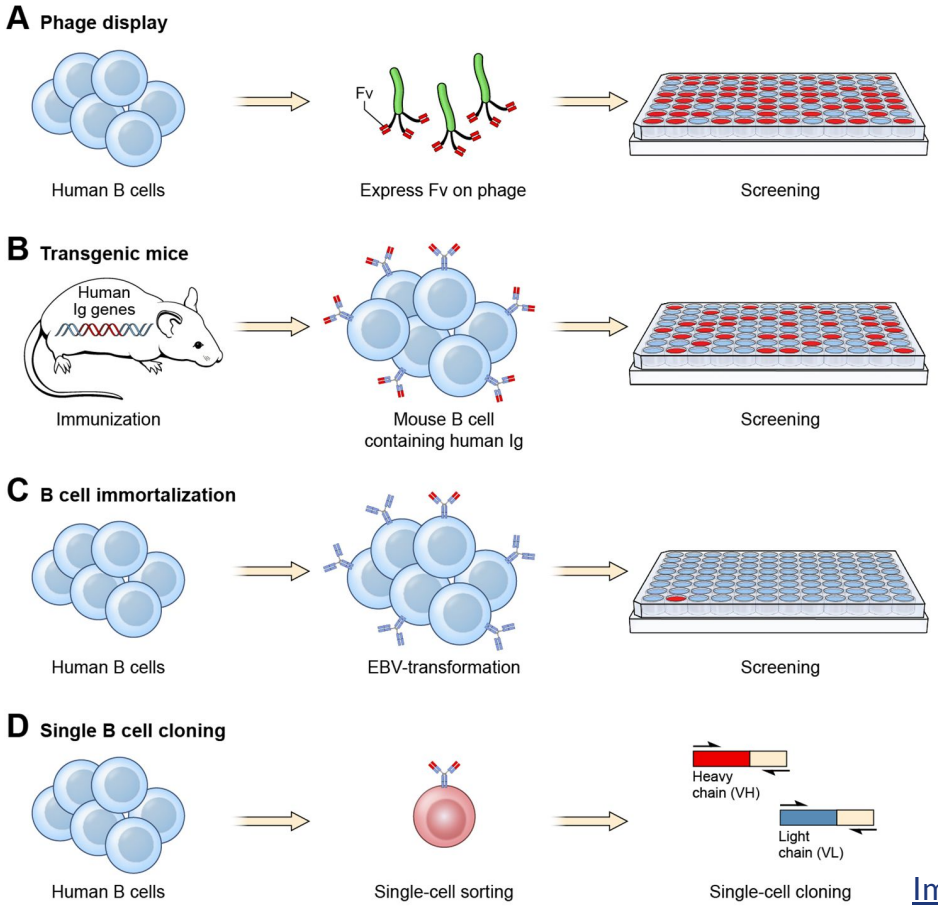
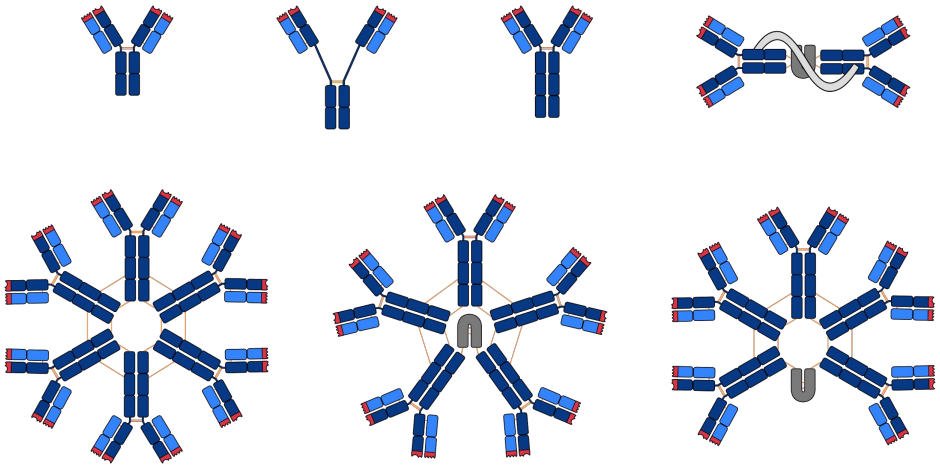
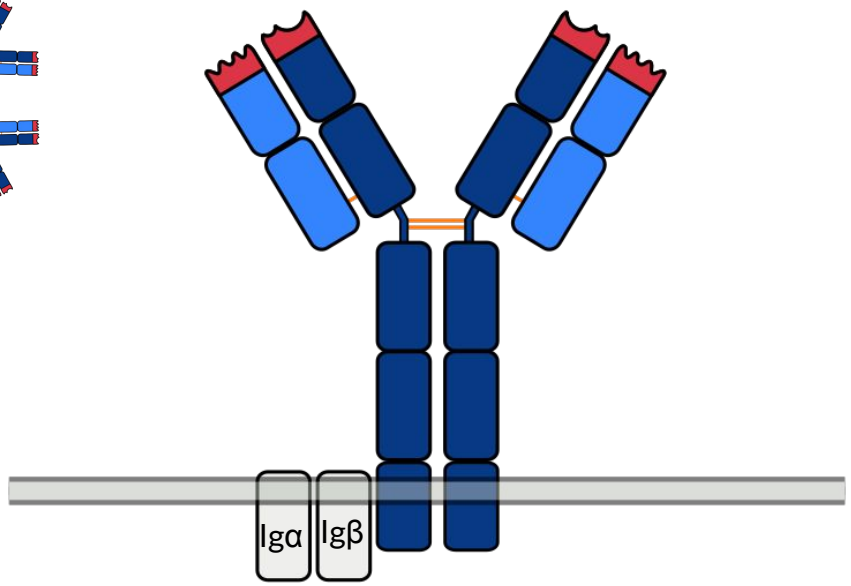


Image credit: Bioshore / CC BY-SA



B cell receptor =
membrane-bound version of IgM



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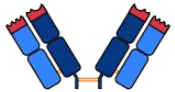
Engineered antibody formats

Fab
fragment antigen binding



$F(ab')_2$

Fab'

scFv
single chain variable fragment

di-scFv
dimeric single chain variable fragment, tandem scFv

diabody

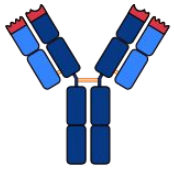
bispecific diabody

trispecific triabody

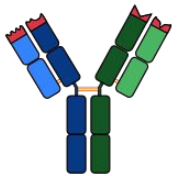
BiTE
bispecific T cell engager

sdAb/V_HH/V_{NAR}/ nanobody
single domain antibody, heavy-chain antibody VH (camelids), variable new antigen receptor (sharks)

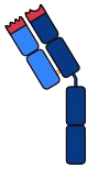
chemically linked $F(ab')_2$



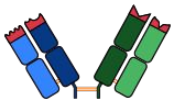
$IgGF_C$
full antibody



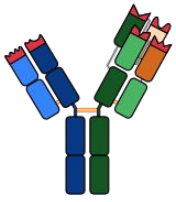
bispecific antibody



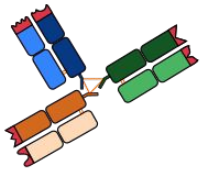
monovalent $IgGF_C$



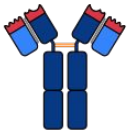
bispecific $F(ab')_2$



trispecific antibody



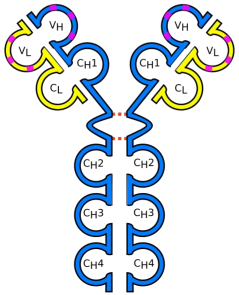
trispecific $F(ab')_3$



scFv-Fc

hcIgG (camelids)

minibody



IgG$_1$

scFv
single chain
variable fragment

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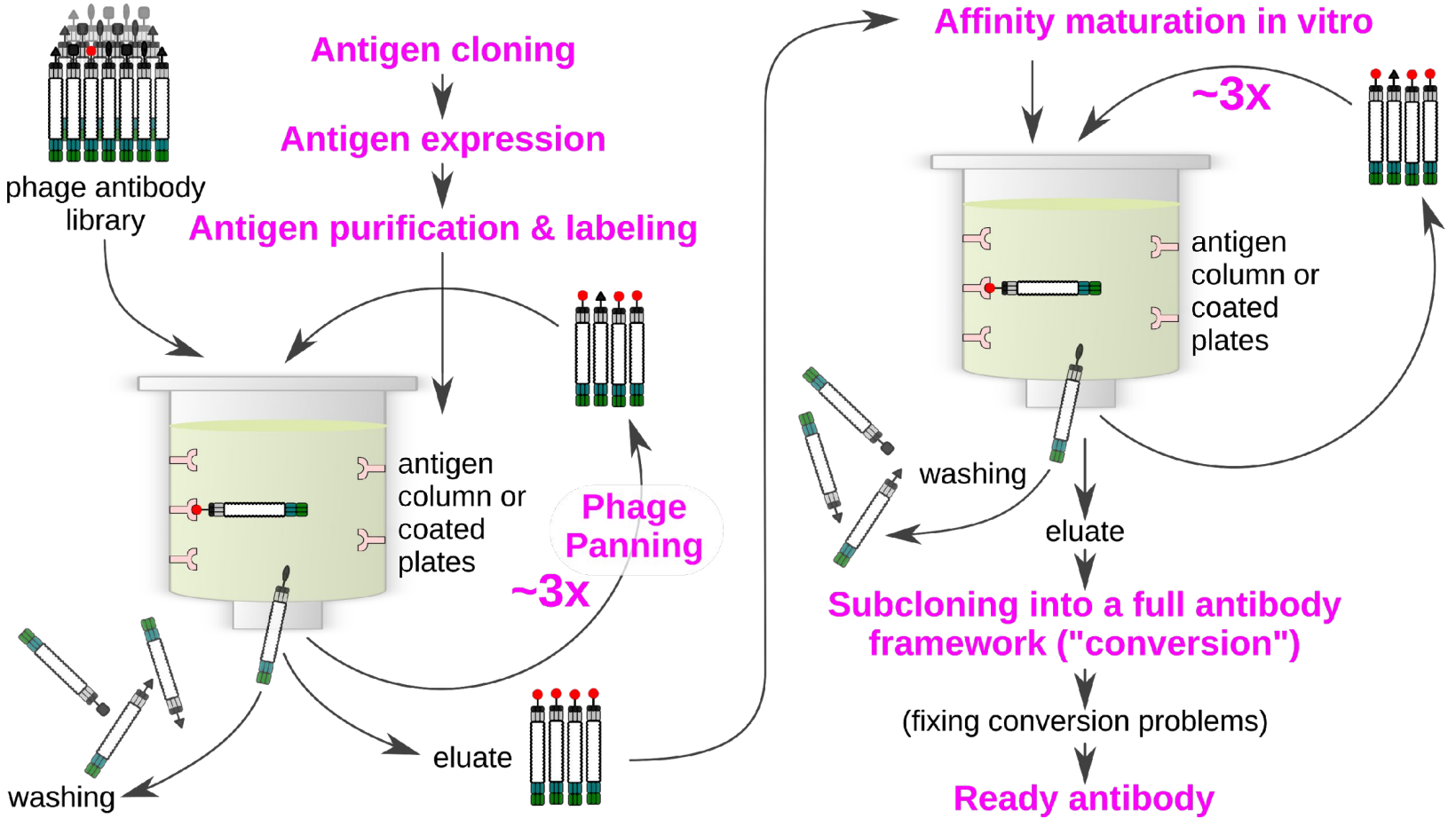

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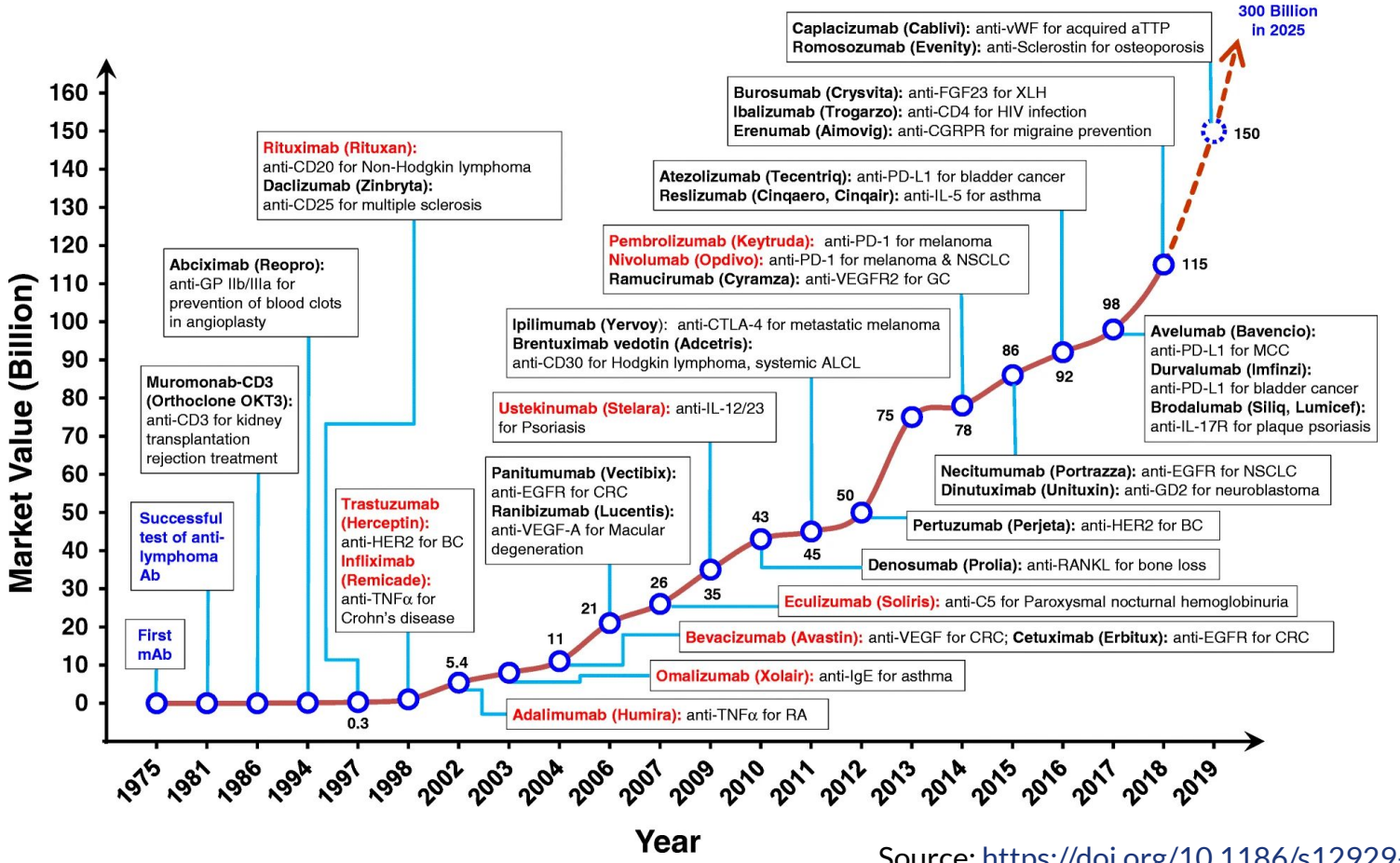
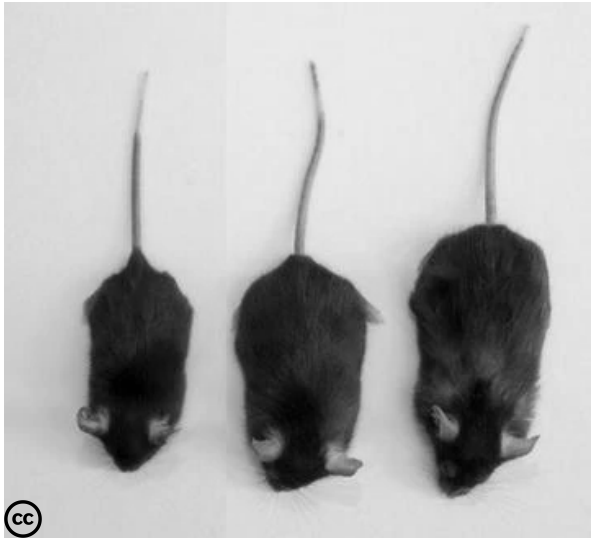
- No affinity maturation by somatic hypermutation (counter-measure: mega-libraries)
- No elimination of antibodies with disfavorable physical attributes (aggregation, protease-sensitive, low protein expression levels, etc.)



Source: https://doi.org/10.1186/s12929-019-0592-z

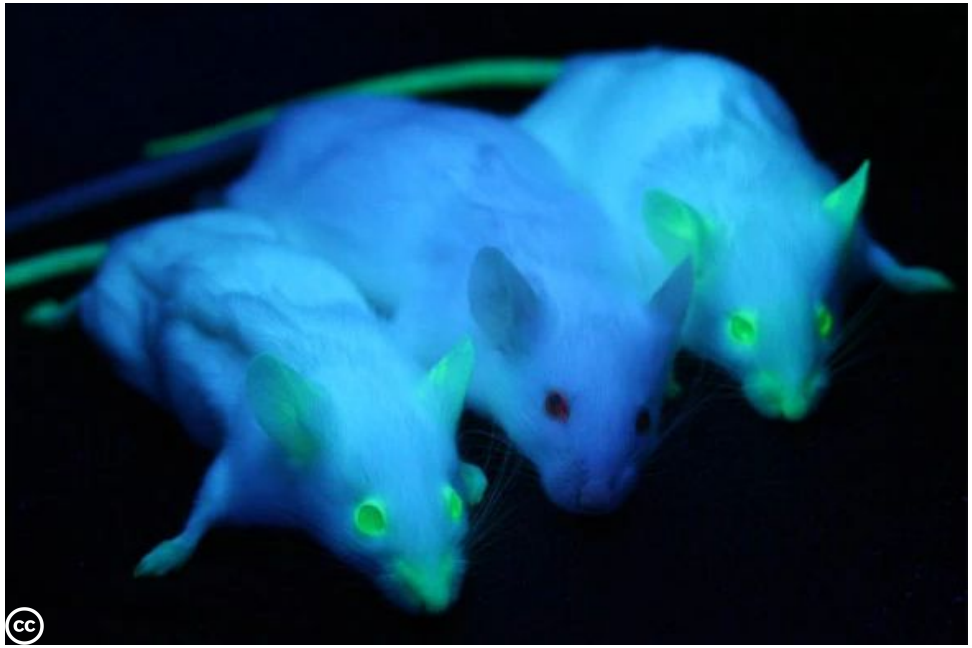


First transgenic mouse*

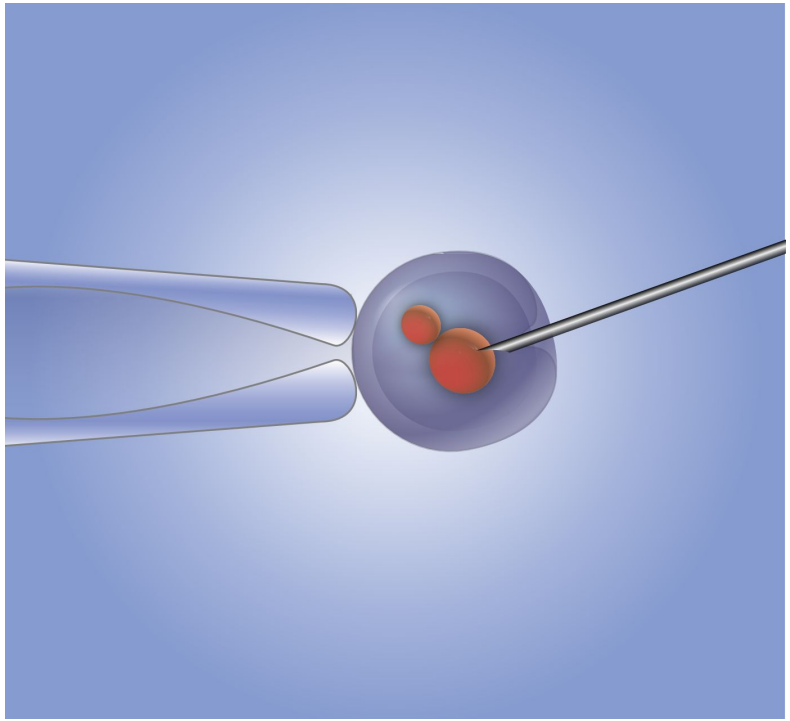
MT-hGH, 1982

First transgenic mouse in Finland**

K14-hVEGF-C, 1997



- eGFP mice for research
- GloFish for consumers
- Only available in the USA and Taiwan
- Banned in the EU



- In vitro fertilization
- Injection of purified, linear DNA into male pronucleus
- Implantation into pseudopregnant females
- F1 offspring is screened by PCR for DNA integration
- Success rate: 5-20% of F1 are positive
- Transfer of large DNA fragments possible
- Transgene integrates randomly as a tandem array

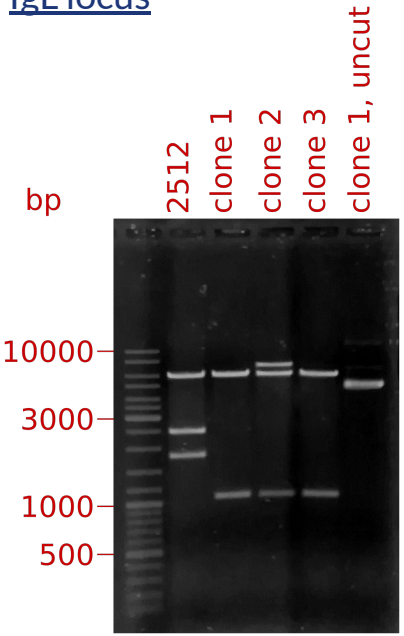
Modified from https://commons.wikimedia.org/wiki/File:Microinjection_of_a_human_egg.svg

Genes & genomes: https://ensembl.org/

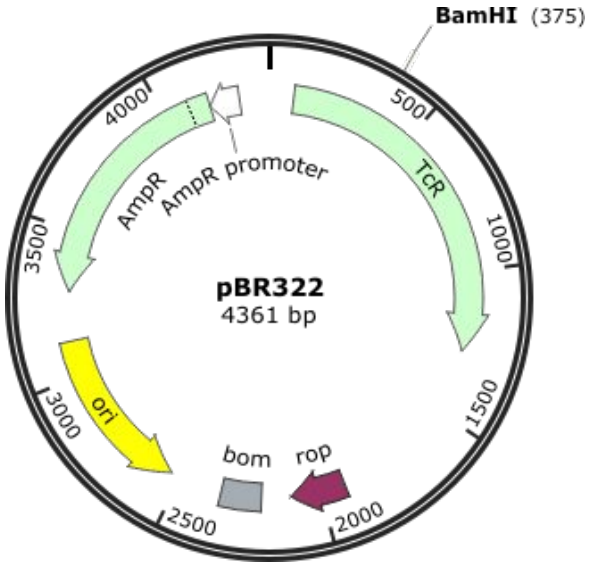
Proteins: https://uniprot.org

IgH locus
IgK locus
IgL locus

~1-1.5 Mb



- Plasmids: max. ~20 kb
- Cosmids: 28-45 kb
- λ (Lambda) vectors: 8-24 kb
- Bacterial Artificial Chromosomes (BAC): max. 350 kb
- Yeast Artificial Chromosomes (YAC): max. 1 Mb



- XenoMouse: Cell Genesys/Amgen, https://doi.org/10.1038/nbt1337
- HuMab mouse: GenPharm/Medarex/Bristol Myers Squibb
- VelociImmune mouse (Regeneron): piece by piece in-place replacement, https://doi.org/10.1073/pnas.1324022111
- OmniRat® (OmniMouse®/OmniChicken®): OMT/Pfizer/Ligand: human V + rat C regions https://doi.org/10.1038/s41598-020-57764-7
- Alloy Gx™: Alloy Therapeutics Inc., royalty-free and proprietary, new player
- Kymouse™: Kymab Ltd./Wellcome Trust, human V + mouse C regions, https://doi.org/10.1038/nbt.2825
- Harbour Antibodies™: Harbour BioMed, normal (“H2L2”) and heavy chain only (“HCAb”), https://doi.org/10.1073/pnas.0601108103
- Trianni Mouse™: Trianni Inc., in-place replacement of V regions, https://www.nature.com/articles/d42473-018-00011-5

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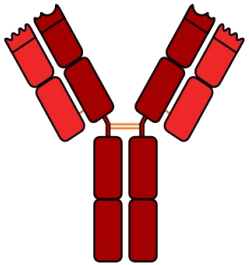
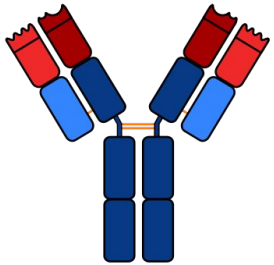
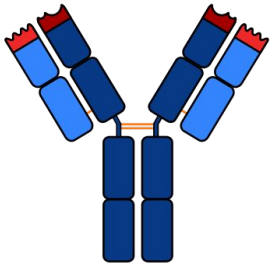
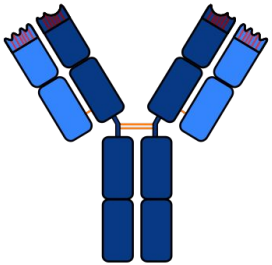
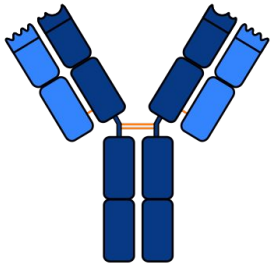
Eliminating immune response to therapeutic mAbs

	Mouse monoclonal	Chimeric monoclonal	Humanized/ CDR-grafted monoclonal	Phage display synthetic monoclonal	Fully human/ transgenic human monoclonal
					
e.g.	Muromonab ("Muromomab") (Orthoclone OKT3)	Rituximab (Rituxan)	Bevacizumab (Avastin)	Adalimumab (Humira)	Panitumumab (Vectibix)
	1975 Köhler & Milstein	1984 Morrison et al.	1986 Jones et al.	1990 McCafferty et al.	1994 Lonberg et al. & Green et al.

https://en.wikipedia.org/wiki/Nomenclature_of_monoclonal_antibodies

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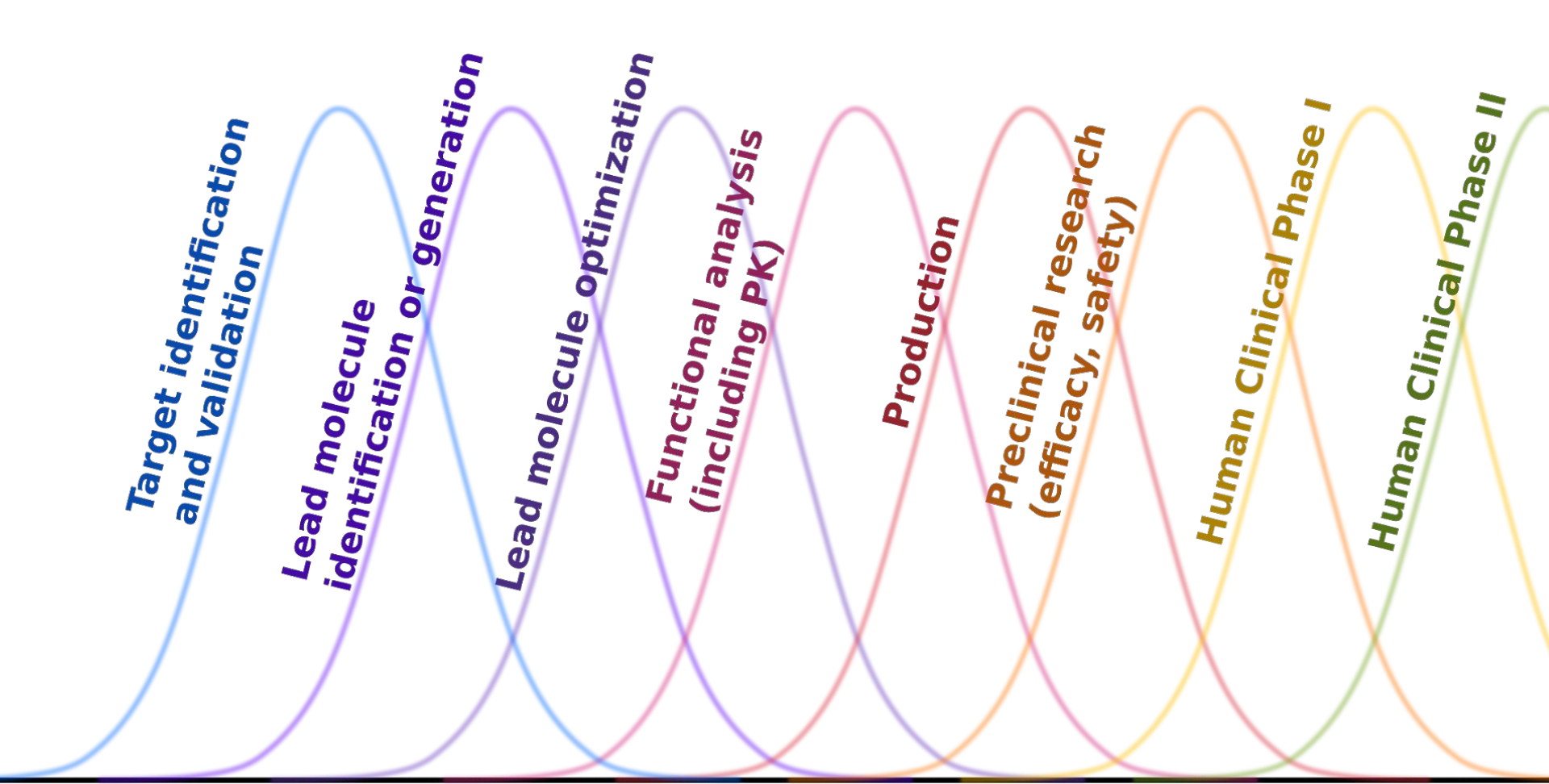

- Basics about B cells: https://www.ibiology.org/immunology/b-cell-development/#part-1
- Erichsen Geld und Gold E158, in German 😕: Impfstoff-Aktien vor hohen Verlusten (27.08.2020): https://podcasts.google.com/feed/aHR0cHM6Ly9lcmljaHNlbi5wb2RpZ2VlLmlvL2ZlZWQvbXAz
- Freakonomics Radio E430: Will a Covid-19 vaccine change the future of medical research? (27.08.2020): https://freakonomics.com/podcast/vaccine/
- The (in)complete list of all coronavirus vaccine development efforts: https://www.who.int/docs/default-source/coronaviruse/novel-coronavirus-landscape-covid-19-(3).pdf
- Very good, but old review about Ig-humanized mice: https://doi.org/10.1038/nbt1135

- My laboratory: mjlab.fi (https://www.helsinki.fi/en/researchgroups/lymphangiogenesis-research-and-antibody-development)
- Core facility for protein production and purification: b3p.it.helsinki.fi
- jeltsch.org (private rumblings)
- jeltsch.org/science (private rumblings without the non-scientific stuff)
- Questions to: michael@jeltsch.org
- or via Skype: jeltsch
- This presentation: mjlab.fi/pddd, (jeltsch.org/teaching)