An example of how to approach this project:

Liquid biopsies and NIPT

- Circulating nucleic acids discovered free floating in blood plasma in 1940's by Mandel and Mëtais, but origin of these molecules and their significance was unknown, therefore the paper did not attract much attention
  - *P. Mandel, P. Metais. Les acides nucleiques du plasma sanguin chez l'homme. CR Acad. Sci. Paris, 142 (1948), pp. 241-243*
- Cell-free nucleic acids are found to be generated by dying cells in 1970 by Williamson (described then as "cytoplasmic DNA"), and later shown to be a result of degraded nuclear DNA. The cell-free DNA sizes formed a "ladder" of multiples of 200bp, due to protection from degradation by nucleosome proteins.
  - *Williamson R., 1970, Properties of rapidly labelled deoxyribonucleic acid fragments isolated from the cytoplasm of primary cultures of embryonic mouse liver cells. J. Mol. Biol. 51: 157–168. doi:10.1016/0022-2836(70)90277-9*
- Cell-free DNA is found to be elevated in patients with disease, including Lupus and cancer patients. How did they show this? What techniques did they use?
  - *E.M. Tan, P.H. Schur, R.I. Carr, H.G. Unkel. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J. Clin. Invest., 45 (1966), pp. 1732-1740*
  - *G.D. Sorenson, D.M. Pribish, F.H. Valone, V.A. Memoli, D.J. Bzik, S.L. Yao. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol. Biomar. Prev., 3(1994), pp. 67-71*

An example of how to approach this project:

Liquid biopsies and NIPT

- Dennis Lo (CUHK) first demonstrated that fetal DNA is present as free-floating cell-free DNA in maternal blood in 1997. How did they show this? What experimental designs/techniques did they use? What were the implications of these findings?
  - *Y.M. Lo, N. Corbetta, P.F. Chamberlain, V. Rai, I.L. Sargetn, C.W. Redman. Presence of fetal DNA in maternal plasma and serum. Lancet, 350 (1997), pp. 485-487*
- Digital PCR is used to count the molecules of cell-free DNA in the maternal blood, in order to detect fetal aneuploidy. Two groups simultaneously published this technique. What were the experimental designs? Any drawbacks? What are the implications of this?
  - Fan, H. C., & Quake, S. R. (2007). Detection of aneuploidy with digital polymerase chain reaction. *Analytical chemistry*, *79*(19), 7576-7579.
  - *Lo, Y. D., Lun, F. M., Chan, K. A., Tsui, N. B., Chong, K. C., Lau, T. K., ... & Chiu, R.W. (2007). Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proceedings of the National Academy of Sciences, 104(32), 13116-13121.*
- As the cost of next generation sequencing decreases and becomes widely available, NGS is used to perform sequencing of cell-free DNA and can accurately quantitate fetal copy numbers for diagnosis of aneuploidy. How was this demonstrated? What were the main challenges? Why is this an improvement on the previous method?
  - *Fan, H. C., Blumenfeld, Y. J., Chitkara, U., Hudgins, L., & Quake, S. R. (2008). Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proceedings of the National Academy of Sciences, 105(42), 16266-16271.*
  - *Chiu, R.W., Chan, K.A., Gao, Y., Lau, V.Y., Zheng, W., Leung, T.Y., ... & Zee, B. C. (2008). Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proceedings of the National Academy of Sciences, 105(51), 20458-20463.*

An example of how to approach this project:

Liquid biopsies and NIPT

- Target amplification/preamplification and NGS approaches vs.
- Non-targeted, whole genome approach
  - *Fan, H. C., Gu, W., Wang, J., Blumenfeld, Y. J., El-Sayed, Y. Y., & Quake, S. R. (2012). Non-invasive prenatal measurement of the fetal genome. Nature, 487(7407), 320.*
  - *Lo, Y. D., Chan, K. A., Sun, H., Chen, E. Z., Jiang, P., Lun, F. M., ... & Chiu, R. W. (2010). Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Science translational medicine, 2(61), 61ra91-61ra91.*
  - *Kitzman, J. O., Snyder, M. W., Ventura, M., Lewis, A. P., Qiu, R., Simmons, L. E., ... & Tabor, H. K. (2012). Noninvasive whole-genome sequencing of a human fetus. Science translational medicine, 4(137), 137ra76-137ra76.*
- This technique was only useful for large chromosomal aberrations like aneuploidy. What about single gene mutations?
  - *Li, Y., Di Naro, E., Vitucci, A., Zimmermann, B., Holzgreve, W., & Hahn, S. (2005). Detection of paternally inherited fetal point mutations for β-thalassemia using size-fractionated cell-free DNA in maternal plasma. Jama, 293(7), 843-849.*
  - *Gu, W., Koh, W., Blumenfeld, Y. J., El-Sayed, Y. Y., Hudgins, L., Hintz, S. R., & Quake, S. R. (2014). Noninvasive prenatal diagnosis in a fetus at risk for methylmalonic acidemia. Genetics in Medicine, 16(7), 564.*

An example of how to approach this project:

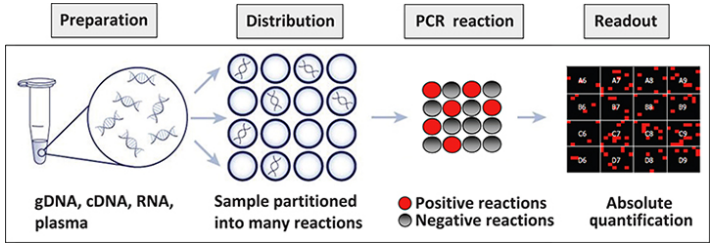
Liquid biopsies and NIPT

- cfDNA and cfRNA correlate with tissue damage/tissue renewal/cell death
- Are there different approaches being used to perform NIPT and liquid biopsies?
- It can predict transplantation rejection
  - DNA/RNA that circulates in the blood comes from not only human cells, but microbial origin as well (bacteria, viruses, fungi, etc)
- It turns out liquid biopsies can predict cancer, preterm birth, and other diseases
  - *Koh, W., Pan, W., Gawad, C., Fan, H. C., Kerchner, G. A., Wyss-Coray, T., ... & Quake, S. R. (2014). Noninvasive in vivo monitoring of tissue-specific global gene expression in humans. Proceedings of the National Academy of Sciences, 111(20), 7361-7366.*
  - *Tsang, J. C., Vong, J. S., Ji, L., Poon, L. C., Jiang, P., Lui, K. O., ... & Lo, Y. M. D. (2017). Integrative single-cell and cell-free plasma RNA transcriptomics elucidates placental cellular dynamics. Proceedings of the National Academy of Sciences, 114(37), E7786-E7795.*
  - *Ngo, T. T., Moufarrej, M. N., Rasmussen, M. L. H., Camunas-Soler, J., Pan, W., Okamoto, J., ... & Tibshirani, R. (2018). Noninvasive blood tests for fetal development predict gestational age and preterm delivery. Science, 360(6393), 1133-1136.* ***MIT Tech Review Top Breakthroughs 2019!***

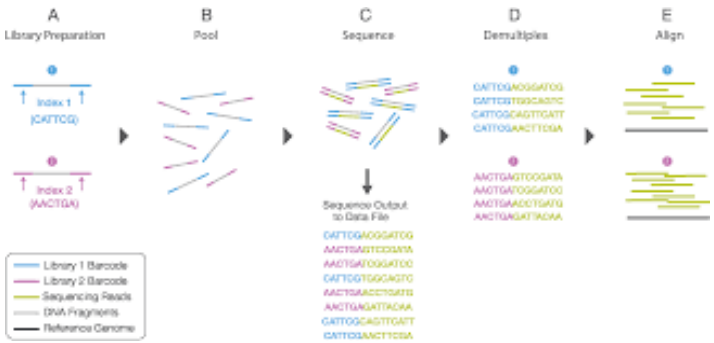
Make sure to highlight and explain key technologies

- Overall, what were the main challenges/hurdles in the development of this technique? What technologies had to be invented/to be substantially improved in order for this to come to fruition?
- Make sure to detail/explain any relevant technical points, such as molecular biology and mathematical relationships

- Digital PCR



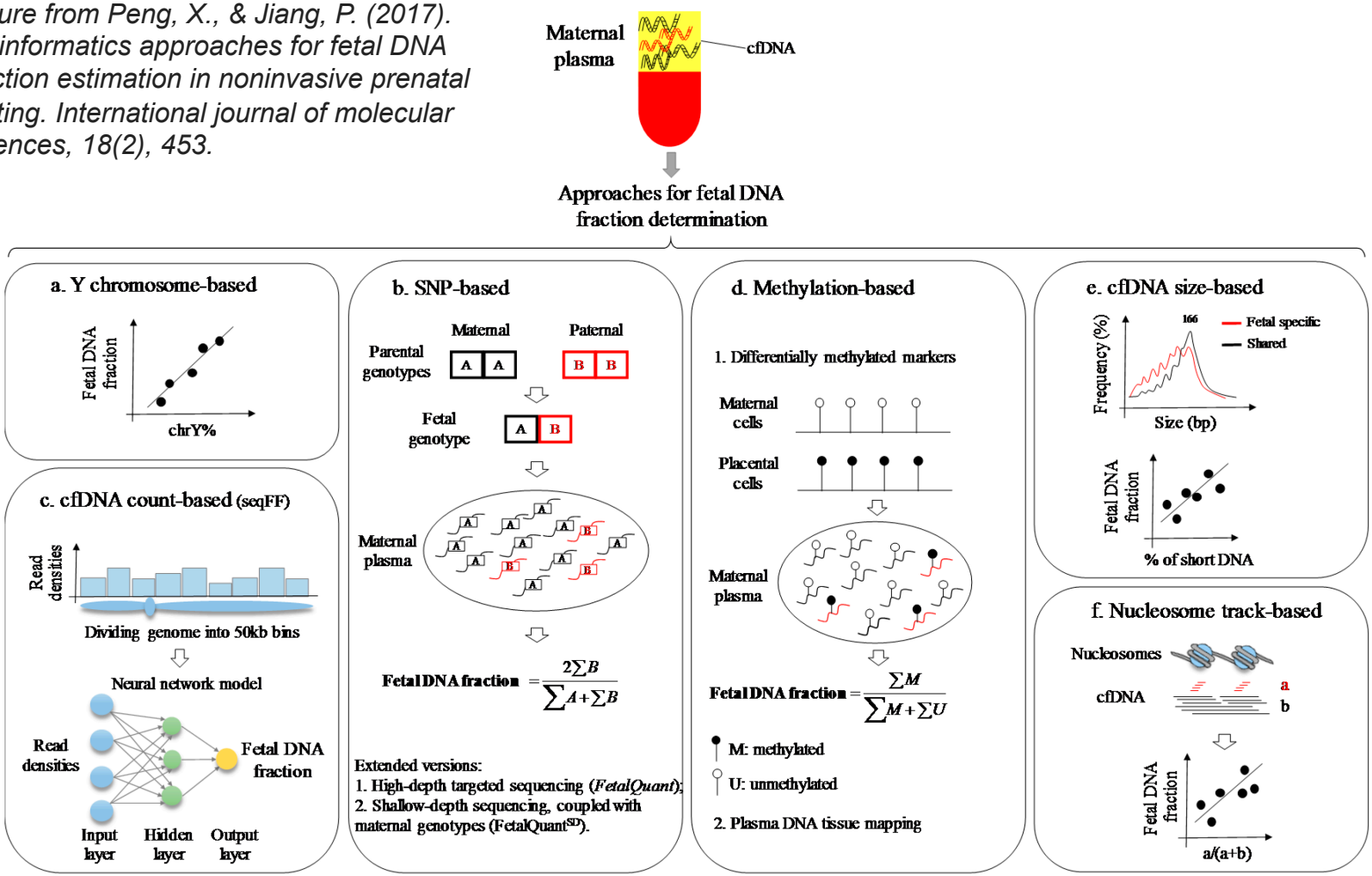
- Next gen sequencing

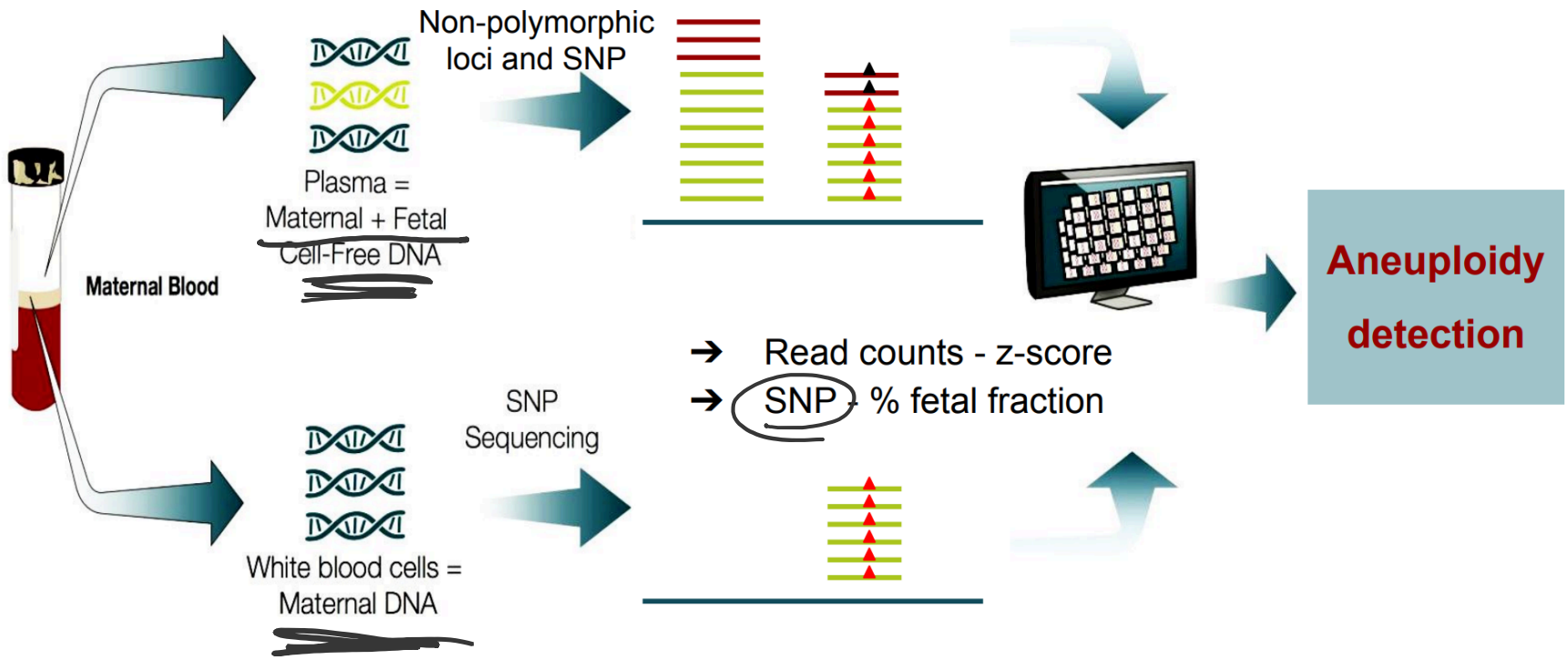


- Improved computational power
- Novel computational tools

Illustrate difficult concepts with figures

Figure from Peng, X., & Jiang, P. (2017). Bioinformatics approaches for fetal DNA fraction estimation in noninvasive prenatal testing. International journal of molecular sciences, 18(2), 453.





http://bioinformaticsinstitute.ru/sites/default/files/kozyulina_20170916.pdf

Don't forget to highlight the significance of the technology!

- What is the significance? Is it saving lives? Is it reducing suffering? Does it make a lot of money? Does it let us do/see/learn something new?

- **Safety:** NIPT replaces amniocentesis and chorionic villus sampling (CVS):
  - Both procedures are painful, has 1-3% risk of miscarriage, and other risks such as injury to fetus or mother, infection, and preterm birth, etc.
- **Accuracy:** More accurate than ultrasound methods
- Over 3 million tests done per year
- "NIPT reduced total testing costs per year by €4.1 million (from million €43.7 to €39.6), due to the decrease of testing costs (by €3.5 million), and ancillary-care costs (by €665,418)"
  - *Paolini, D., Camurri, L., Dionisi, M., Speranza, G., & Prefumo, F. (2017). Economic Analysis of The Use of Non-Invasive Prenatal Test (NIPT) For Prenatal Screening of Trisomy 21, 18, 13 In Pregnant Women. Value in Health, 20(9), A578.*

VIRUSES, VIRAL VECTORS, AND GENE TRANSFER – CONTINUED…

What are viruses? How do they cause infection and disease? How do we harness them in biology?

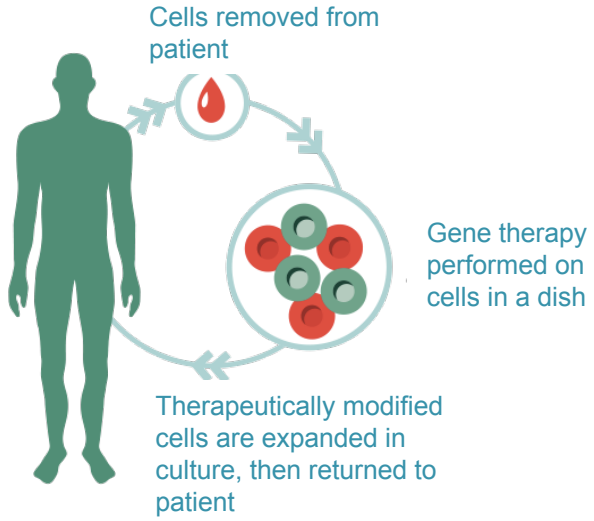
Delivery of therapeutic genes

- https://youtu.be/Ez560GnkSrE
- What?
  - What are the things that need to be delivered? (Single plasmid? Multiple plasmids? Viral vector? RNA? Protein?)
  - What are the cell type(s) it needs to target?
- Where?
  - Where in the body should it be targeted?
  - Where should it absolutely NOT go?
  - Where should the procedure take place, inside or outside the body?
- How?
  - How to deliver the payload? Viral? Non-viral?
  - How to introduce the vector? Injection? Cream? Incubation?

Delivery approaches

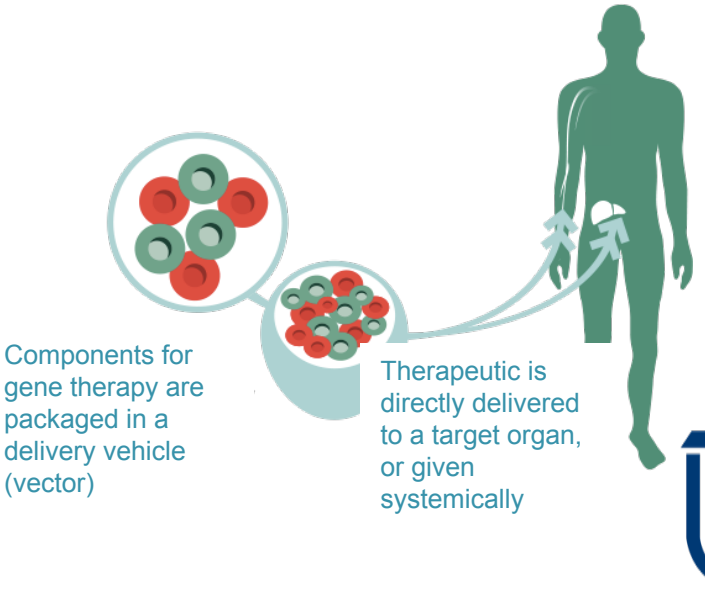
Ex-vivo delivery

- Target cells treated **outside** body
- Reduces safety risk; can screen for tumorigenic cells before giving to patient; but cannot be applied for many cell types



In-vivo delivery

- Target cells treated **inside** body
- Useful if target cells are hard or impossible to culture (e.g. brain); but cell-specific targeting is hard

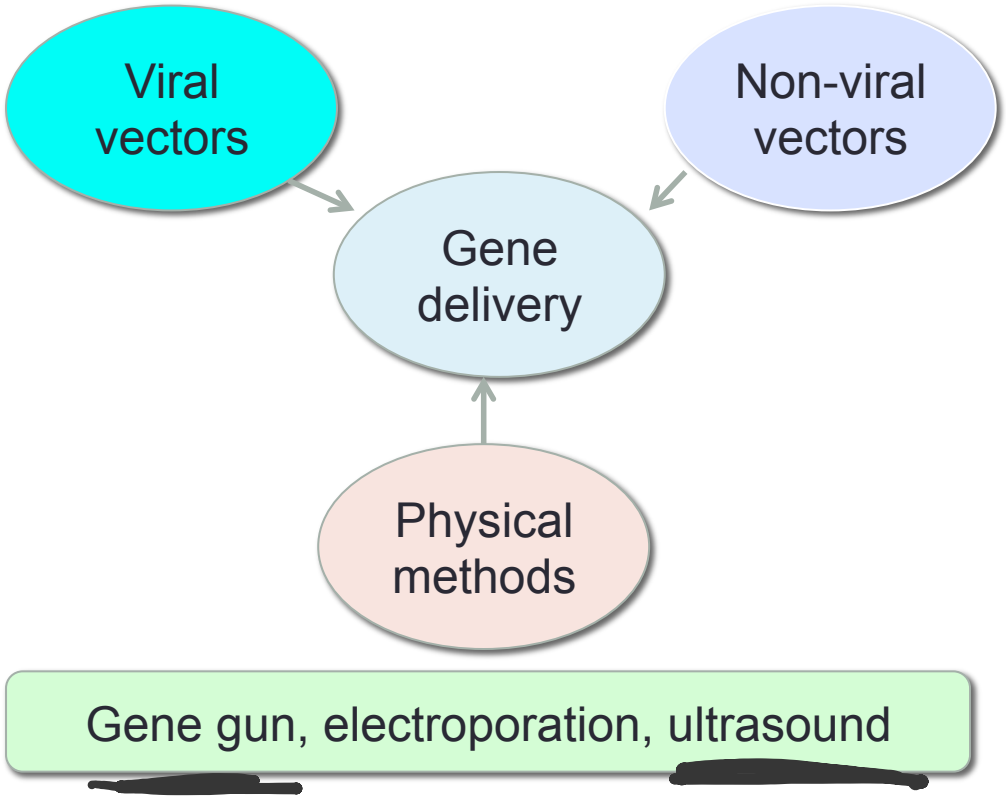


Case study – Leber congenital amaurosis trial, first in-vivo CRISPR treatment

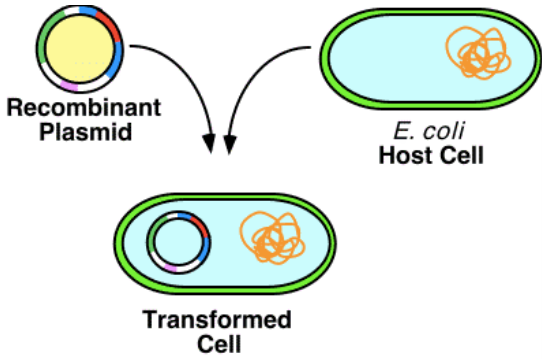
EDITAS

- "AGN-151587 (EDIT-101) is an experimental medicine delivered via sub-retinal injection under development for the treatment of Leber congenital amaurosis 10 (LCA10), an inherited form of blindness caused by mutations in the centrosomal protein 290 (CEP290) gene. The BRILLIANCE clinical trial is a Phase 1/2 study to evaluate AGN-151587 for the treatment of patients diagnosed with LCA10 and is the world's first human study of an in vivo, or inside the body, CRISPR genome editing medicine. The trial will assess the safety, tolerability, and efficacy of AGN-151587 in approximately 18 patients with LCA10."
- "LCA10, is a monogenic disorder caused by mutations in the CEP290 gene and is the cause of disease in approximately 20-30 percent of all LCA patients."
- Uses AAV – adeno-associated virus
- Uses Cas9

Delivery approaches



Delivery vectors

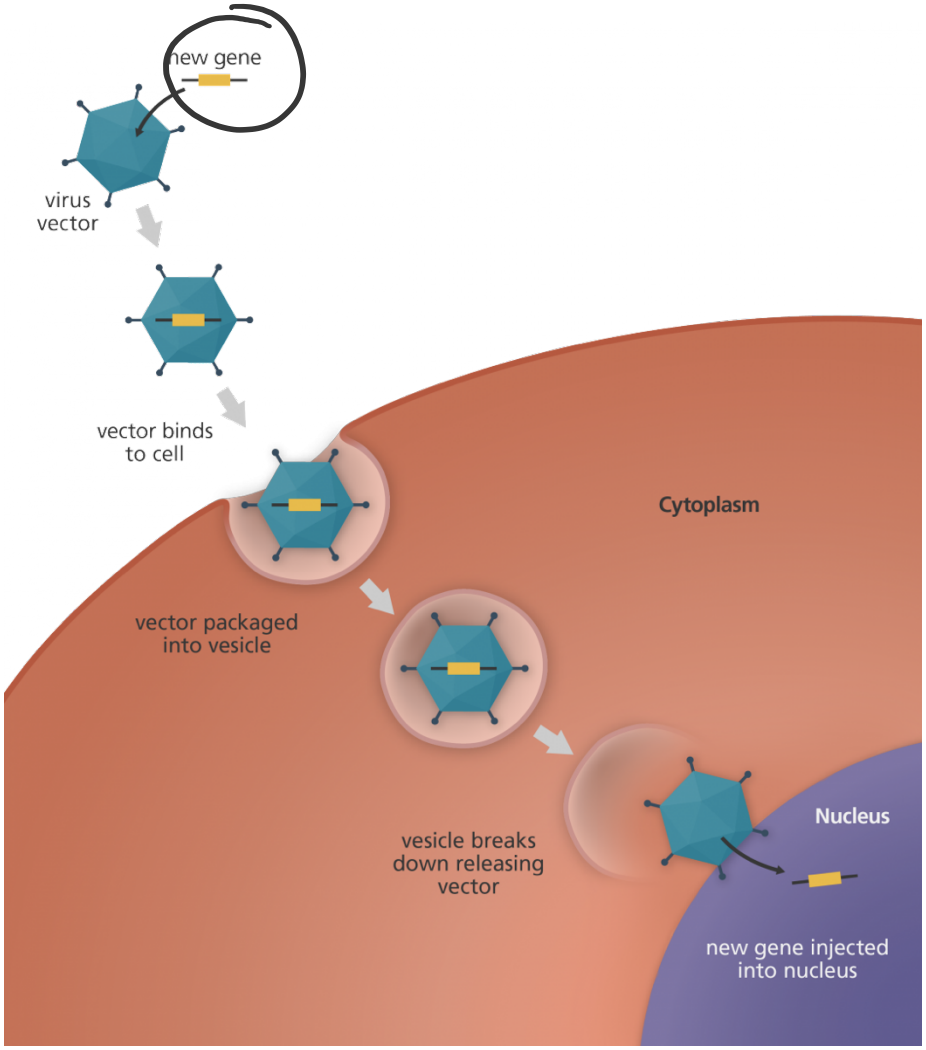


- Criteria of delivery vectors:
  - Target the right cells
  - Able to transfer and integrate genes into cells
  - Minimal harmful side effects
- Examples of types of vectors:

Adenovirus
Retrovirus
Vaccinia virus
Poxvirus
Adeno-associated virus
Herpes simplex virus
Lentivirus

Naked/plasmid DNA (gene gun)
Lipid complex
Liposomes
Peptides/proteins
Polymers
Other non-viral vehicles

Viral vectors



Choosing a viral vector

Table 1 | **The main groups of viral vectors**

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main limitations	Main advantages
Enveloped							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
HSV-1	dsDNA	40 kb* 150 kb‡	Strong for neurons	High	Episomal	Inflammatory; transient transgene expression in cells other than neurons	Large packaging capacity; strong tropism for neurons
Non-enveloped							
AAV	ssDNA	<5 kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenovirus	dsDNA	8 kb* 30 kb§	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

Thomas, Ehrhardt & Kay, Nature Reviews Genetics, 2003

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Main application: short term gene expression, for proof of concept studies

Thomas, Ehrhardt & Kay, Nature Reviews Genetics, 2003

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*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-assoc
stranded DNA.

Main application: long term expression of small genes

Note: AAV is not known to cause disease in humans, therefore lower immune risk

Thomas, Ehrhardt & Kay, Nature Reviews Genetics, 2003

Choosing a viral vector

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*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

Main application: long term expression of small and large genes; ex-vivo applications

Note: lentivirus vs retrovirus – dividing cells

Thomas, Ehrhardt & Kay, Nature Reviews Genetics, 2003

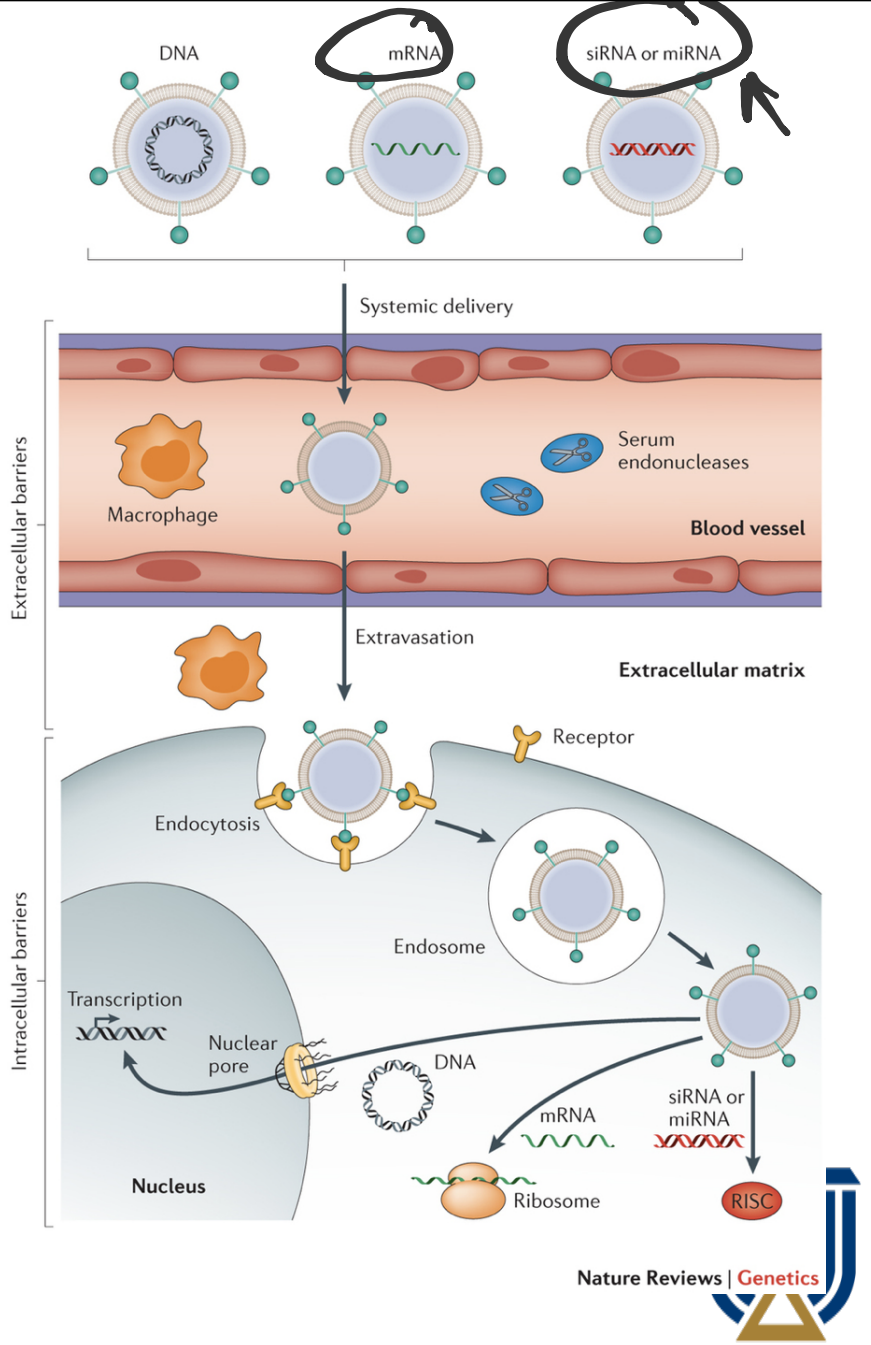
Choosing a viral vector

		Adenovirus	Adeno-associated virus	Alphavirus	Herpesvirus	Retrovirus / Lentivirus	Vaccinia virus
Particle characteristics	Genome	dsDNA	ssDNA	ssRNA (+)	dsDNA	ssRNA (+)	dsDNA
	Capsid	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Complex
	Coat	Naked	Naked	Enveloped	Enveloped	Enveloped	Enveloped
	Virion polymerase	Negative	Negative	Negative	Negative	Positive	Positive
	Virion diameter	70 - 90 nm	18 - 26 nm	60 - 70 nm	150 - 200nm	80 - 130 nm	170 - 200 X 300 - 450nm
	Genome size	39 - 38 kb	5 kb	12 kb	120 - 200 kb	3 - 9 kb	130 - 280 kb
Gene Therapy Net .com							
	Family	*Adenoviridae*	*Parvoviridae*	*Togaviridae*	*Herpesviridae*	*Retroviridae*	*Poxviridae*
Gene Therapy Properties	Infection / tropism	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing cells*	Dividing and non-diving cells
	Host genome interaction	Non-integrating	Non-integrating*	Non-integrating	Non-integrating	Integrating	Non-integrating
	Transgene expression	Transient	Potential long lasting	Transient	Potential long lasting	Long lasting	Transient
	Packaging capacity	7.5 kb	4.5 kb	7.5 kb	> 30 kb	8 kb	25 kb

http://sgugenetics.pbworks.com/f/1301871554/virus%20classification.jpg

Non-viral vectors

- Non-viral vectors can be used to deliver DNA, mRNA and short double-stranded RNA
  - siRNA and miRNA mimics must be loaded into the RNA-induced silencing complex (RISC)
  - mRNA must bind to the translational machinery
  - DNA has to be further transported to the nucleus to exert its activity

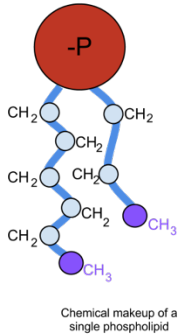


Yin et al., "Non-viral vectors for gene-based delivery", Nature Review Genetics, 2014

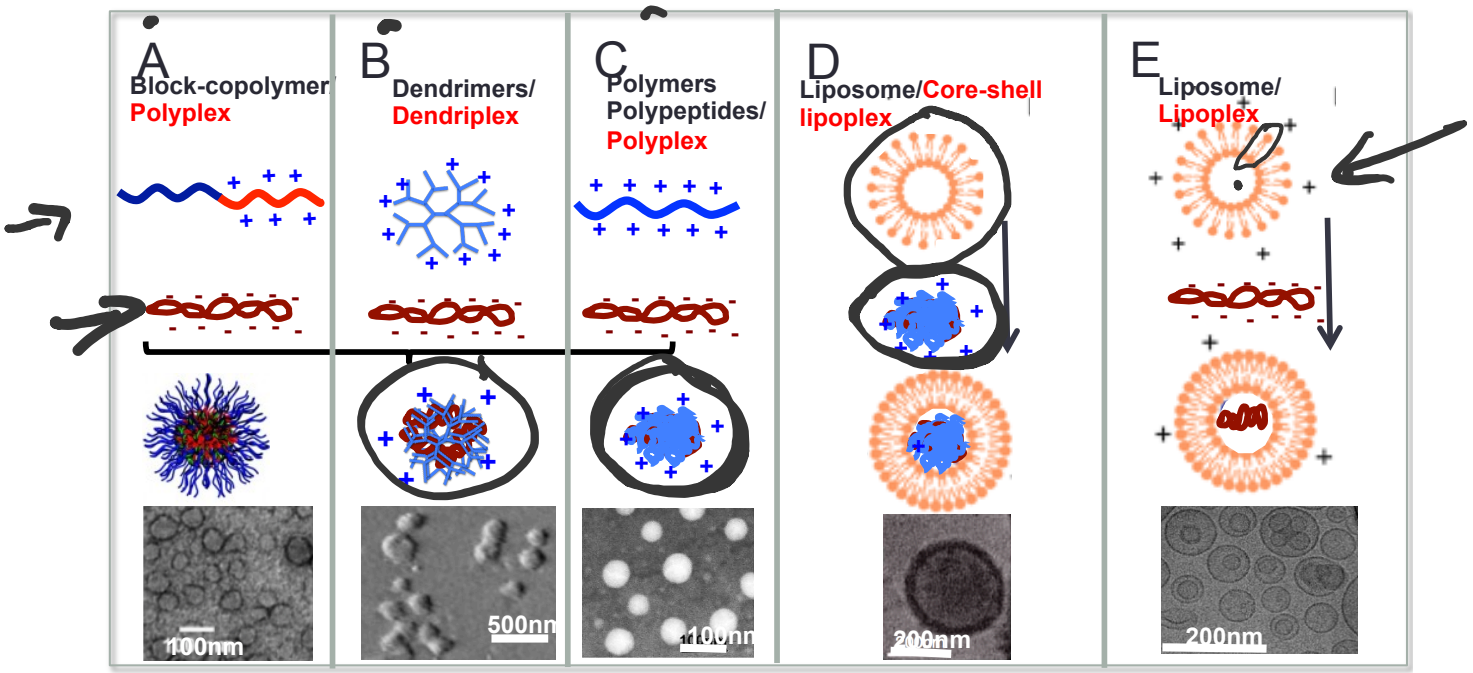
Creation of non-viral vectors

- Non-viral vectors form due to charge interactions
- https://youtu.be/RBjWwlnq3cA?t=10s
- https://youtu.be/04SP8Tw3htE?t=2m10s

A phospholipid with a hydrophilic head and a hydrophobic tail



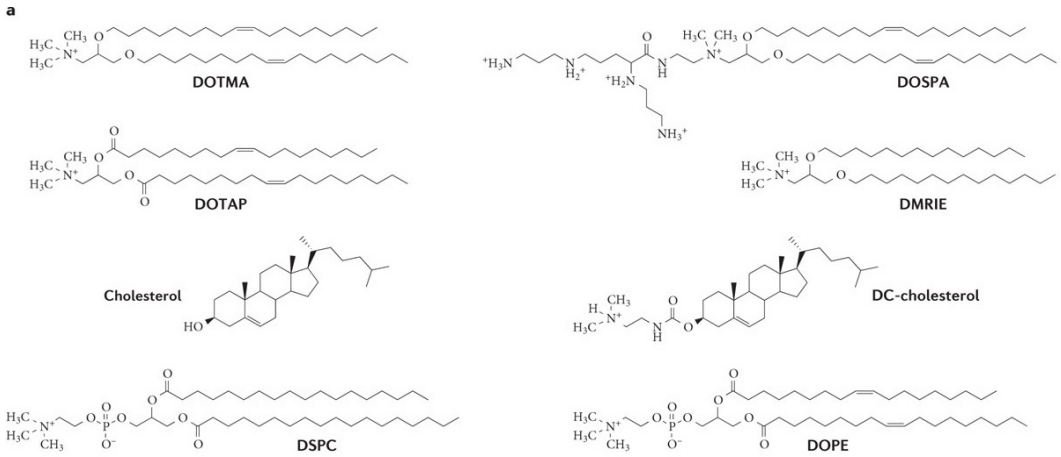
Chemical makeup of a single phospholipid

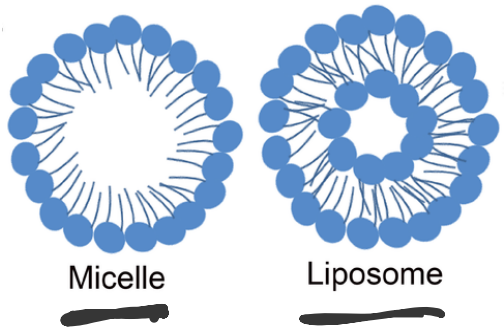


Lipoplex; liposome; lipid nanoparticle; micelle

Lipid-based vectors

- Lipid-based vectors are among the most widely used non-viral gene carriers.
- Limitations of cationic lipids include low efficacy (poor stability and rapid clearance), and tendency to generate inflammatory or anti-inflammatory responses





Yin et al., "Non-viral vectors for gene-based delivery", Nature Review Genetics, 2014
Wang et al, "Lipid Nanoparticles for Ocular Gene Delivery", J. Funct. Biomater. 2015

Polymeric vectors

- Cationic polymers are attractive due to their immense chemical diversity and potential for functionalization

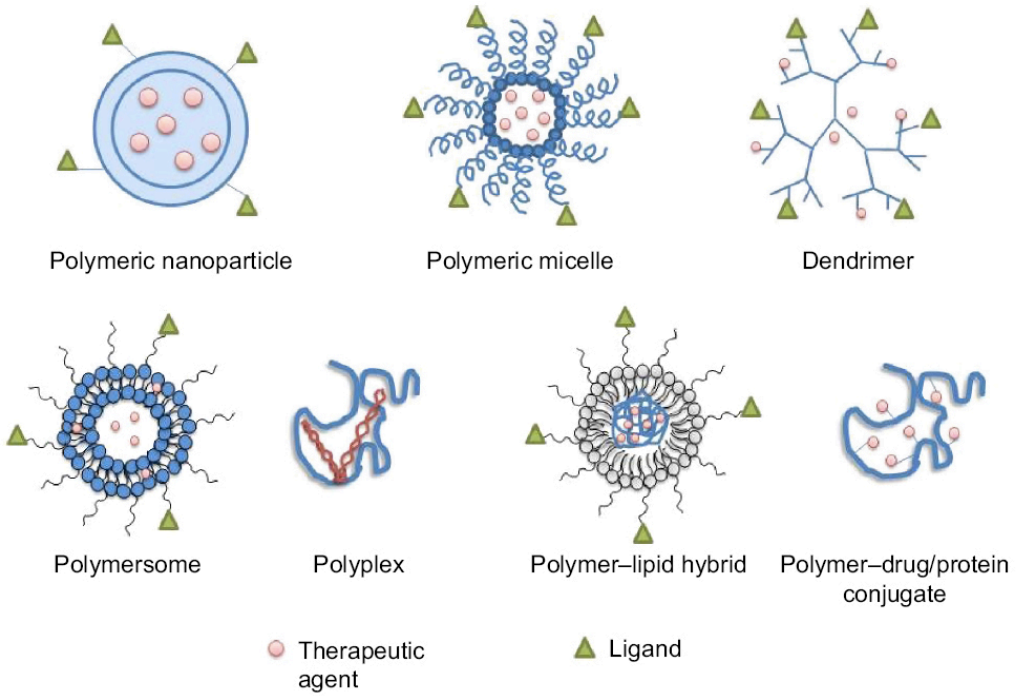
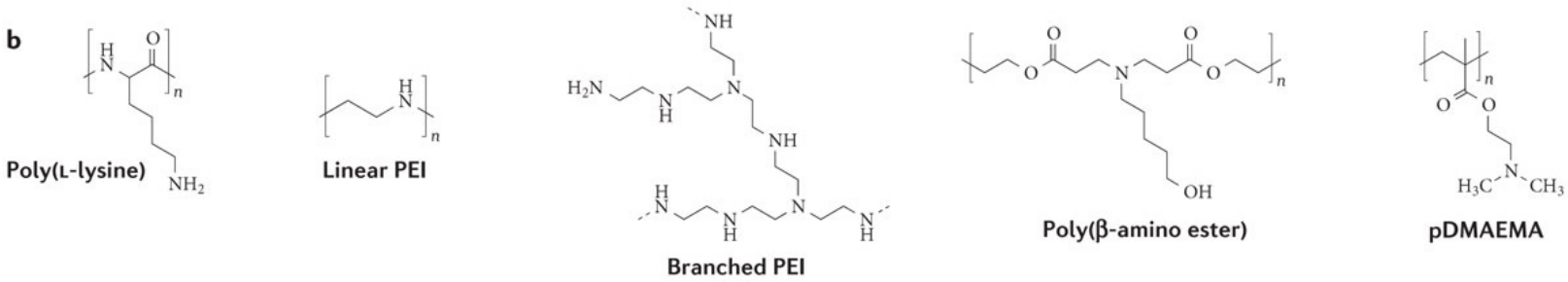


Figure 1 Schematic illustration of polymeric nanoparticle platforms.
Note: Blue color represents the polymeric platform.

Prabhu et al. "Polymeric nanoparticles for targeted treatment in oncology: current insights", Intl J Nanomedicine, 2014

Polymeric vectors

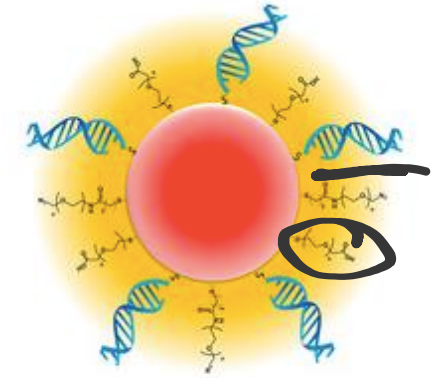
- Early examples of polymeric vectors: poly(L-lysine) (PLL) and polyethylenimine (PEI) – PEI and its variants are among the most studied polymeric materials for gene delivery
- A nitrogen atom at every third position along the polymer means PEI has a high charge density at reduced pH, which seems to aid in condensation of DNA and endosomal escape
- PEI can actually induce cytotoxic, so requires chemical modifications to improve biocompatibility and biostability



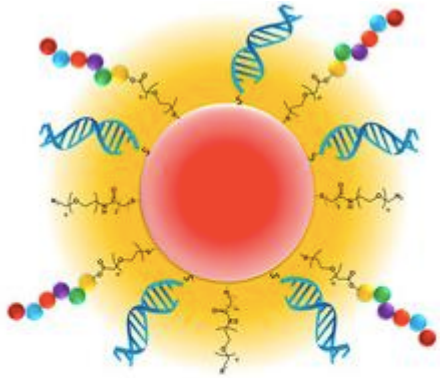
Yin et al., "Non-viral vectors for gene-based delivery", Nature Review Genetics, 2014

Inorganic and mechanical delivery

- Gold nanoparticles/nanoshells
  - Au-S bond covalently linked nucleic acids - cargo can be released from the particle by light-inducible mechanisms (e.g. pulse laser)
- Direct injection of naked DNA plasmid into the cell/tissue
- Electroporation
  - Uses short pulses of high voltage to temporarily form pores in the cell membrane so DNA can pass through



Au-siRNA



Au-Tat-siRNA

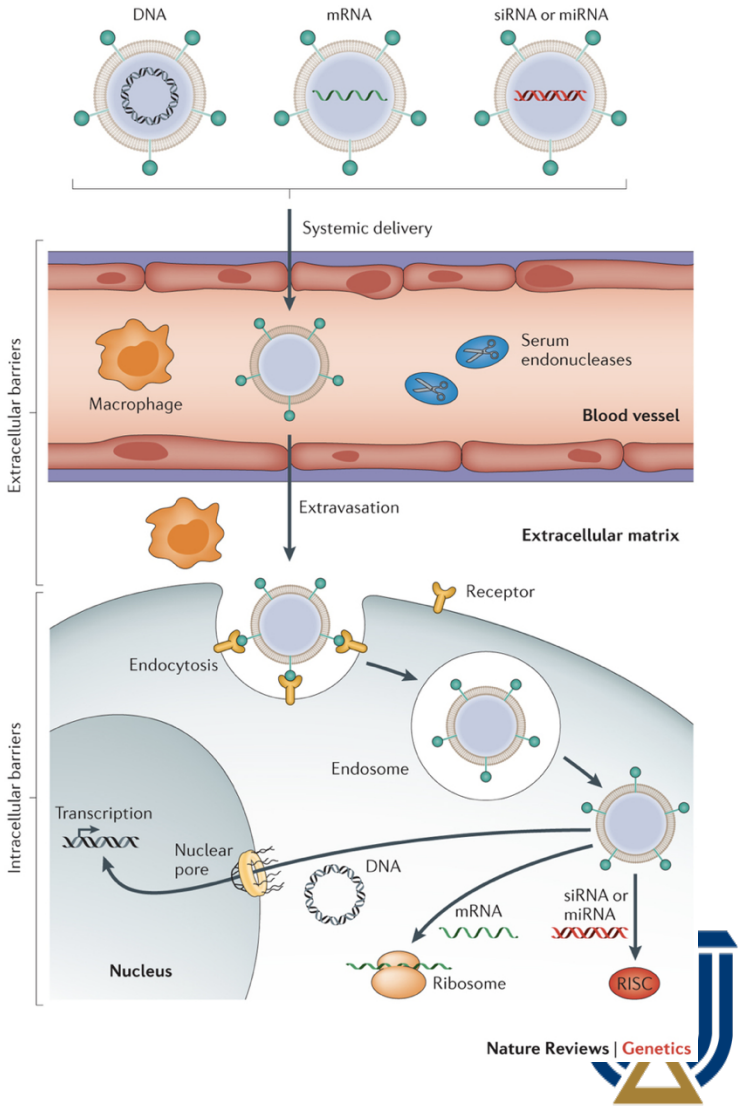
Child et al."Gold Nanoparticle-siRNA Mediated Oncogene Knockdown at RNA and Protein level, with associated Gene effects", Nanomedicine (Lond.), 2015

Inorganic and mechanical delivery

- Gene gun
  - DNA is coated onto gold particles and loaded into a device which generates a force to achieve penetration of the DNA into the cells
- Sonoporation
  - Uses ultrasound to deliver DNA into cells. The process of acoustic cavitation is thought to disrupt the cell membrane and allow DNA to move into cells
- Hydrodynamic delivery
  - Rapid injection of a high volume of a solution containing DNA/RNA into vasculature; elevated hydrostatic pressure helps molecules enter the cell

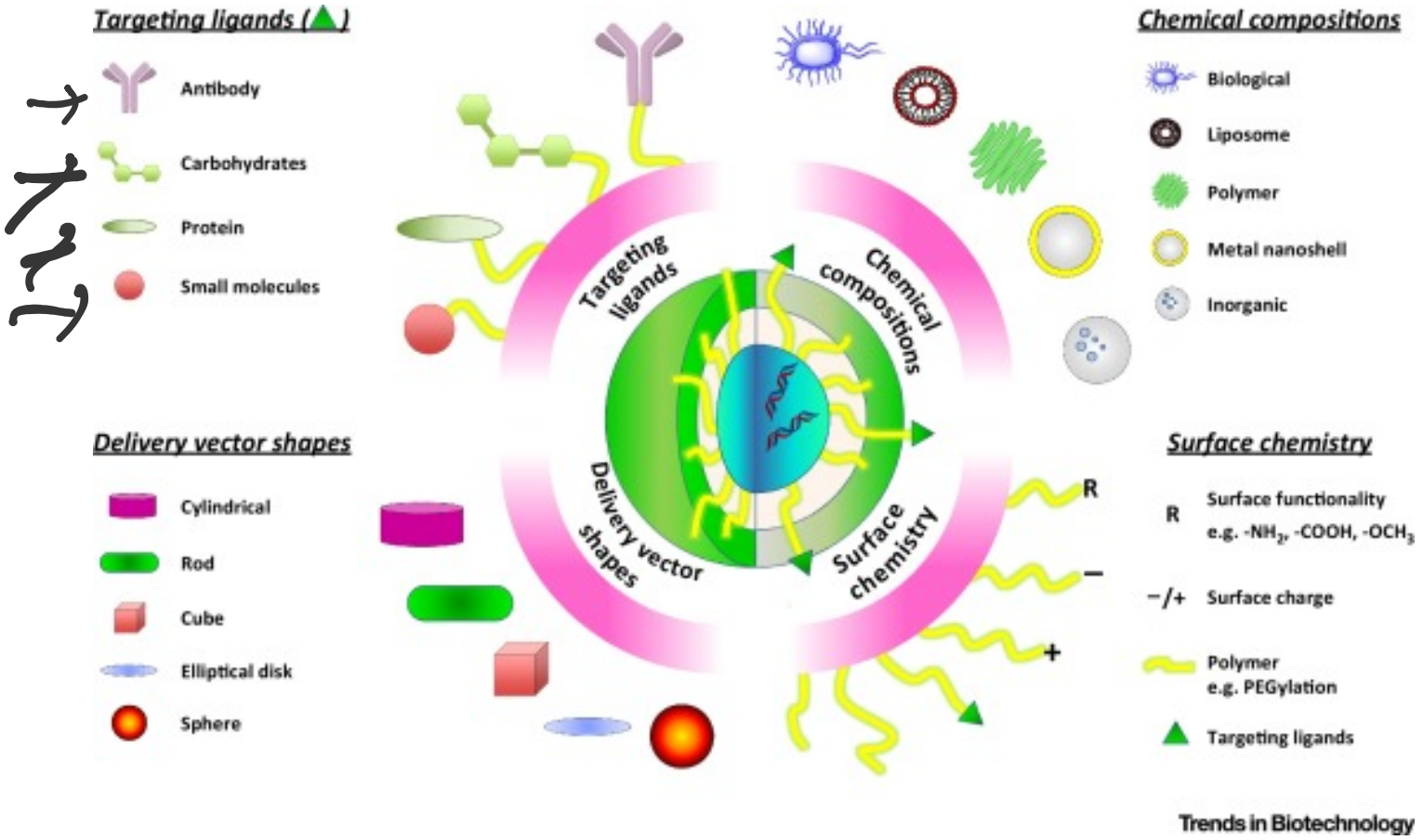
Designing non-viral vectors

- To survive from outside to cell target, non-viral vectors need to:
  - Avoid degradation by serum endonucleases and evade immune detection, e.g. by chemical modifications of nucleic acids/encapsulation of vectors
  - Avoid renal clearance from the blood and prevent nonspecific interactions, e.g. using polyethylene glycol (PEG) or through specific characteristics of particles
  - Extravasate from bloodstream to target tissues, e.g. by using certain characteristics of particles and specific ligands
  - Mediate cell entry and endosomal escape, e.g. by specific ligands and key components of carriers



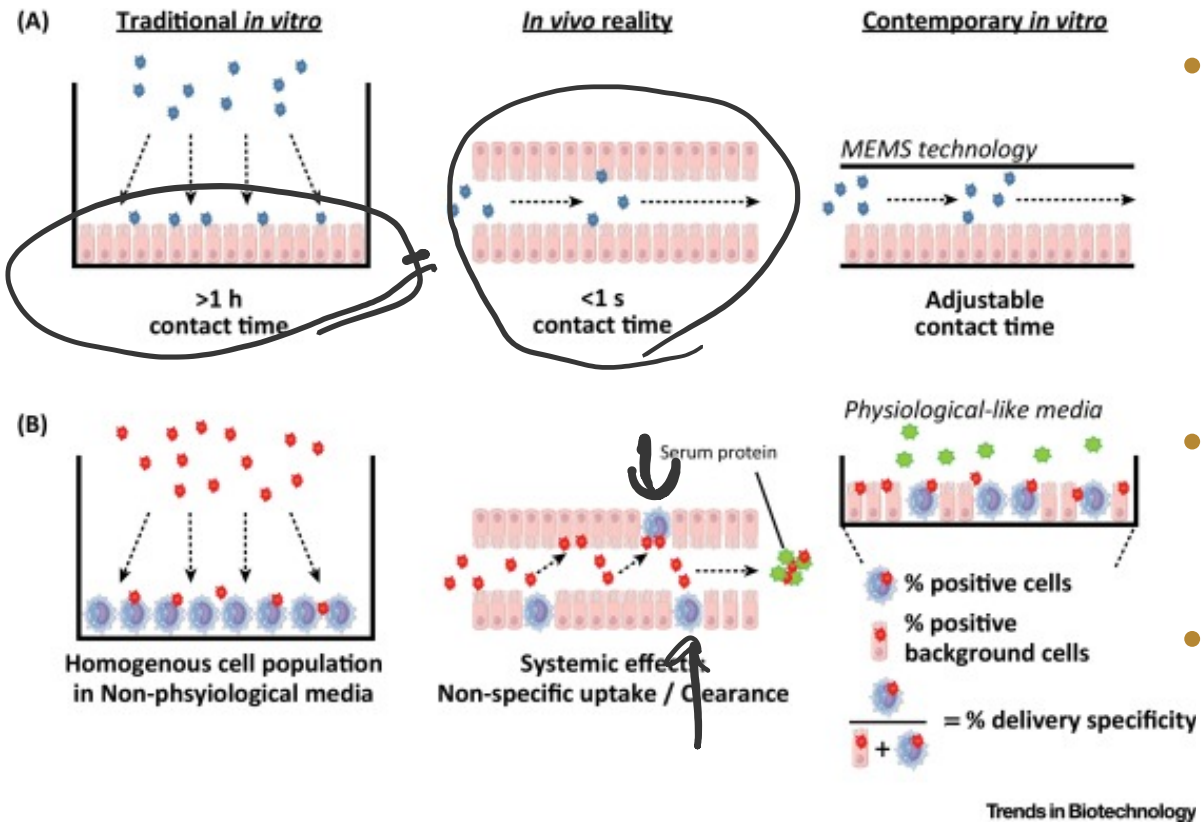
Yin et al., "Non-viral vectors for gene-based delivery", Nature Review Genetics, 2014

Designing non-viral vectors



Hill et al. "Overcoming Gene-Delivery Hurdles: Physiological Considerations for Nonviral Vectors", Trends in Biotechnology, 2015

Challenges in designing non-viral vectors



- Balancing protecting vs. releasing the cargo
- Endosome escape
- Nuclear entry (DNA)

Hill et al. "Overcoming Gene-Delivery Hurdles: Physiological Considerations for Nonviral Vectors", Trends in Biotechnology, 2015

Pros and cons of viral vs. non-viral vectors

Viral vectors

Pros

1. They are very efficient, and the rate of successful gene expression is very high
2. Naturally, we can select viruses to target specific cell-types

Cons

1. Size of cargo is restricted
2. They can cause immune response in patients which reduce treatment effectiveness, or worst case case death
3. Integration mechanism could cause mutations/cancer

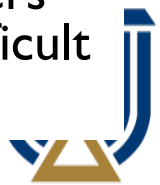
Non-viral vectors

Pros

1. Low immune risk
2. No cargo size limitation
3. Can design intelligently according to needs
4. More cost-effective and available because they are easier to make

Cons

1. Efficiency is much lower than viral systems
2. Difficult to design parameters precisely/accurately and difficult to model in-vivo dynamics



MODEL SYSTEMS

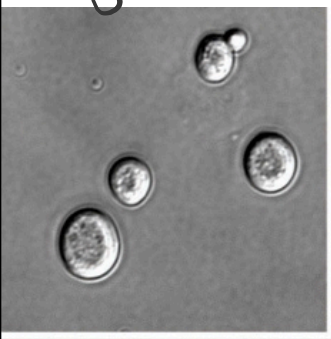
Cell models and animal models

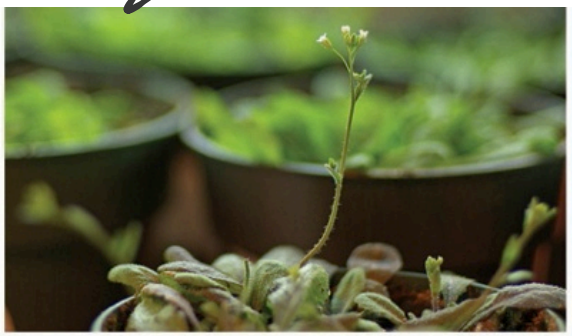
Xenopus (frog) — Genome available, similarities to human; small, easy to breed, large # per generation. — Not human.

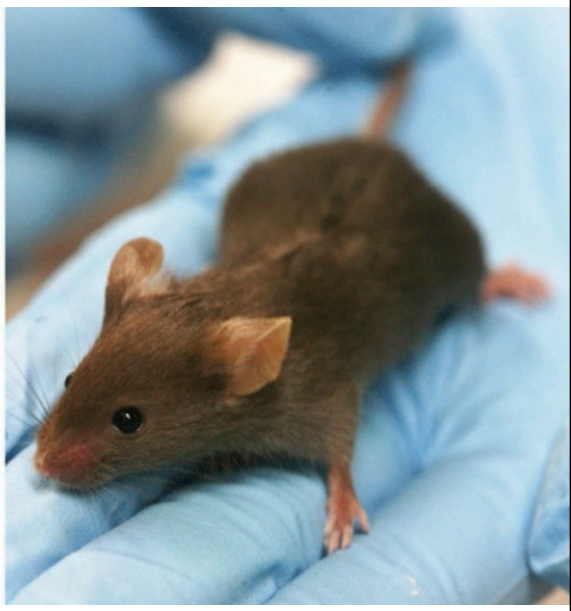
Model organism	Why they are useful	Pros	Cons
Mouse	similarity to human biology; easy to raise (comp to monkey); we can make genetic mod.		Keep them – cost handling us. cells or fish for example; ethical considerations.
Zebrafish	Similarities in gene structure; embryo develop outside + can image/observe directly.	they breed quickly. Small + easy to raise.	v. small; fish still v. diff from human.
Drosophila (fruit fly)	Genetic mutation studies; easy to cross + see phenotype. quick to breed.		Not like human.
E. coli	Make lots of them + study genetic evolution. Very easy to manipulation.		Prokaryotes, v. diff from human.

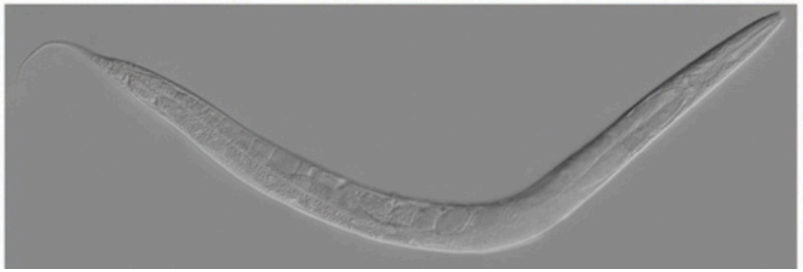
yeast

arabidopsis









C. elegans

Model organism	Why they are useful	Pros	Cons
E. coli (bacteria)	Ask basic questions in very simple system; understand evolution; use as a tool (e.g. making plasmids)	Grows quickly, easily; well understood, lots of prior data; easy to manipulate	No nucleus, no chromatin; lacking some key processes e.g. glycosylation
S. cerevisiae (yeast)	Ask basic questions in very simple eukaryotic system (e.g. homologous recombination)	Similar as E. coli, euks, have nuclei, same basic cell processes as human cells (mitochondria etc.)	NOT HUMAN
C. elegans (roundworm)	Simplest organism with a nervous system; nervous system is completely mapped; development/cell lineage also fully mapped, good for studying development; aging	Similar to above; also transparent (imaging)	NOT HUMAN

Model organism	Why they are useful	Pros	Cons
D. melanogaster (fruit fly)	Sexual reproduction; rapid generation time; homologs of human disease genes; vision; more complex organs	Most convenient for genetics, extensive knowledge of development	NOT HUMAN
D. rerio (zebrafish)	Visual-brain connection; developmental toxicity; full immune system	Transparent during development	NOT HUMAN
A. thaliana (plant)	Useful for genetics; small genome for plant	Short life cycle for plants; robust organism	NOT HUMAN
M. musculus (mouse)	Most common mammalian animal model; easily manipulated genetically	Quickly reproduce, can manipulate genetics; can make xenografts.	NOT HUMAN
Non-human primates (monkey, chimpanzee, etc)	Most similar to humans	Much more closely resemble human than all other models, however-	NOT HUMAN; very expensive; potential ethical issues

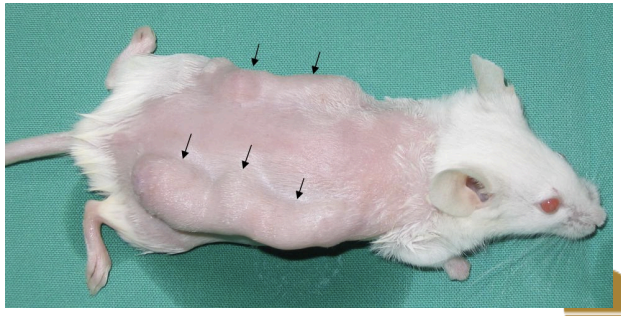
lemur


© Mike Baldwin / Cornered
Don't worry, I had the same thing, and they cured me!
CARTOONSTOCK .com
Search ID: mba0892

Various mouse models

- By Breeding
  - **NOD** – non-obese diabetic; model of Type I diabetes (juvenile diabetes, autoimmune)
  - **Nude** – immunodeficient mice, no hair, no thymus, no T-cells, so no immune response; cannot fight infection, transplants, xenografts
  - **SCID** – severe combined immunodeficient: almost no immune system; crossing with other mutations helps to create more efficient strains for specific study
    - Prkdc deficient mice
    - RAG1/RAG2 knockout mice

Nude immunodeficient mouse



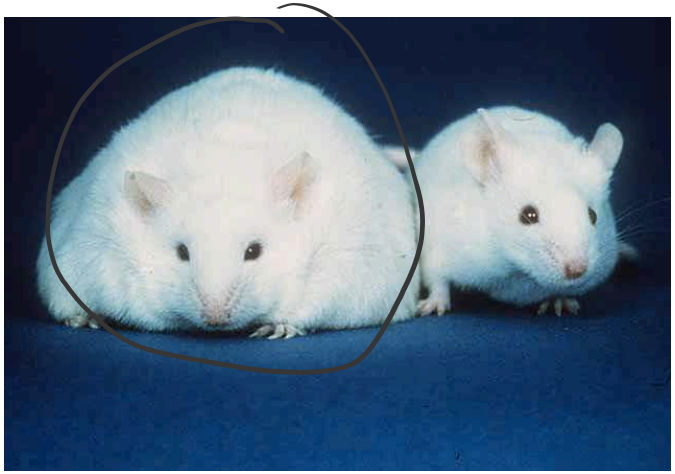
SCID mouse with xenografts

Nude mouse: By Kuebi = Armin Kübelbeck (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons

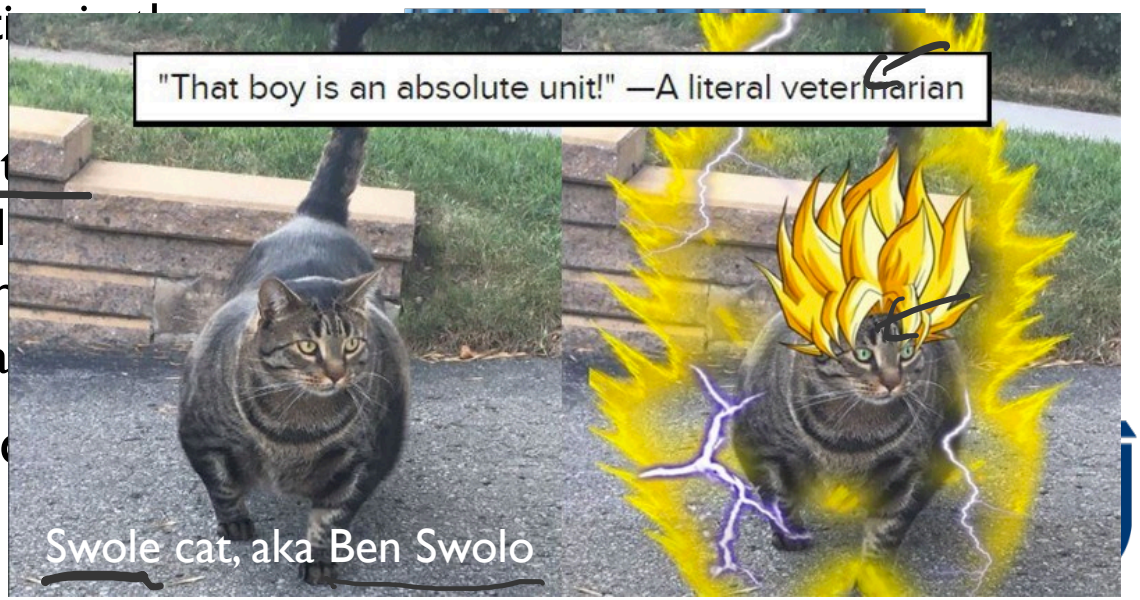
SCID mouse: http://ksvm.agri.huji.ac.il/personalpages/ny-shpigel/XenograftModel.html

Various mouse models

- **Knock-out** – loss of function of a specific gene
  - **Obese mouse**: a model of Type II diabetes (metabolic disorder); mutation in gene Lep results in no production of the hormone leptin, which leads to obesity and excessing eati
    mouse
  - **"Mighty mouse"**: myost
    that results in uninhibited
    growth and differentiation
    an effort to treat muscula
- **Knock-in** – gain of fun
  new gene

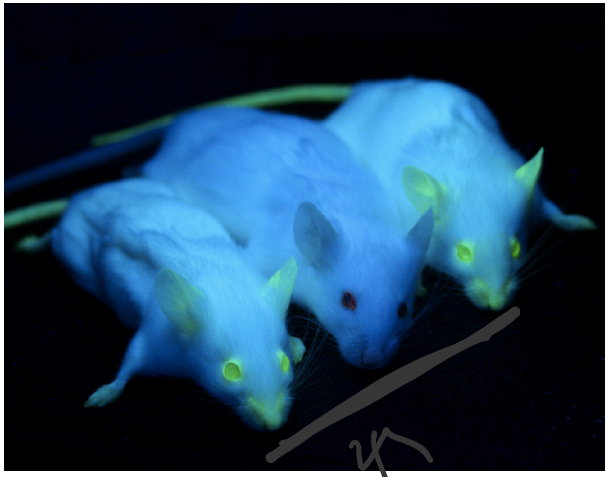


obese mouse next to wild type



Swole cat, aka Ben Swolo

Various mouse models



- Transgenic organism – obtained by introducing nucleic acid that modified the genome of the organism, and resulted in STABLE change to the organism, i.e. modification was passed down in the germline.
  - GFP mice
  - Oncomice – introducing activated oncogenes into the mouse to increase chance of mouse getting cancer

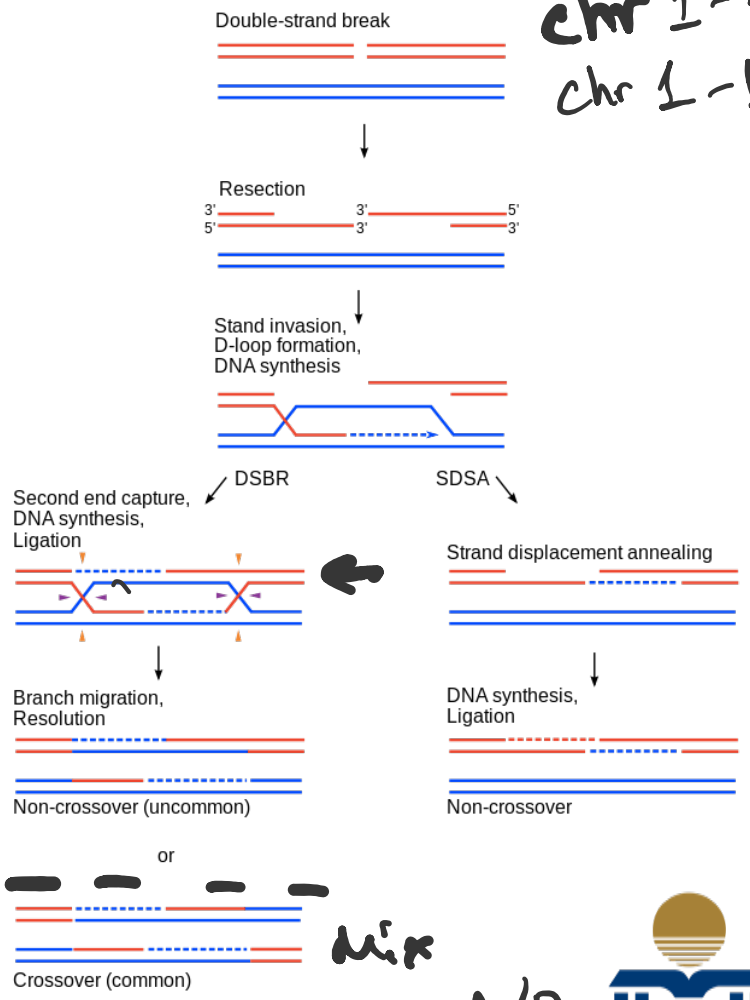
Green fluorescent protein.

Remember homologous recombination?

- Requires homologous DNA to be present
- Helps repair double strand breaks
- Can also happen during eukaryotic meiosis and in bacteria/viruses to generate genetic diversity (cuts are made 'on purpose')
  - Meiosis: process of generating gametes
  - Gametes: e.g. sperm or egg
- Facilitated/regulated by proteins
- https://www.youtube.com/watch?v=86JCMM5kb2A

HDR

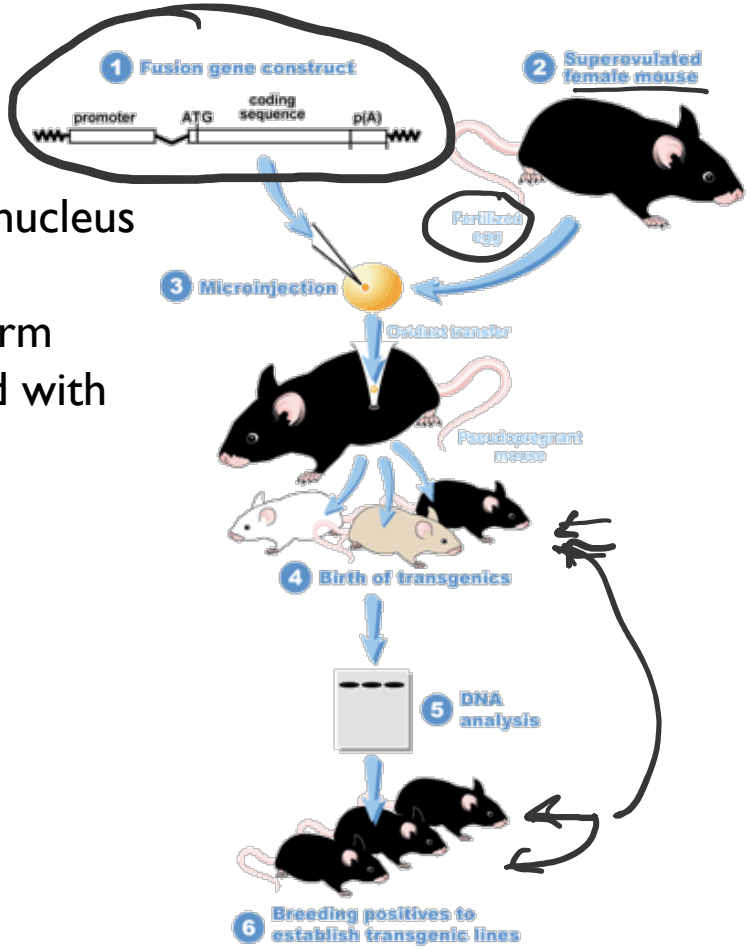
chr 1-A
chr 1-B



mix
copy A/B

Making a transgenic mouse

Injection into pronucleus of fertilized egg (nucleus from sperm before it has fused with nucleus of egg)

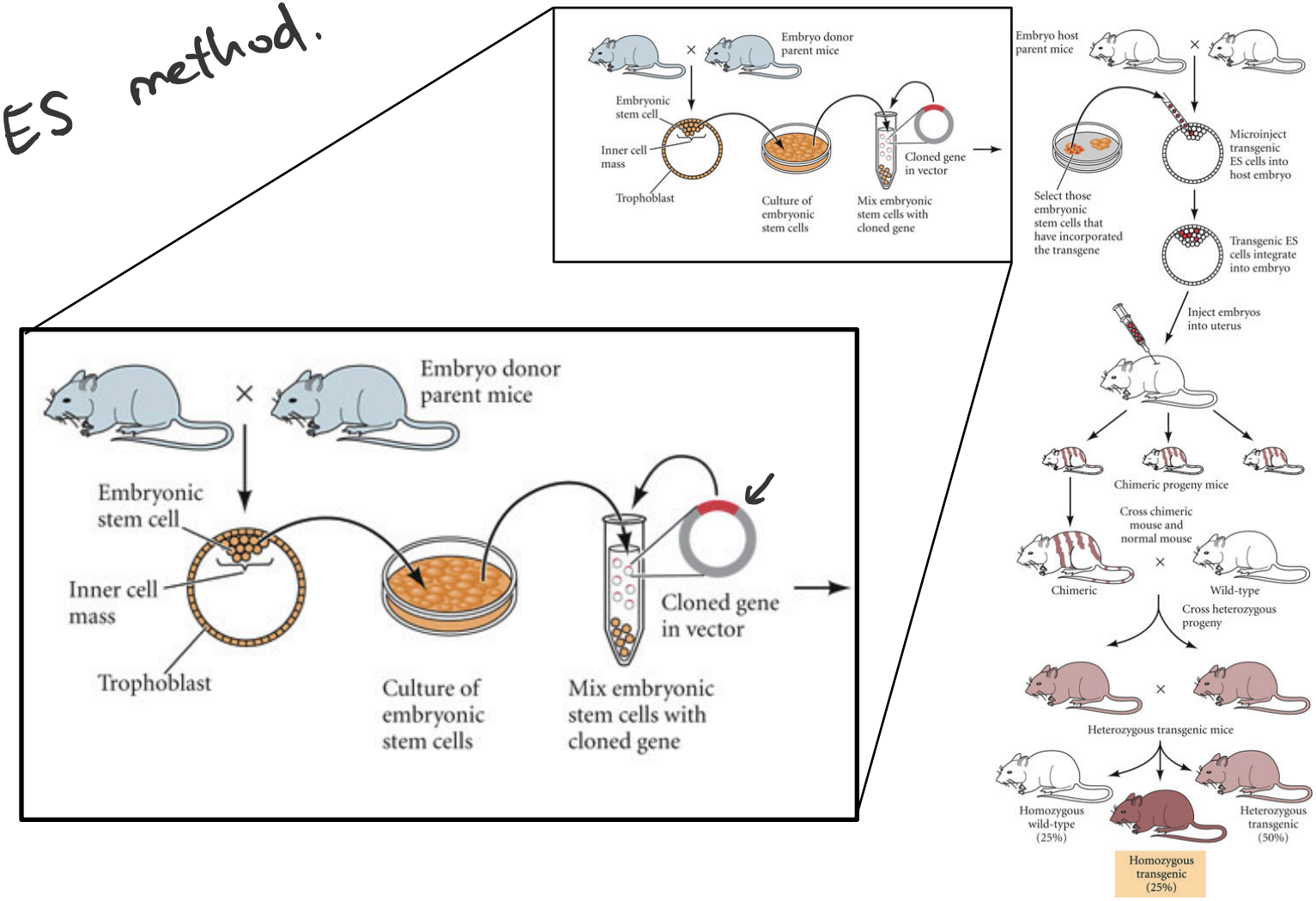


pronuclear injection.

https://healthsciences.ucsd.edu/research/moores/shared-resources/transgenic-core/services/Pages/pronuclear-injection.aspx

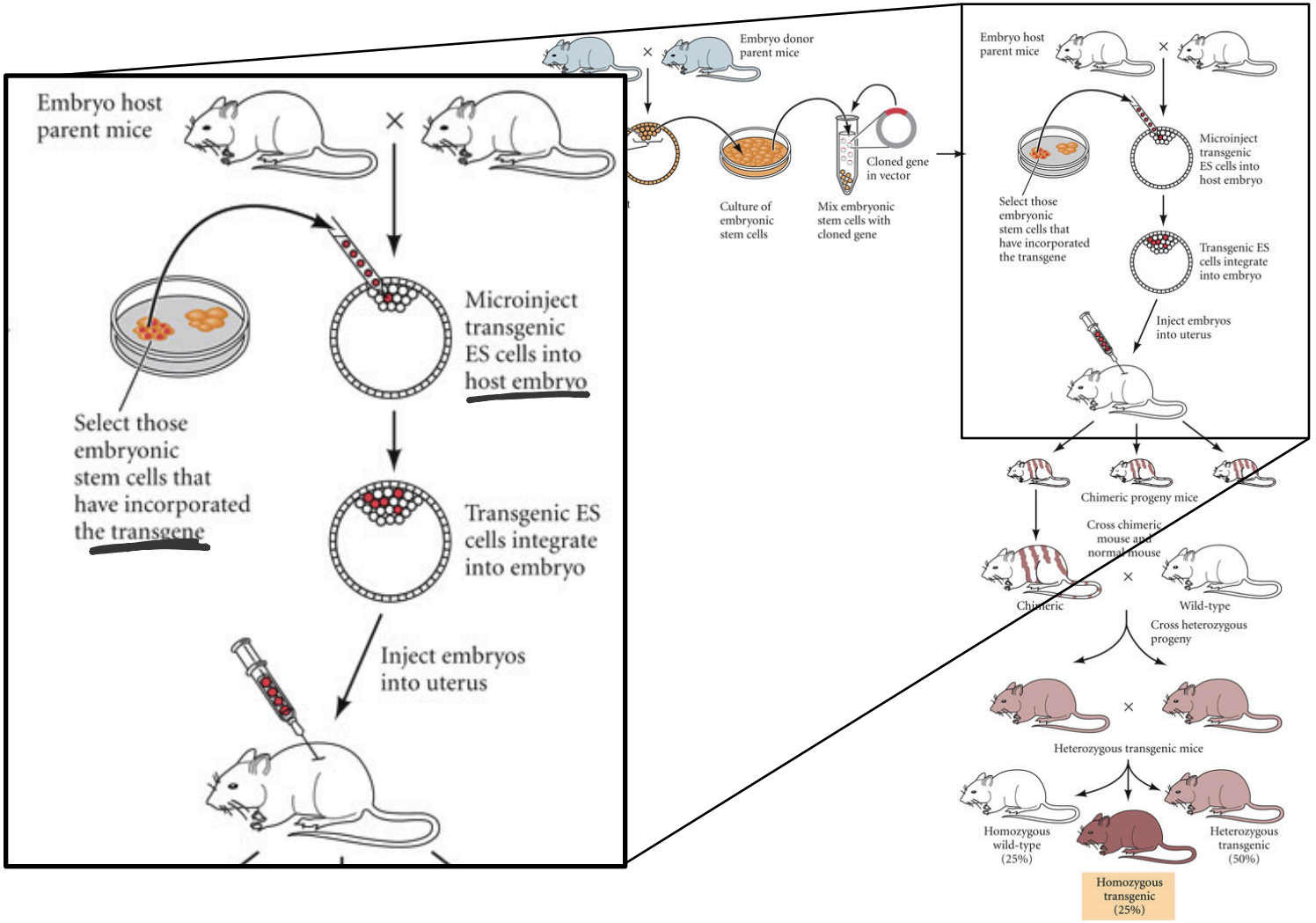
Making a transgenic mouse

ES method.



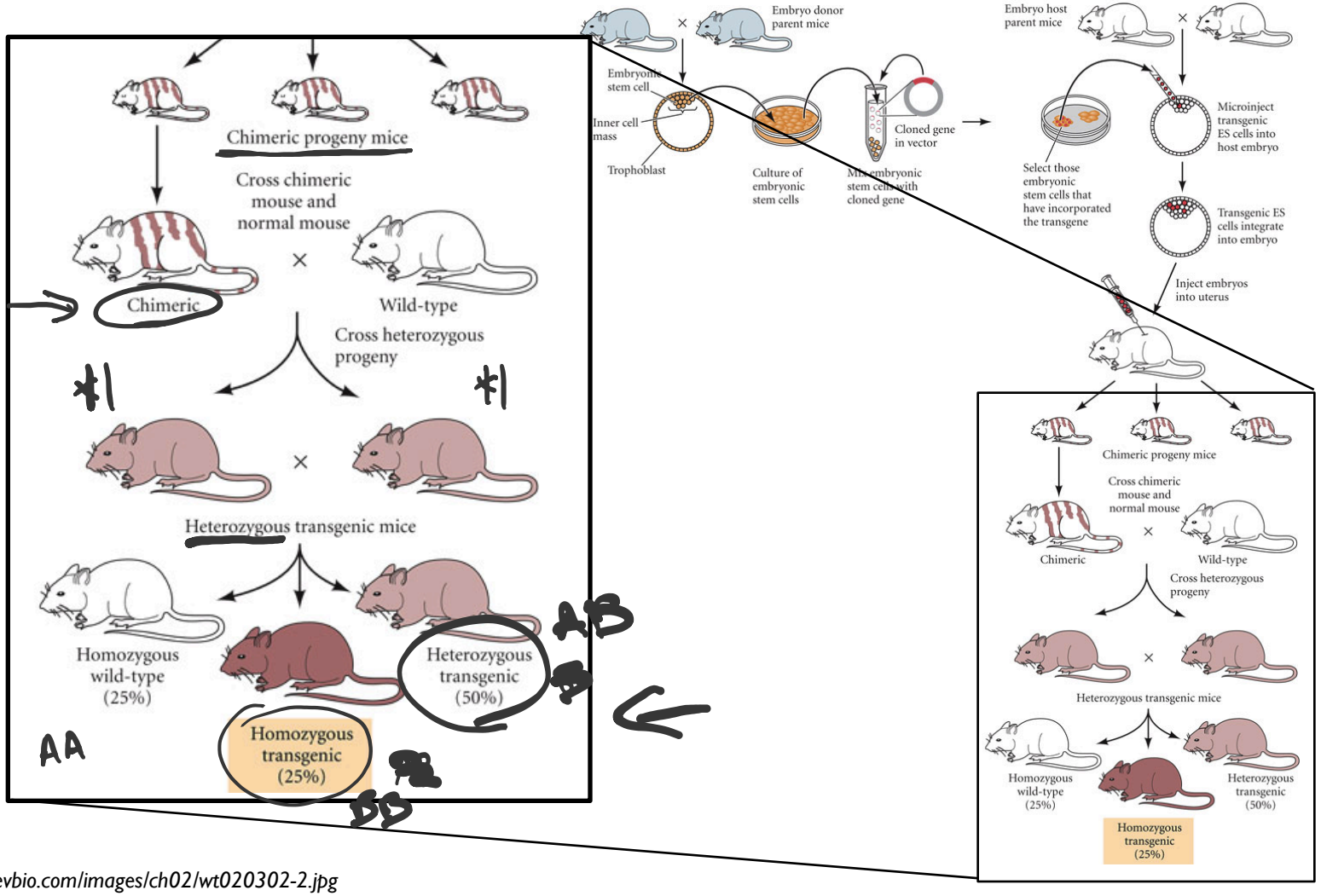
http://10e.devbio.com/images/ch02/wt020302-2.jpg

Making a transgenic mouse



http://10e.devbio.com/images/ch02/wt020302-2.jpg

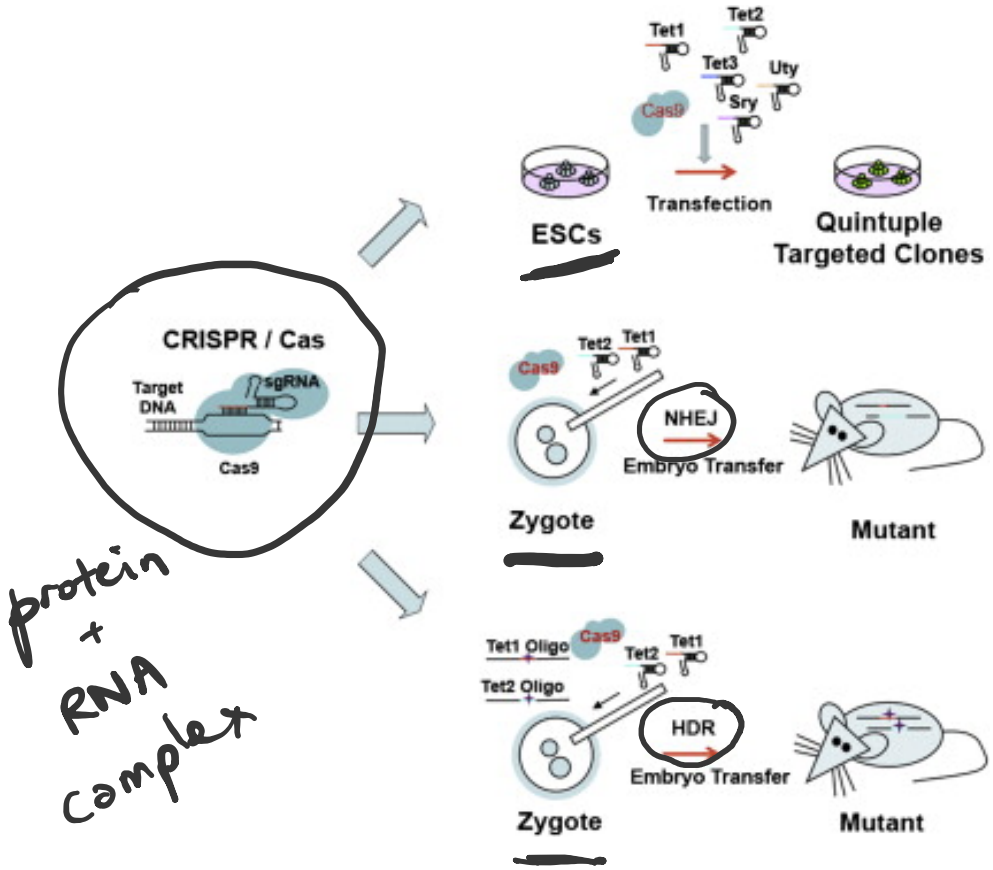
Making a transgenic mouse



http://10e.devbio.com/images/ch02/wt020302-2.jpg

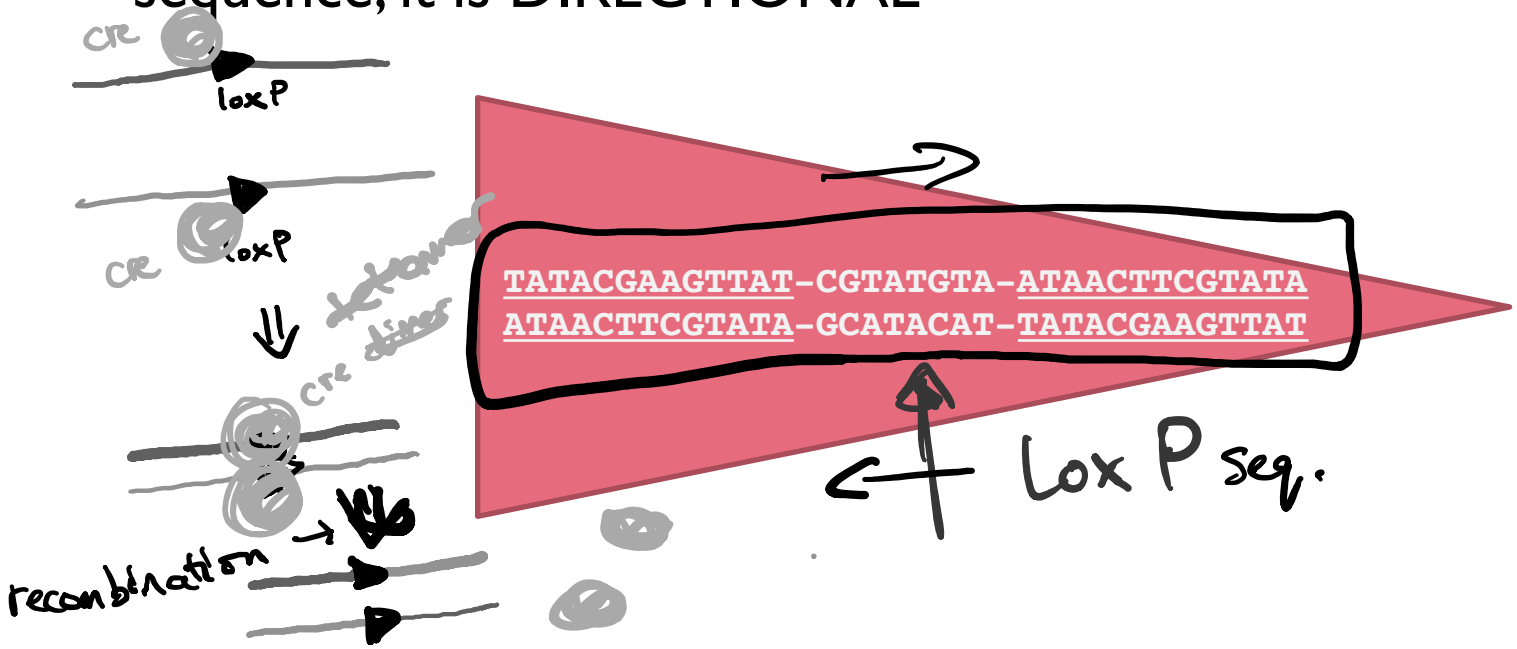
Making a transgenic mouse

- One-step generation of transgenic animal using CRISPR/Cas9



Site-specific recombinase technology

- Cre-loxP system (https://youtu.be/zOStRhccn6M?t=4s)
- Cre recombinase is an enzyme that can induce recombination at specific location, namely at:
- LoxP flanked sites. LoxP is simply a 34bp long recognition sequence; it is DIRECTIONAL



Site-specific recombinase technology

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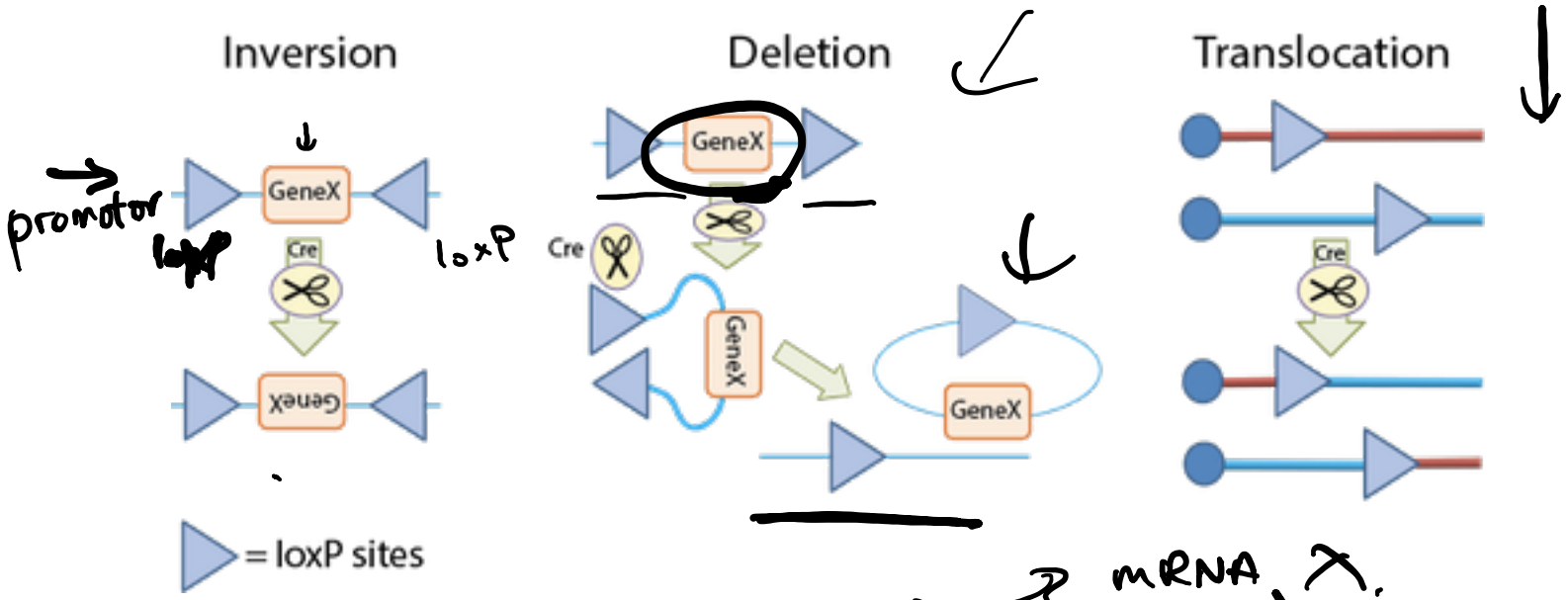
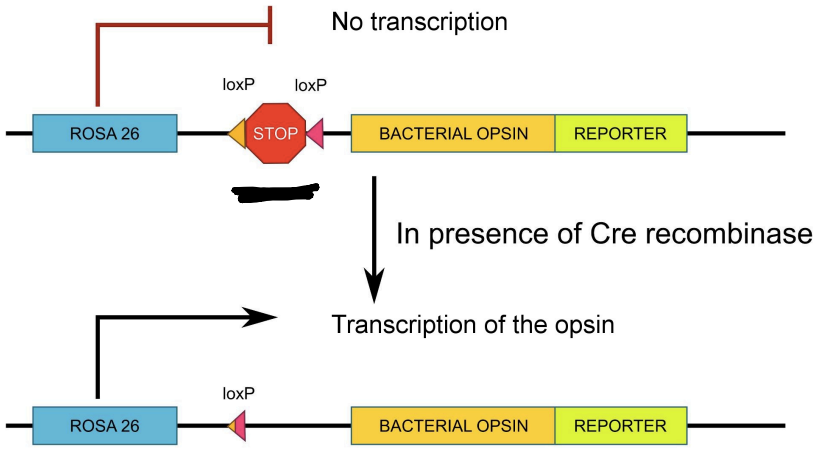


https://www.addgene.org/cre-lox/

Site-specific recombinase technology

- **Cre-dependent gene expression:** If you put a stop codon flanked by loxP in front of a gene (lox-stop-lox), then transcription of the gene will be stopped unless cre is there to remove the stop codon and allow transcription to proceed



- **Cre-dependent gene knock-out:** If you put a gene flanked by loxP, then the gene will be functional until cre is activated, then it will be removed and not functional.

http://biology.stackexchange.com/questions/8612/optogenetics-how-do-microbial-opsins-work

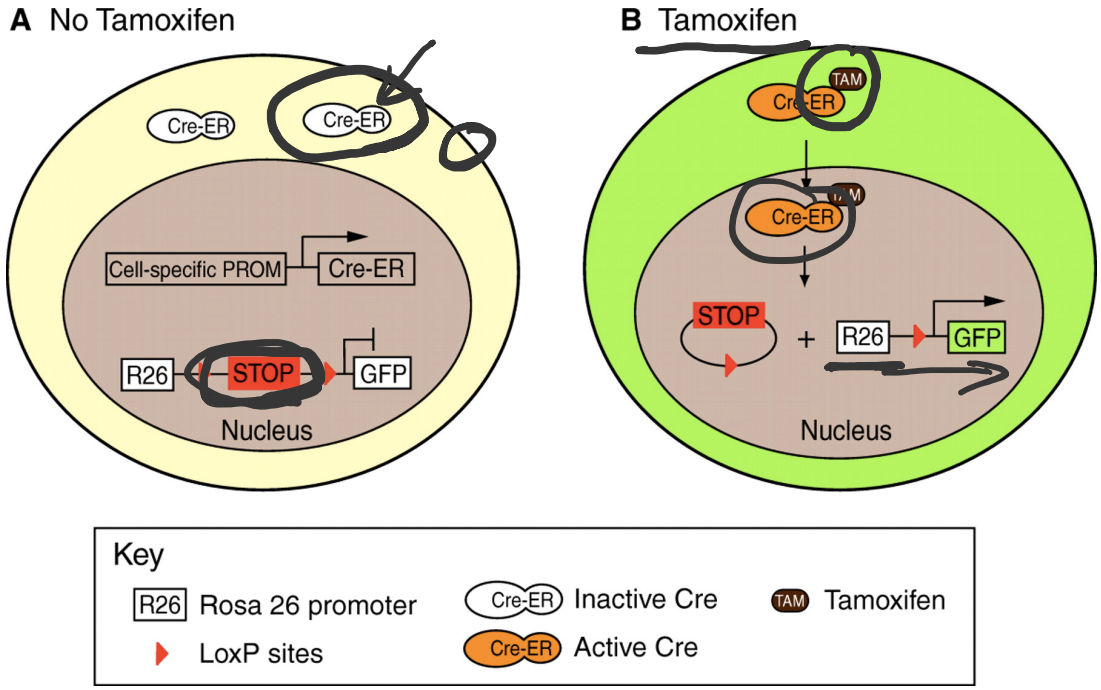
Site-specific recombinase technology

- **Controlling activation of Cre:**
  - **Inducible Cre:** These constructs require the addition of an exogenous ligand (e.g. tamoxifen) to activate Cre. Use this to control precisely when cre is activated
  - **Promoter-regulated Cre:** The promoter region defines the areas in which Cre will be expressed. Control whether Cre is expressed globally under a common promoter, or expressed only in a subset of cells under a more specific promoter (e.g. Rho-Cre is expressed in the retina).
  - **Fluorescent Cre:** The fusion of Cre to a fluorescent reporter enables visualization of Cre expression.

https://www.addgene.org/cre-lox/

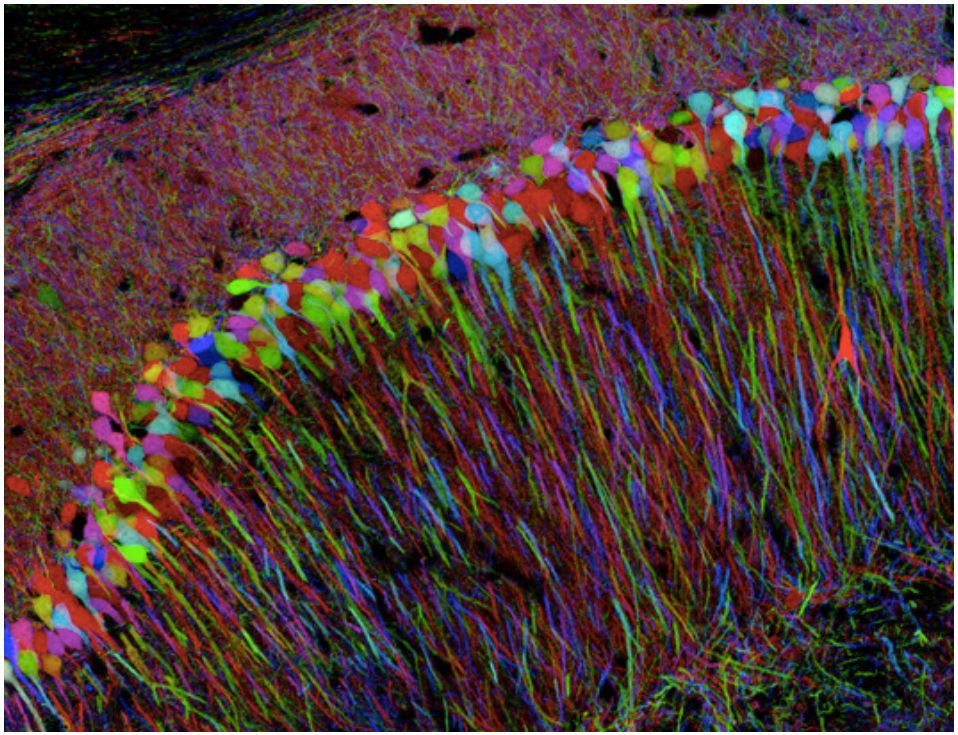
Site-specific recombinase technology

Rosa 26 – promoter in embryonic stem cells



Site-specific recombinase technology

- BRAINBOW
- https://www.youtube.com/watch?v=XeIFMI19VME



Additional resources

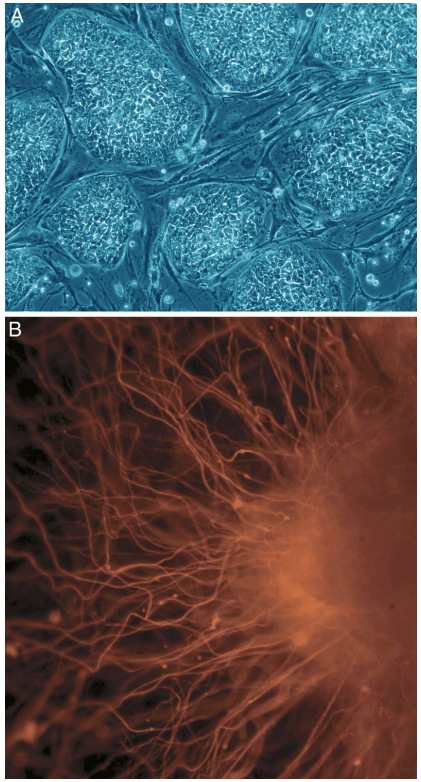
- How to choose the right model organism?
  - Literature reviews
  - Jackson labs guide for mice: https://www.jax.org/news-and-insights/2006/march/choosing-an-immunodeficient-mouse-model
- Using computational models instead?
  - Karr JR, Sanghvi JC, et al., Cell, 2012, A whole-cell computational model predicts phenotype from genotype, https://www.ncbi.nlm.nih.gov/pubmed/22817898
- More info about site specific recombination:
  - Addgene website: http://blog.addgene.org/plasmids-101-cre-lox
  - THE CRE-LOX AND FLP-FRT SYSTEMS: https://www.jax.org/news-and-insights/2006/may/the-cre-lox-and-flp-frt-systems
  - CRE/LOX BREEDING FOR DUMMIES: https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding-for-dummies
  - 12 things you don't know about Cre-lox: https://www.jax.org/news-and-insights/jax-blog/2013/september/a-dozen-facts-you-didnt-know-about-cre-lox

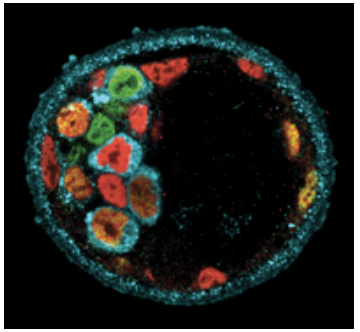
STEM CELLS AND REGENERATIVE MEDICINE

Stem cell potency, induced pluripotency, cancer stem cells, stem cell niches, and challenges of regenerative medicine

What is "potency"?

- Potency – ability of cell to take on different fate
- Totipotent – has potential to take on all fates
  - Zygote, very very early embryo
- Multipotent/Pluripotent – fates are narrowed down, restricted to a few fates
  - Commitment – process of committing to some lineage; choices of fates are reduced
  - Embryonic stem cells, adult stem cells
- Bipotent – can be one of two fates
- Unipotent – can only have one fate
  - Differentiated/fully committed cells



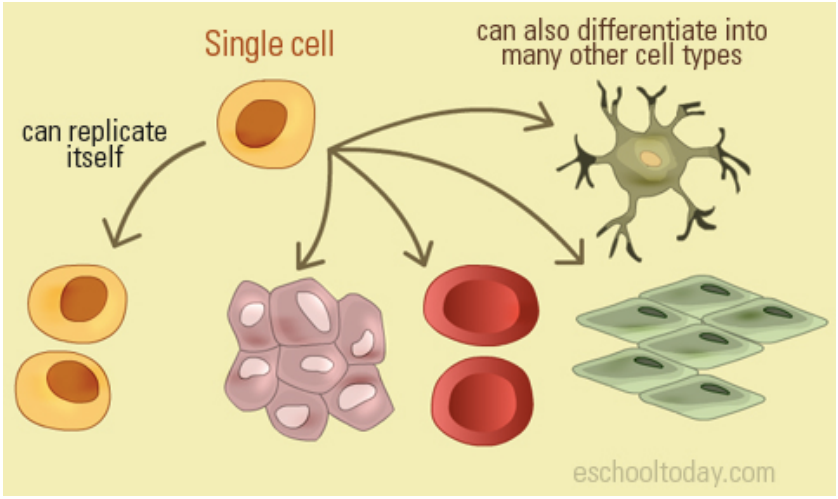


Molecular heterogeneity during mouse blastocyst patterning. Cells expressing Nanog (green), Gata6 (red) or Serpinh1 (blue).

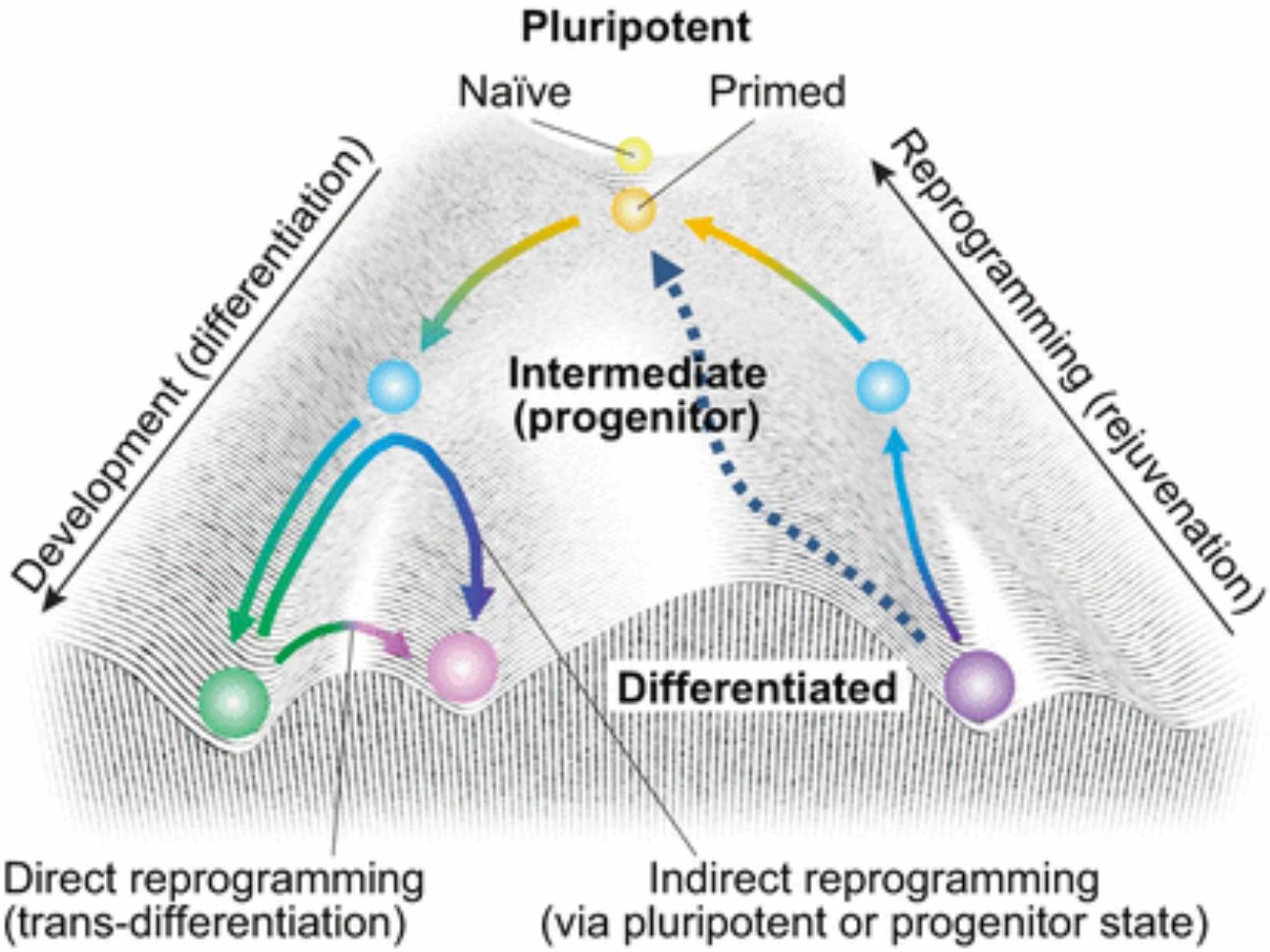
http://www.embl.de/research/units/dev_biology/hiiragi/

How to define a stem cell

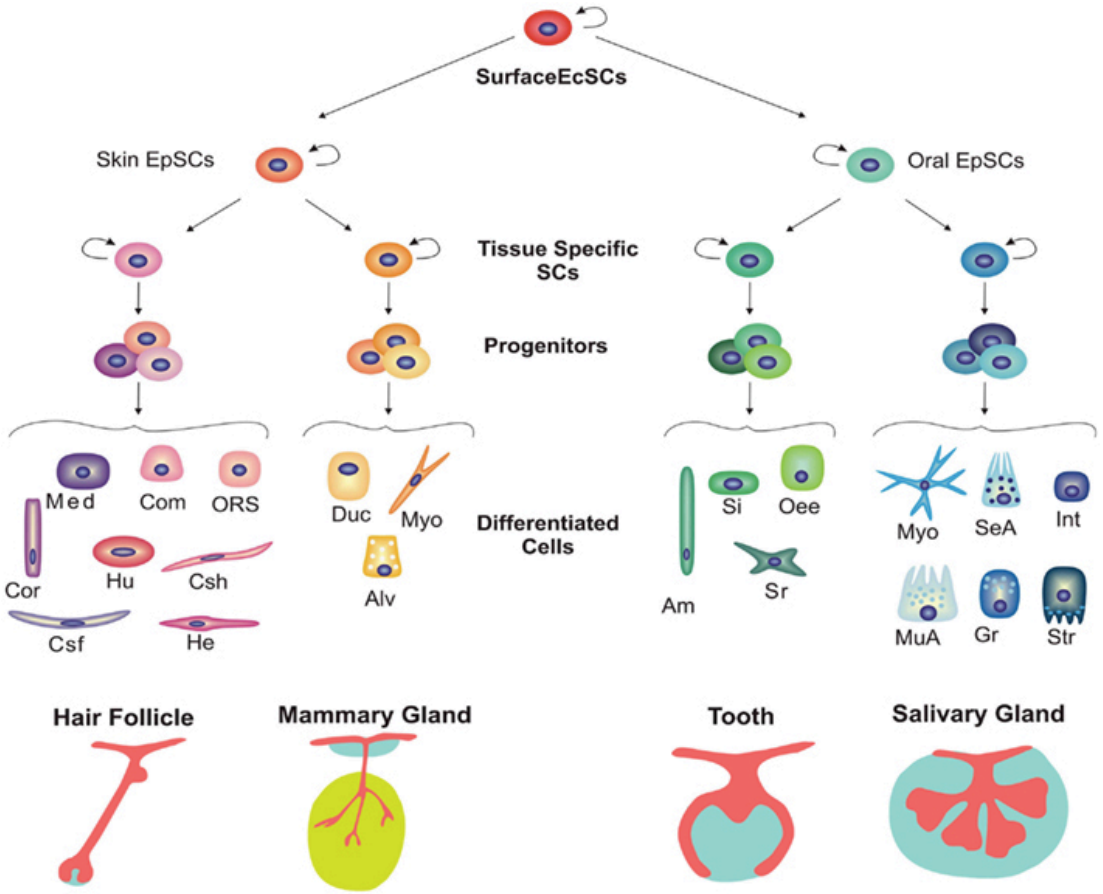
- Self-renewal
- Differentiate (potency)
- E.g. in HSC, a single HSC must be able to re-populate the whole blood system
- http://ed.ted.com/lessons/what-are-stem-cells-craig-a-kohn
- Fun facts about blood (~120 days); intestine (~1 week); hair (~4 years); skin (~2-4 weeks)



Commitment and differentiation



Commitment and differentiation



Least committed

Differentiation into pluripotent SC

Somewhat committed

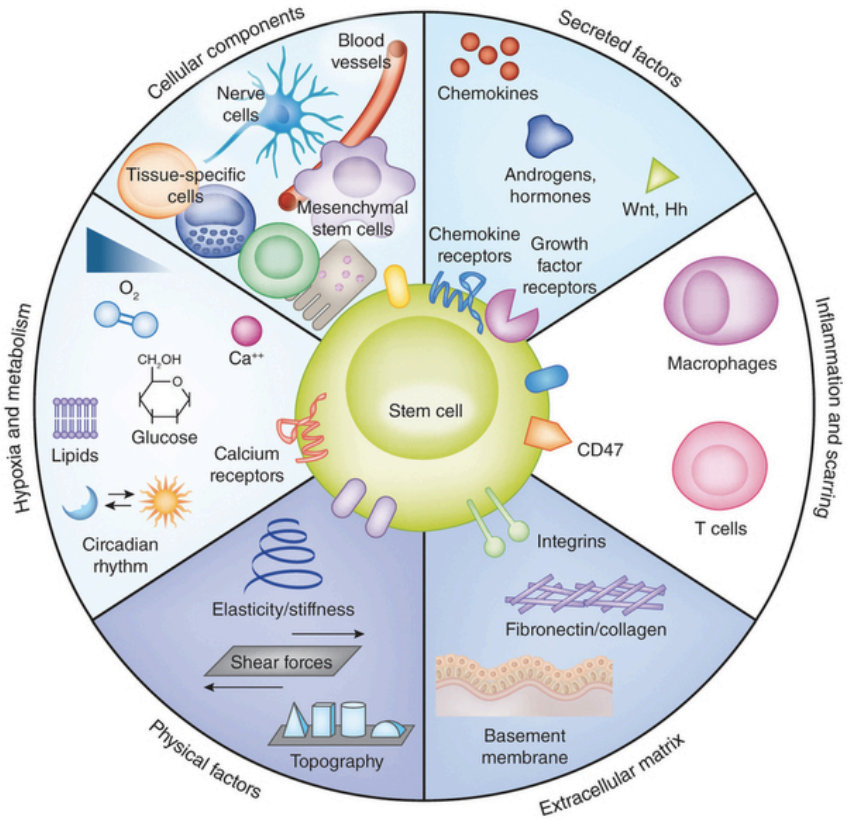
Full differentiation

Fully committed and differentiated

Jiménez-Rojo et al., "Stem cell fate determination during development and regeneration of ectodermal organs", Frontiers in physiology, 2012

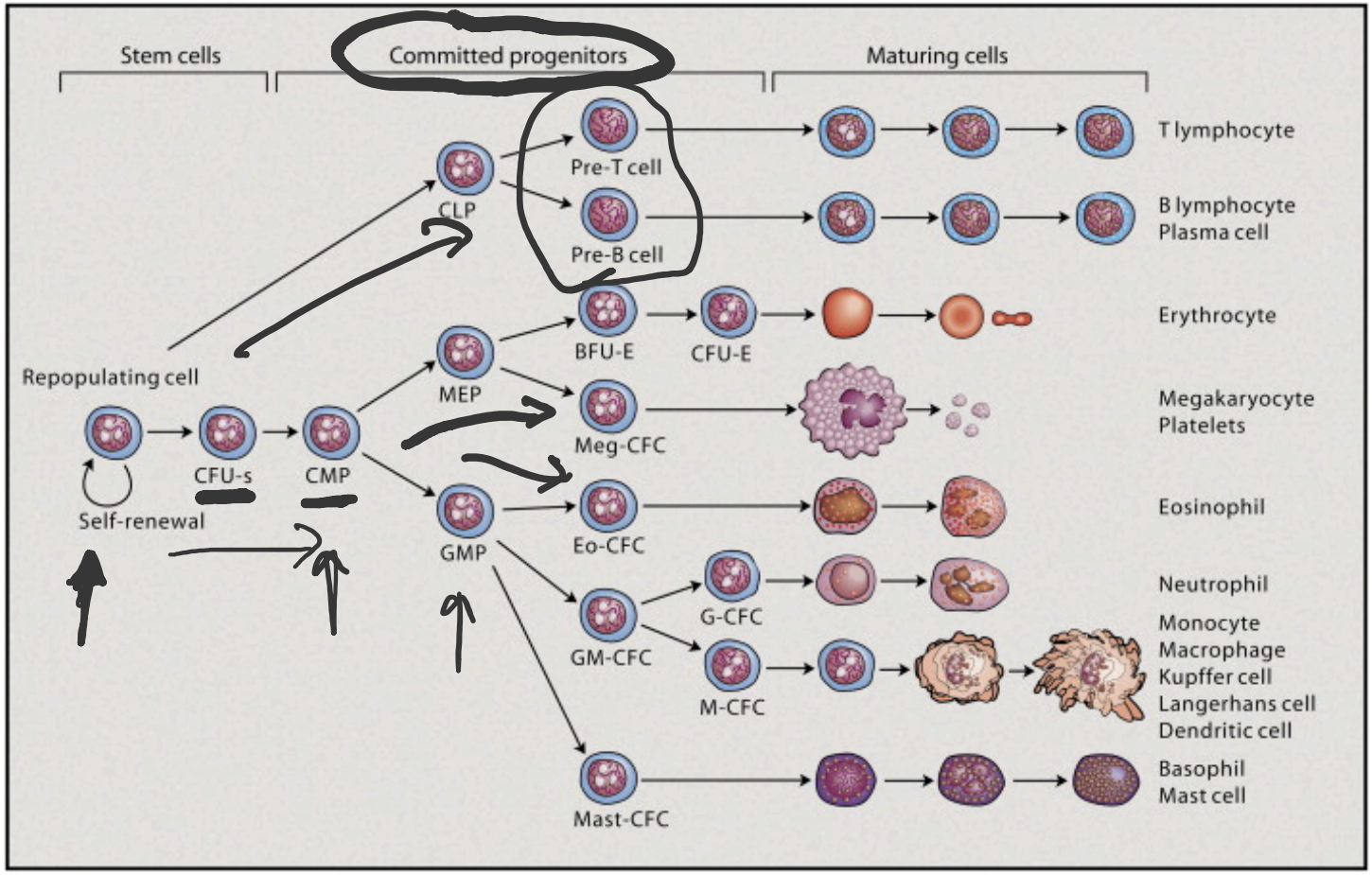
Stem cell niche

- Regulation and homeostasis is important for stem cells
- Normally SCs are kept quiescent (in a dormant/resting state) and not dividing or differentiating
  - They are kept quiescent by surrounding cells (the niche)
- Stimulus from the environment may activate/trigger the SC
  - E.g. in muscle, it could be an injury to the tissue; in blood, it could be an immune response to infection



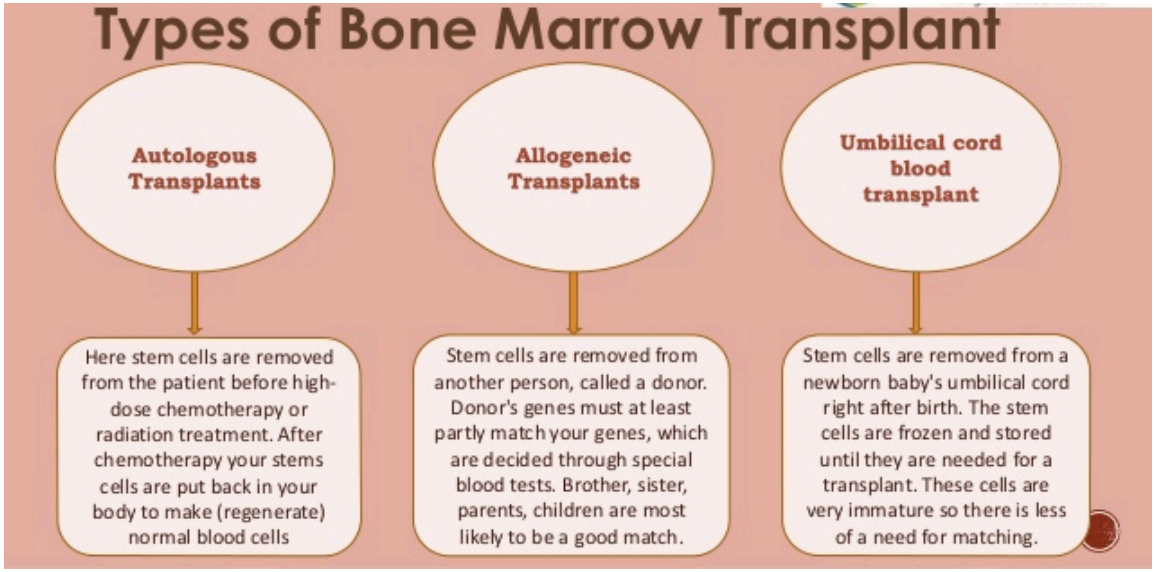
Lane, Williams, & Watt, "Modulating the stem cell niche for tissue regeneration", Nature Biotech, 2014

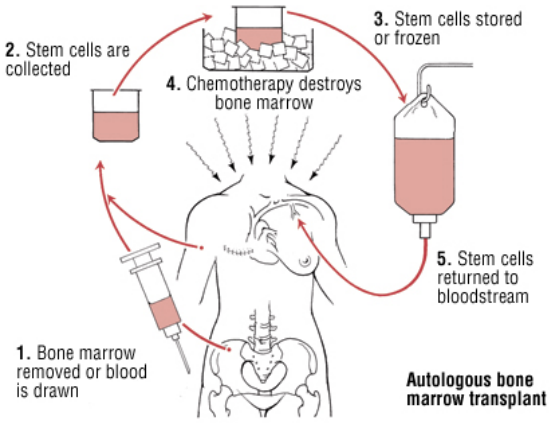
Case study: HSCs

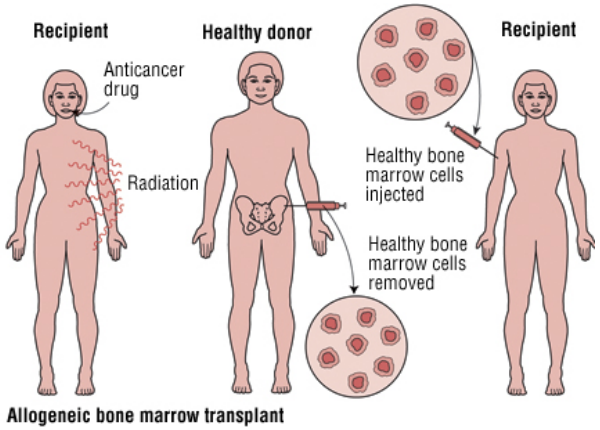


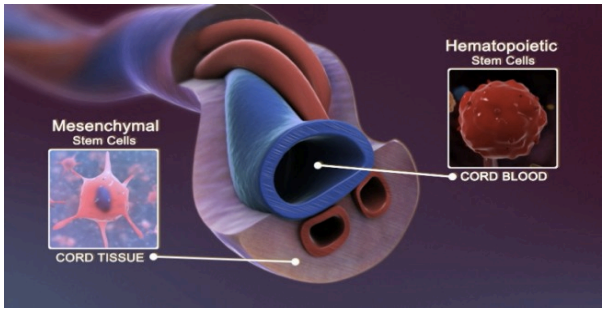
http://www.cell.com/cms/attachment/593400/4586019/gr1.jpg

Case study: HSCs







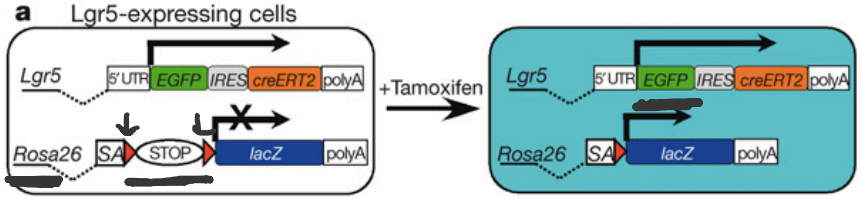


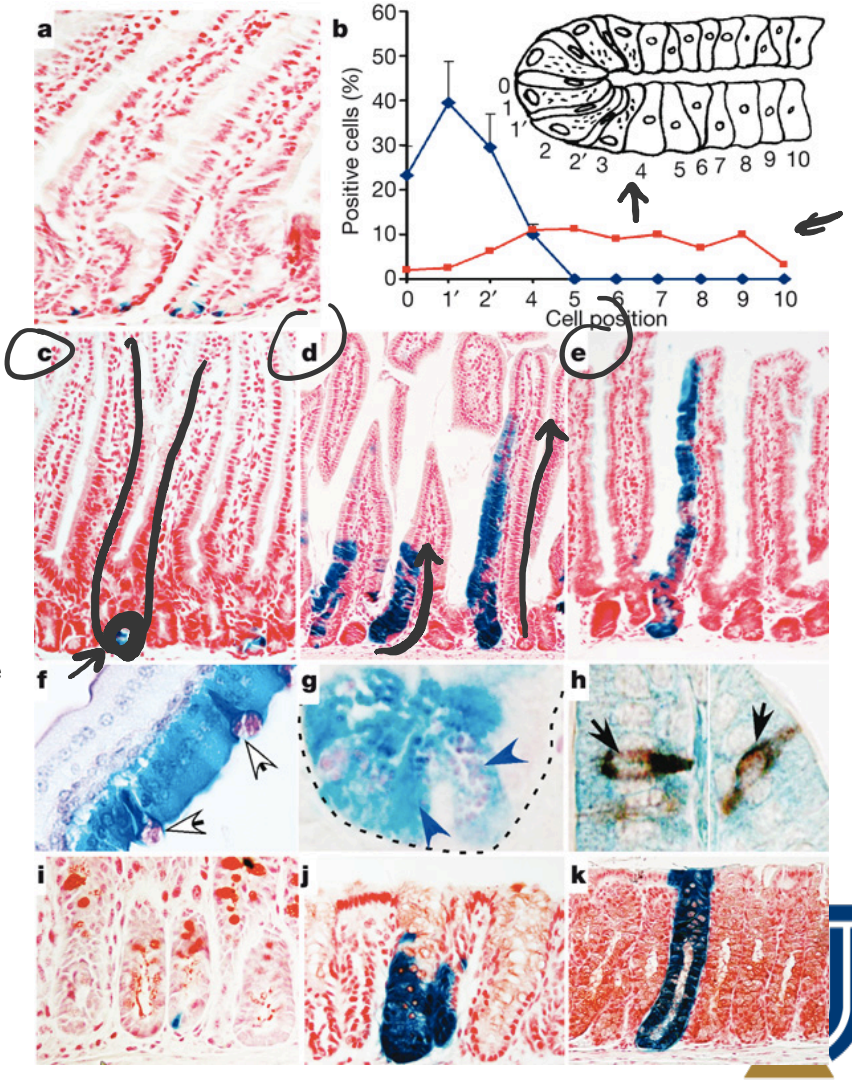
Mesenchymal stem cells, or MSCs, are multipotent stromal **cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells).*

Case study: Intestine and colon

lineage tracing.

- Clevers group discovered the intestinal stem cell – it is marked by the LGR5 protein
  - Difficult marker because it is not a surface marker





a, *Lgr5-EGFP-IRES-creERT2* knock-in mouse crossed with *Rosa26-lacZ* reporter mice 12 h after tamoxifen injection. **b**, Frequency at which the blue cells appeared at specific positions relative to the crypt bottom, according to the scheme in the inset. Results are depicted as means and standard deviations of four independent stretches of proximal small intestine totalling 400 positive crypts. Most of the Cre^+ LacZ-labelled CBC cells occurred at positions between the Paneth cells, whereas only 10% of these cells were observed at the +4 position directly above the cells (blue line). Quantitative data on the position of long-term DNA-label-retaining cells obtained in adult mice after irradiation (marking the '+4' intestinal stem cell) were published recently[17]. The graph shows a comparison of these data (red line) with the position of CBC cells carrying activated Cre. **c–e**, Histological analysis of LacZ activity in small intestine 1 day after induction (**c**), 5 days after induction (**d**) and 60 days after induction (**e**). **f–h**, Double-labelling of LacZ-stained intestine using PAS demonstrates the presence of goblet cells (**f**, white arrows) and Paneth cells (**g**, blue arrows) in induced blue clones. Double-labelling with synaptophysin demonstrates the presence of enteroendocrine cells within the induced blue clones (**h**, black arrows). **i–k**, Histological analysis of LacZ activity in colon 1 day after induction (**i**), 5 days after induction (**j**) and 60 days after induction (**k**).

Regenerative medicine

- Holy grail – inject stem cell to where things are broken or missing, and they get repaired
- In reality there are lots of challenges:
  - Need SCs with the right potency (will generate the right cell types)
  - Adult stem cells are rare and hard to find/make
  - Tissue organization is not yet resolved
- Instead of adult SC, it is easier to access
  - Embryonic SC – but there are ethical issues, and they are not 'self' so there is immune and rejection risk
  - Cord blood SC – low dose; need to expand in-vitro
  - iPSC – issues of tumorigenicity
  - Trans-differentiation from other differentiated cell types - still need more studies