Diabetes and Gene Editing

Cell based therapy for single mutation induced diabetes

GENE THERAPY COURSE – ISABELLA SAGGIO

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Monogenic Diabetes : Two types (1)

- Monogenic forms of pancreatic β-cell dysfunction include maturity-onset diabetes of the young (MODY) and neonatal diabetes.

- MODY is the most common form of inherited diabetes (constitutes 1-5% of all cases of diabetes in industrialized countries)

Symptoms and Features :

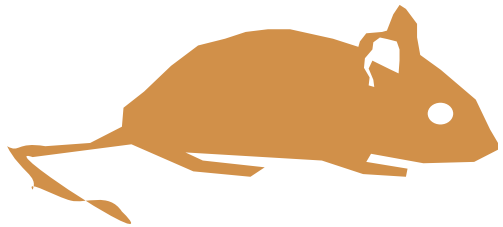
MODY

- High blood glucose, polyuria, polydipsia, fatigue
- Diagnosed with diabetes under the age of 25
- Parent with diabetes plus in two or more generations
- Not necessarily needing insulin.
- HNF1-alpha gene causes about 56 % of cases
- INS mutated in 4% of the cases

NEONATAL

- High blood glucose, polyuria, polydipsia
- Diagnosed with under the age of 6 months
- Possible development delay
- Rare
- Transient of permanent
- 50 % of people do not need insulin
- INS mutated in 16% of permanent neonatal diabetes

Our model : The MODY Mouse [2-3]

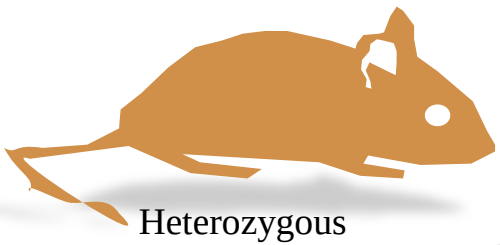


- C57BL/6 - *Ins2*^{Akita}/J Mouse
- Mutation on chromosome 7 in insulin 2 (Ins2) coding gene.
- Single point mutation : TGC → TAC

  Cysteine → Tyrosine
- The mouse autosomal dominant mutation *Mody* develops hyperglycemia with notable pancreatic β-cell dysfunction.
- The mouse develop also, hypoinsulinemia, polydipsia, and polyuria with no obesity and insulitis.

How ? The cell based strategy.

C57BL/6 - *Ins2*^{Akita}/J



Heterozygous

Fibroblasts

Primary Culture

In vitro

Ectopic expression of Yamanaka's factors

1

Fibroblasts Induced Pluripotent Stem Cells (FiPS Cell)

GOAL : Self-monitoring of blood glucose level

C57BL/6 - *Ins2*^{Akita}/J

Cell injection

2

Point mutation gene Editing via CRISPR/Cas9 and HDR

Corrected cells (FiPSC cells)

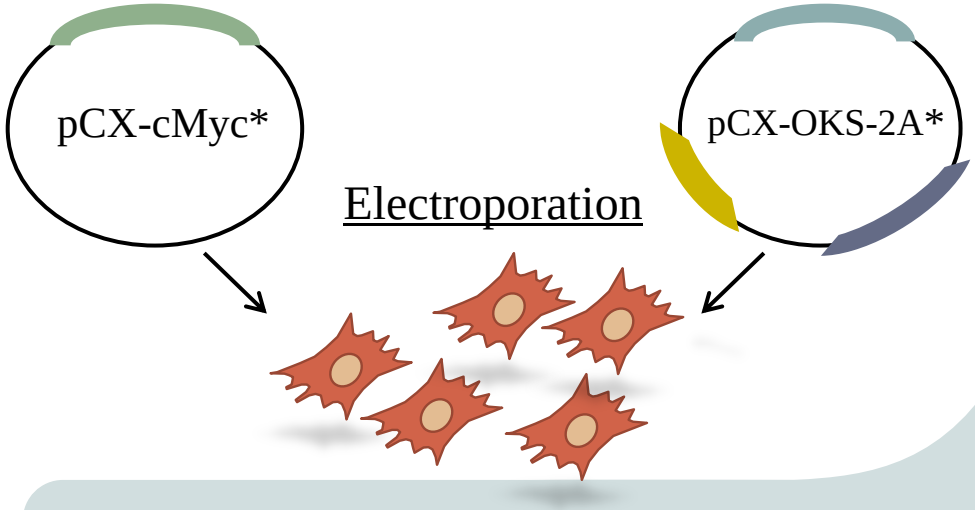
In vitro β-cells Differentiation

3

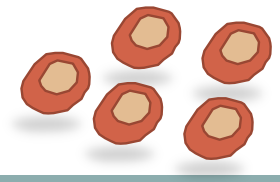
Insulin producing β-cells (FiPSC-β Cells)

1 Generation of fibroblast derived iPS cells

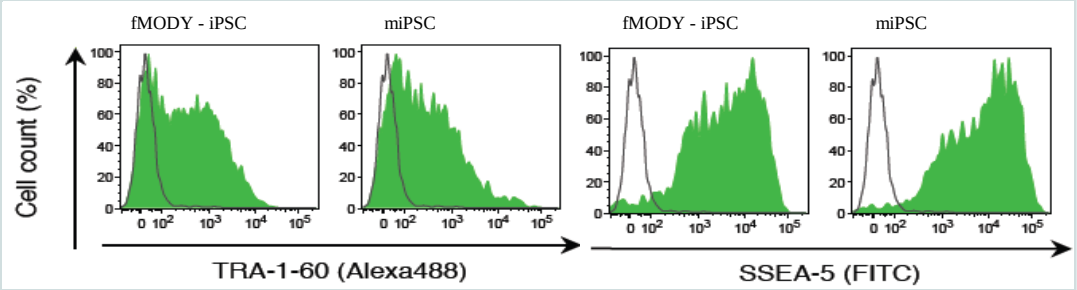
- Ectopic expression of Yamanaka's factors Myc, Oct4, Klf4, Sox2 in MODY mouse fibroblasts **AND** WT C57BL/6J mouse.



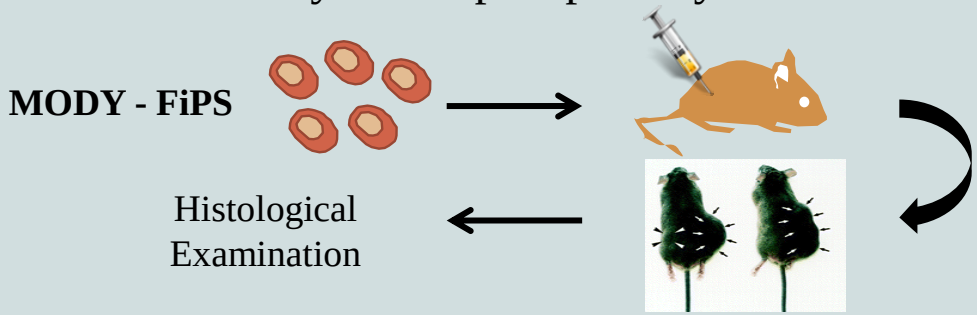
Pipeline from *Hongmei Lisa Li et al 2015* to check for stable, integration free iPSC clones [4]



- Colony with stable karyotype
- Test for plurypotency markers, including Nanog Oct 3/4 , TRA-1-60 and SSEA-5.



- Teratoma assay to test pluripotency

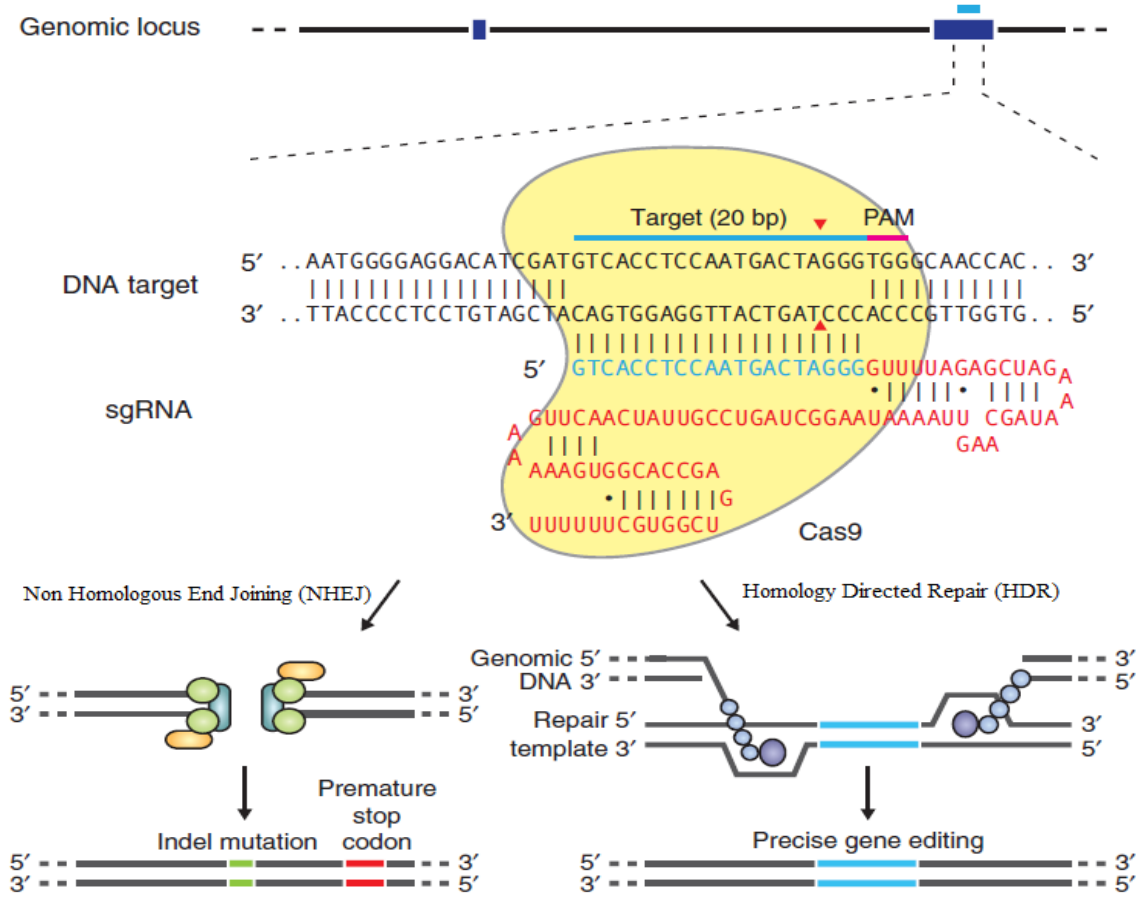


- Integration free clones assessment using qPCR.

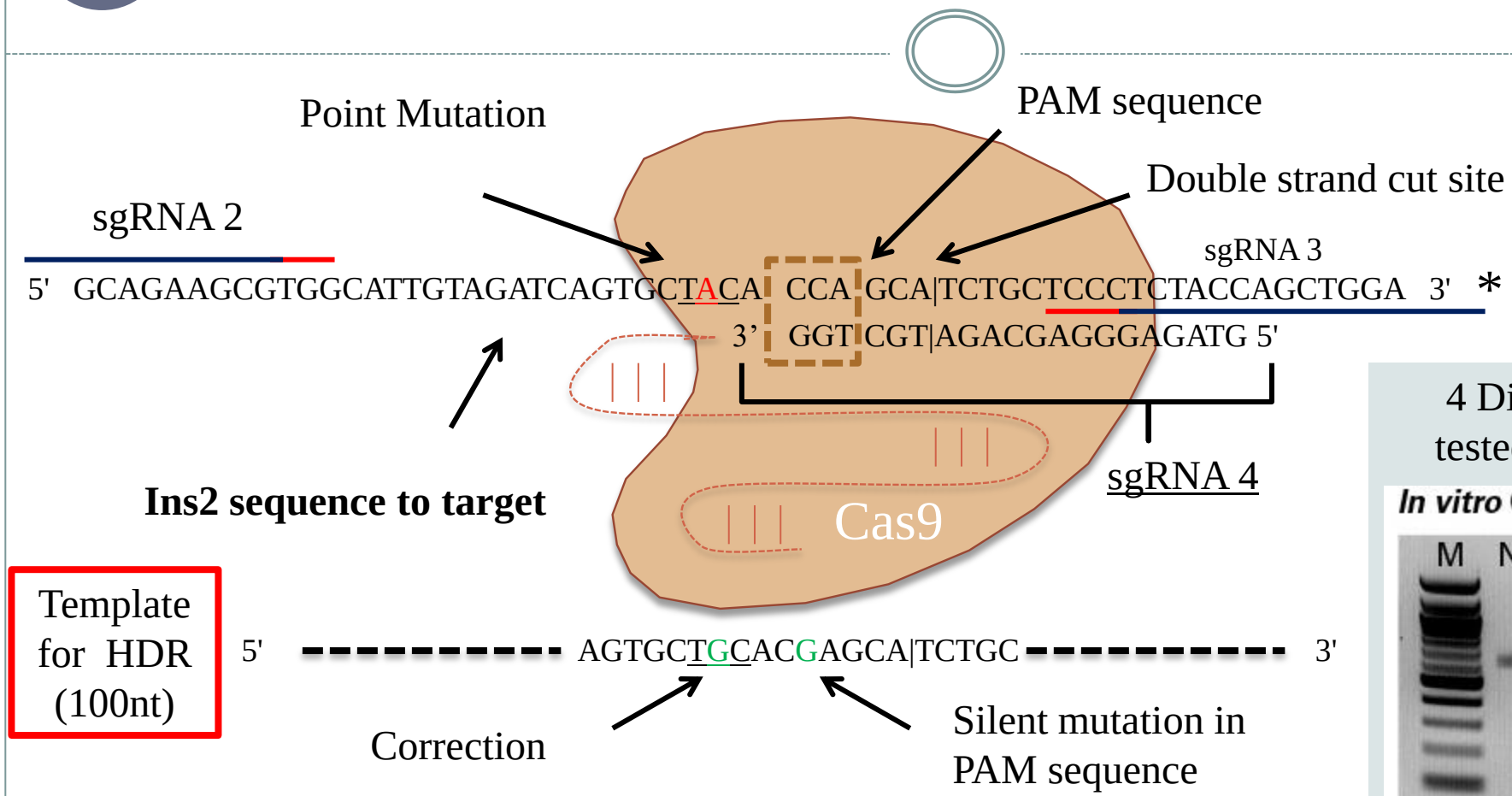
* chose on Addgene for their Non-integrating and mammalian expression of mouse genes.

2 CRISPR/Cas9 mediated engineering [5]

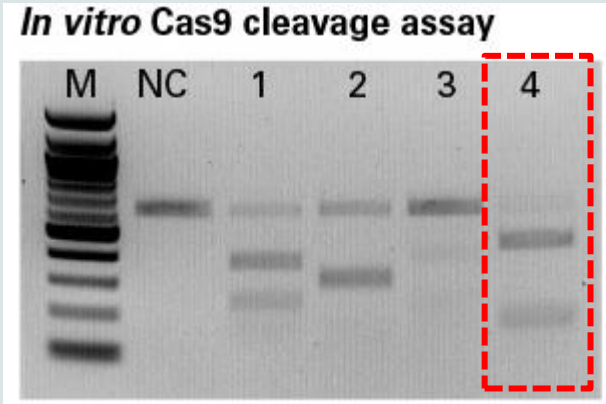
- The RNA guided nuclease Cas9 allows sequence specific double strand breaks.
- DNA repair via homology directed repair (HDR) permit genome editing.
- Require a Protospacer Adjacent Motif (PAM) sequence 5'-NGG-3' in the genome.
- Requires a DNA template sequence for HDR.
- Proved efficient in mouse iPSC and hiPSC[4]



2 CRISPR mediated correction via HDR in MODY mouse



4 Different sgRNA were tested using in vitro assay



N°4 selected for further experiments

➢ The sgRNAs were designed using Broad Institute website [6].

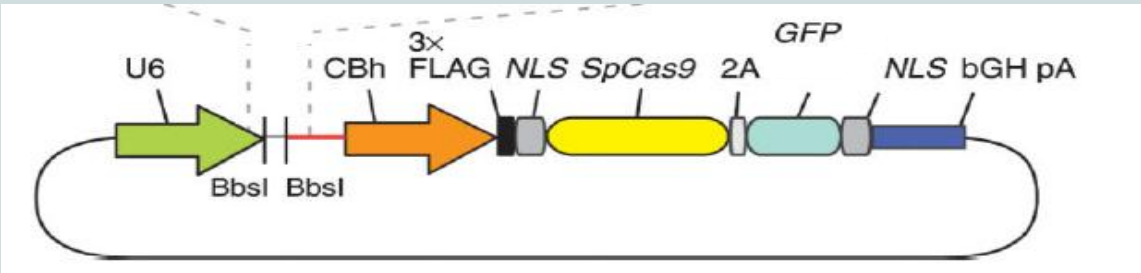
* Ins2 gene sequence obtained on NCBI website (Reference Sequence: NC_000073.6 : C57BL/6J chromosome 7, GRCm38.p3)

2

CRISPR machinery and Template delivery

- Use of the pSpCas9(BB)-2A-GFP (Addgene) recently and successfully used for knockout mESC generation [7]
- Cloning of the guide RNA 4

5' - CACCGGTAGAGGGAGCGAGTGC - 3‘
3' - CCATCTCCCTCGCTCACGCAAA - 5’



- Use of 100 nt long oligonucleotides for HDR (Ultramer® Oligonucleotides IDT)

Coelectroporation in MODY FiPS cells *

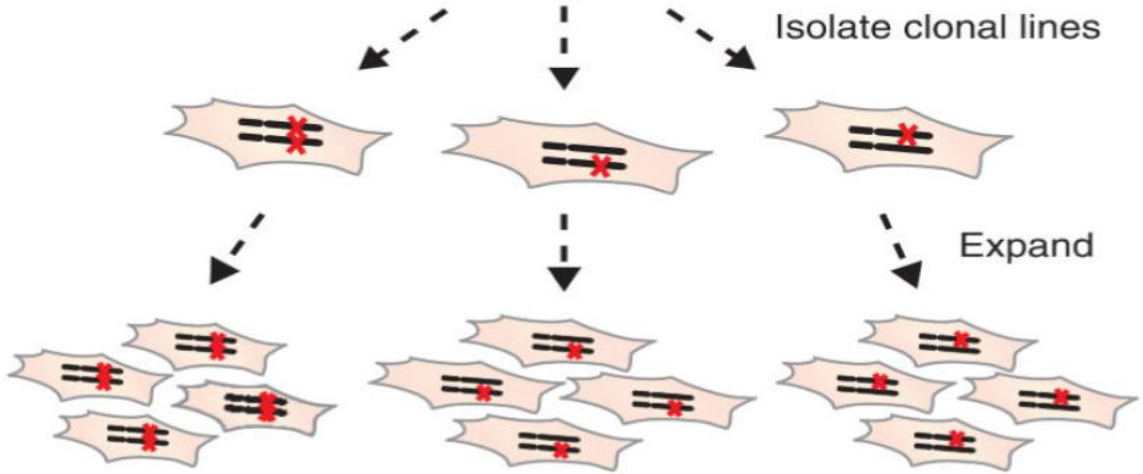
- FACS for GFP positive cells

Commonly used and efficient in miPSC [8] and bovine iPSC [9]

* WT FiPS cell were treated the same way but without plasmids and templates.

2

Selection of safely corrected cells



Deep Sequencing to control Ins2 and Ins1 locus (Chr 19)

↓

Deep Sequencing to control predicted off target (Cas-OFFinder). One potentiel with one mismatch within Chr 19 Position 1,8785,396 (Intron in Trpm6 gene)

↓

Multiple Whole-genome analysis to select clones with low mutation load*

* The selected clone are called MODY FiSC cells for fibroblasts Induced Stem Corrected cells

3 Differentiation into functional pancreatic β cells

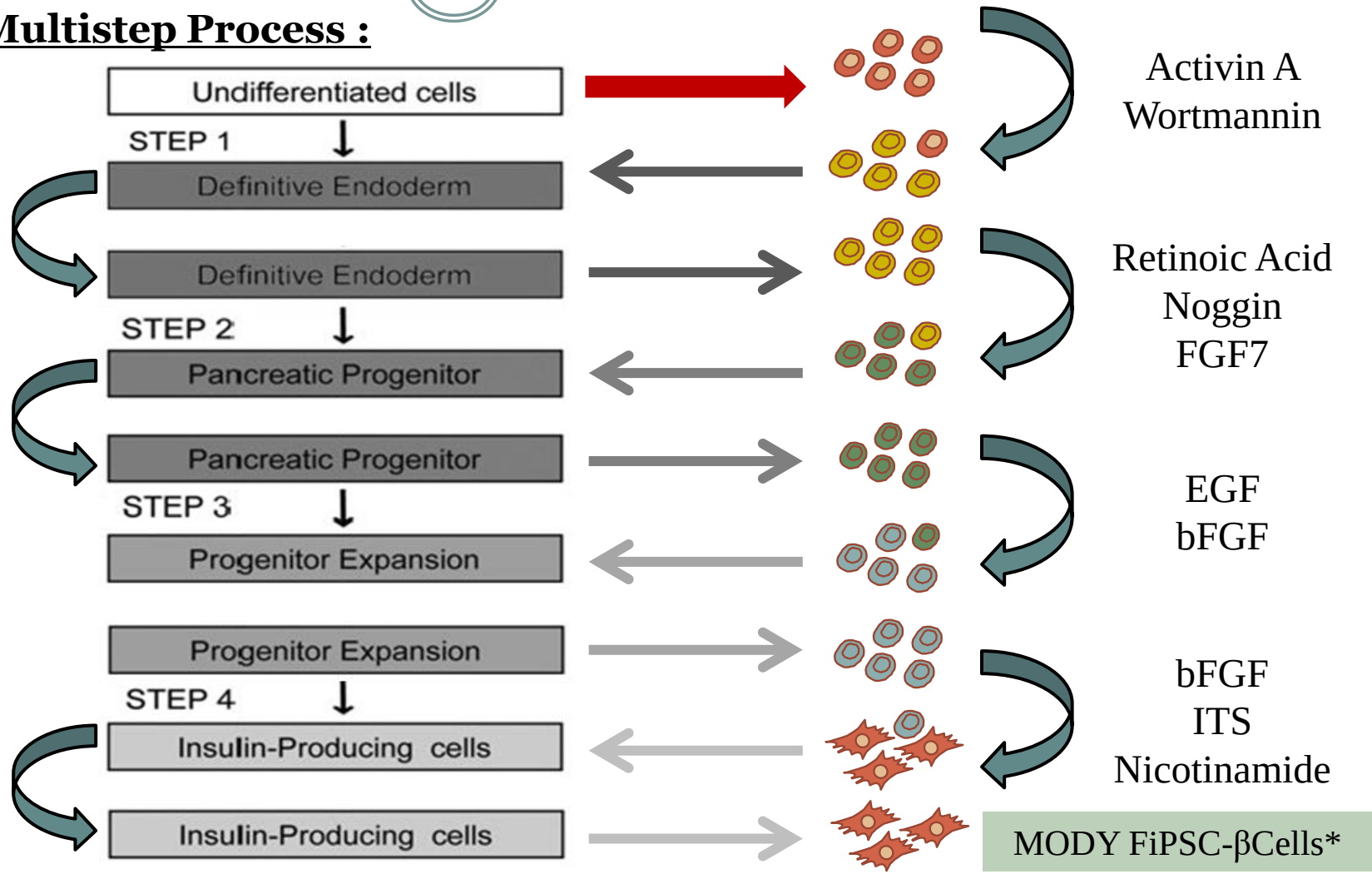
Stage specific extracellular markers for post-step FACS [8-11] :

CXCR4 +

CD133 +

GLUT2 + / CD133-

Multistep Process :



* WT FiPS cell were treated the same way and called WT FiPSC-βCells .

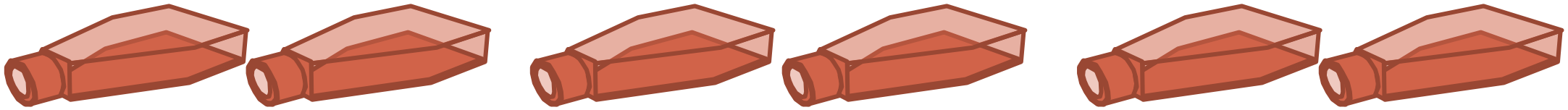
In Vitro Assays : Glucose responsiveness & Insulin expression

Cell Sorting using for GLUT2 $^{+}$ / CD133^{-} cells

WT β cells

MODY FiPSC - β Cells

WT FiPS - β Cells



Glucose 2.5mM

Glucose 25mM

Glucose 2.5mM

Glucose 25mM

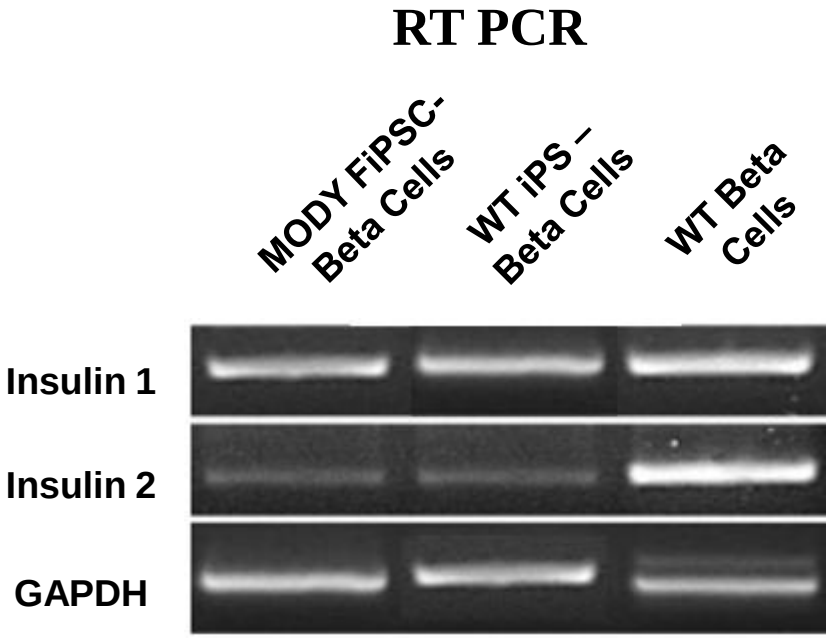
Glucose 2.5mM

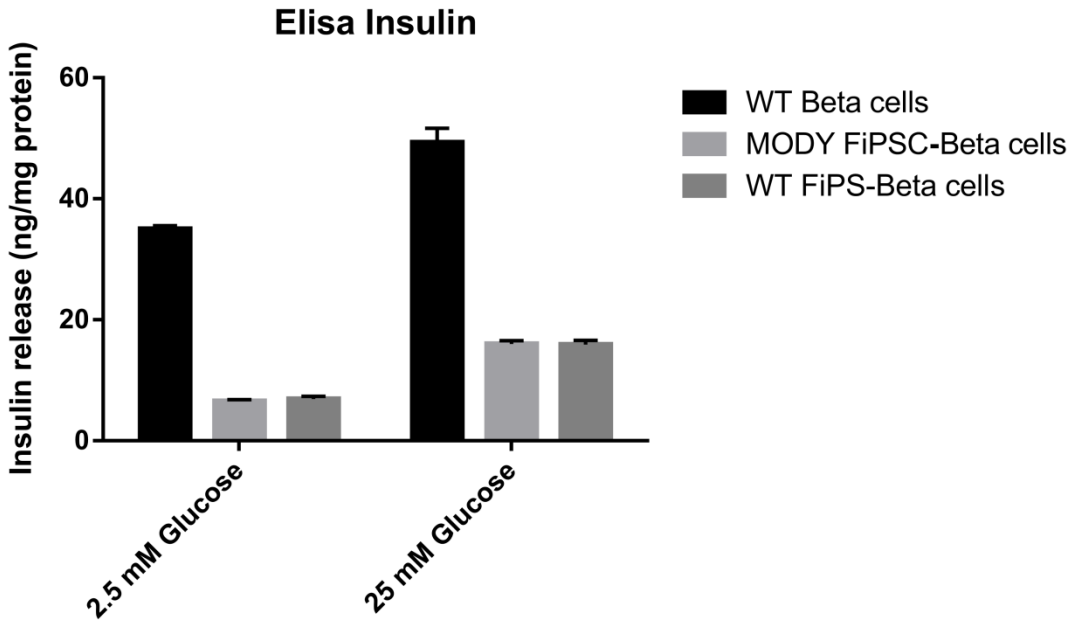
Glucose 25mM

Insulin concentration in supernatant using Elisa to test insulin responsiveness

RT PCR on cells to test Insulin 1 and 2 expression after incubation with 2.5mM

In Vitro Results : Glucose responsiveness & Insulin expression



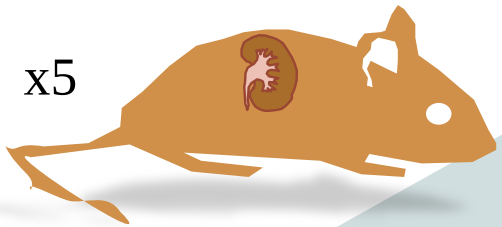


- Expression of insulin 1 and 2 in MODY FiPSC-β Cells and WT iPC-β Cells albeit at a lower level compared to WT β Cells.
- The MODY iPSC differentiated Beta cells are sensitive to glucose concentration.

In Vivo Experiments

C57BL/6 - *Ins2*^{Akita}/J

x5



MODY FiPSC-β Cells right kidney

5 Weeks old mice

D0 D2 D4 D6 D21 D39 D40 D42 D44

Nephrectomy

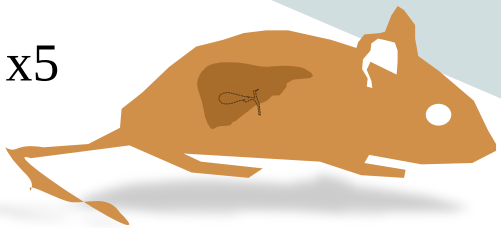
Blood Glucose measurement every 2 days

Circulating insulin

Immunohistochemistry :
- Insulin
- C-peptide

C57BL/6 - *Ins2*^{Akita}/J

x5



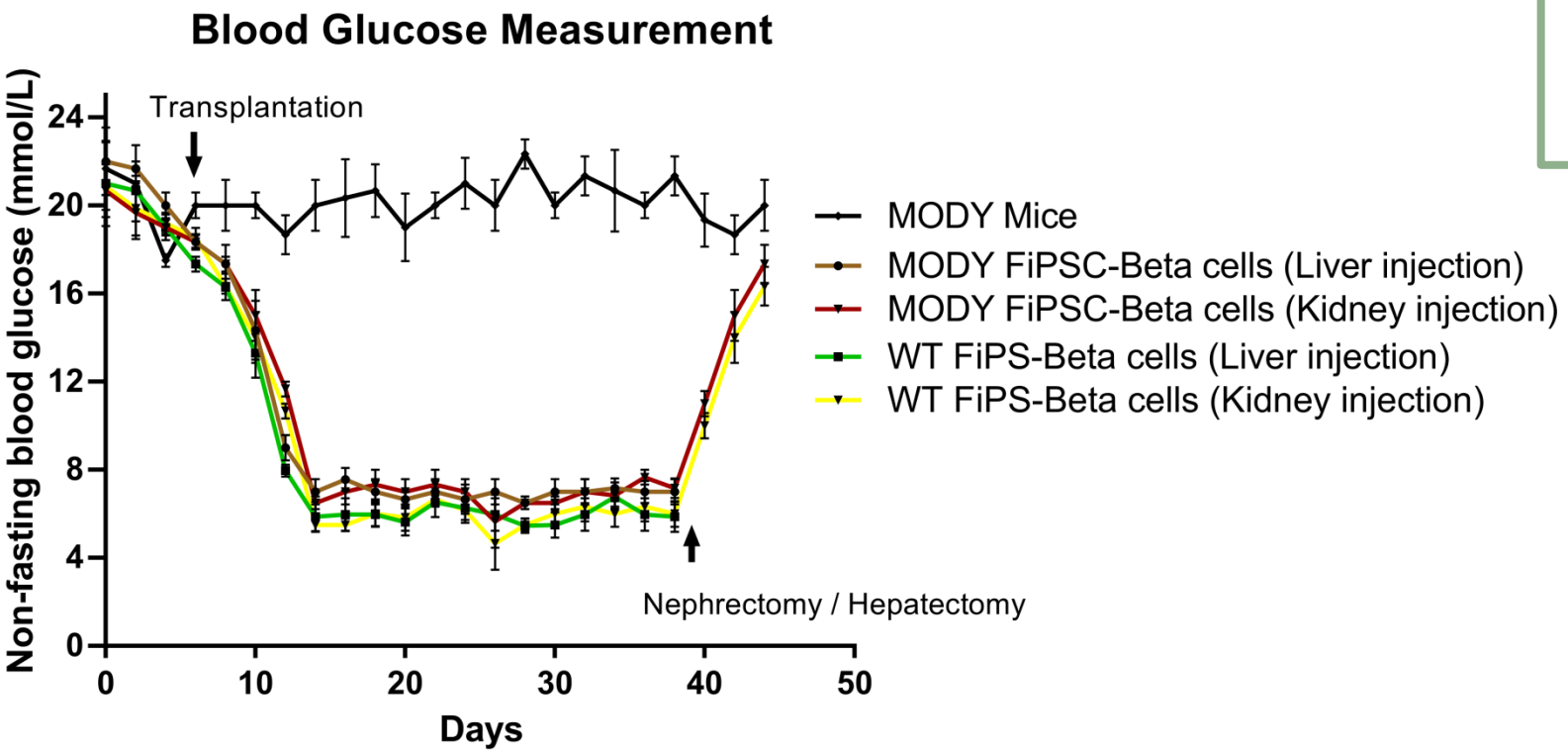
MODY FiPSC-β Cells liver

Hepatectomie

D0 D2 D4 D6 D21 D38 D39

Same experiment in parallel but injecting WT FiPS- βCell treated the same way but without correction.

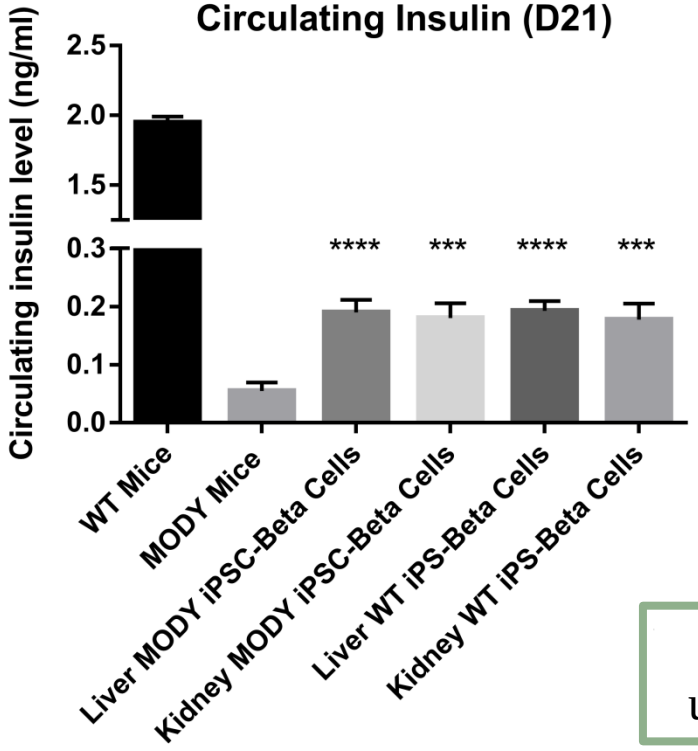
In Vivo Results



Measurement using a portable glucometer (Roche)

- MODY iPSC derived β-cell regulates the glycemia after liver and kidney injection as well as WT iPS derived Beta cells.

In Vivo Results

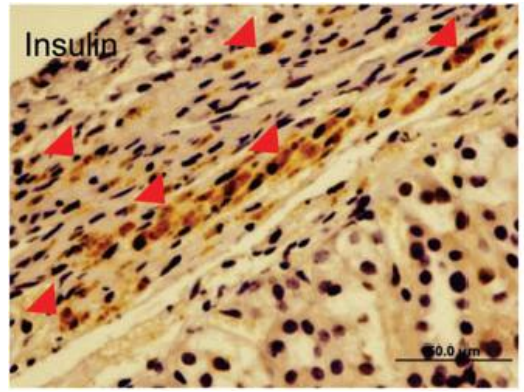


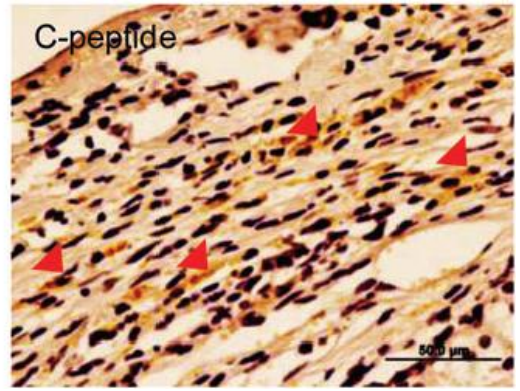
**** : pvalue < 0.0001

*** : pvalue < 0.001

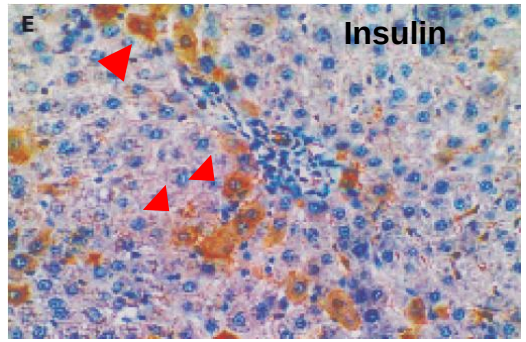
T- test comparing circulating insulin level to untreated MODY mice

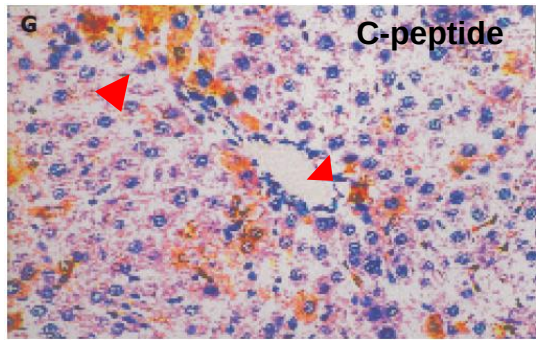
Measurement using Elisa test.





Kidney injected with MODY FiPSC-β cells





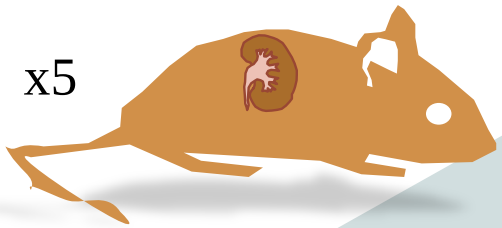
Liver injected with MODY FiPSC-β cells

- The mice show higher circulating insulin after transplantation compared to non treated albeit at a 10 times lower level than wildtype.
- Expression of insulin and C-peptide in the kidney and in the liver. (H&E, diaminobenzidine [8]).

Long term safety

C57BL/6 - *Ins2*^{Akita}/J

x5



MODY FiPSC-β Cells right kidney

5 Weeks old mice

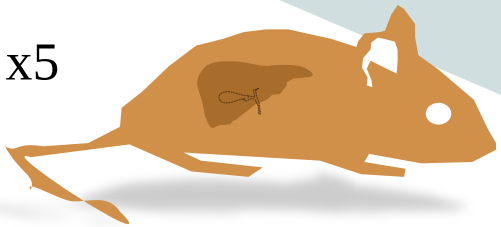
W0 W1 W2 D3 W48 W49 W50 W51

Blood Glucose measurement every Weeks + Health monitoring

The injected mice show stable blood glucose since the injection and no health issues like cancer or tumors

C57BL/6 - *Ins2*^{Akita}/J

x5



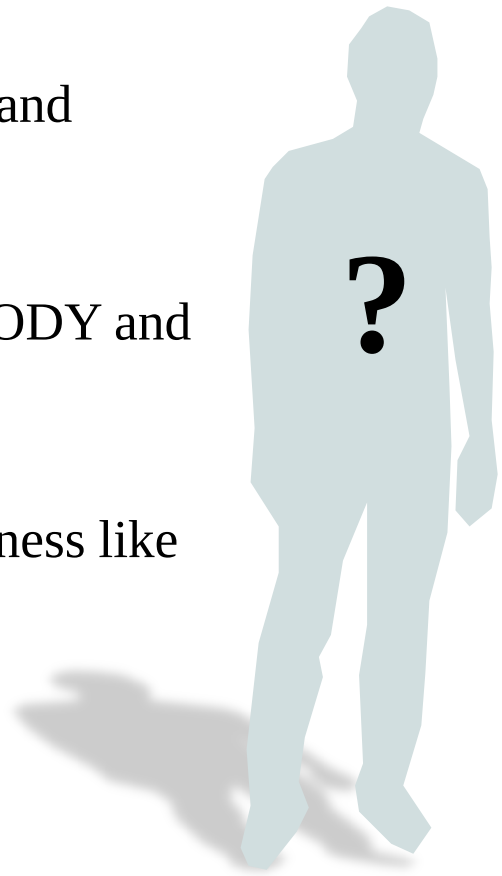
MODY FiPSC-β Cells liver

W1 W2 W3 W4 W48 W49 W50 W51

<u>Same experiment in parallel but injecting WT FiPS- βCell treated the same way but without correction.</u>

Discussion

- Gene editing possible in hiPSC [4]
- Knockin & Knockout doable and reported for other diseases [4]
- Feasible on other forms of MODY and neonatal diabetes
- Potentially useful for other illness like monogenic liver diseases
- Potential long term efficiency



- Improve Crispr safety using bioinformatics tools (Looking for unique sgRNA sequence) [4]
- Challenges still remains for human β cell differentiation. [10]
- Production of a larger amount of cells than for the mouse model
- **Long term safety at a human scale**

Use of a suicide genes before transplantation ?

Cost and Time

PhD Project (4 years) ~ 50 000$

- MODY Mice + WT mice + Stabulation ~ 199$ x 5 males + 25$ x 5 WT females.
- Stabulation for the mice ~ 500$ / months.
- Expression vectors (Addgene) : pCX-cMYC + CX-OKS-2A : 130 $
- CRISPR Vector (Addgene) : pSpCas9(BB)-2A-GFP (PX458) : 65 $
- Ultramer Oligonucleotide (IDT) : 78 - 172 $
- Exome sequencing : 800 -1200 $ times the number of clones tested.
- Multiple control sequencing : ~ 2000$
- FACS antibodies : 8 x 200-300 $ + respective isotype controls .
- Immunohistochemistry antobidies : 200-300$ x Antibodies + secondary antibodies
- Ultra sensitive mouse Insulin ELISA kit (biorbyt): 580 $ / plates
- Non evaluable costs includes routine lab experiments and trouble shootings.

References

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