In vivo Rbp4 down regulation by CRISPRi: a new strategy for insulin resistance in Type 2 diabetes

Gene therapy course – Isabella Saggio
A. A. 2018/2019

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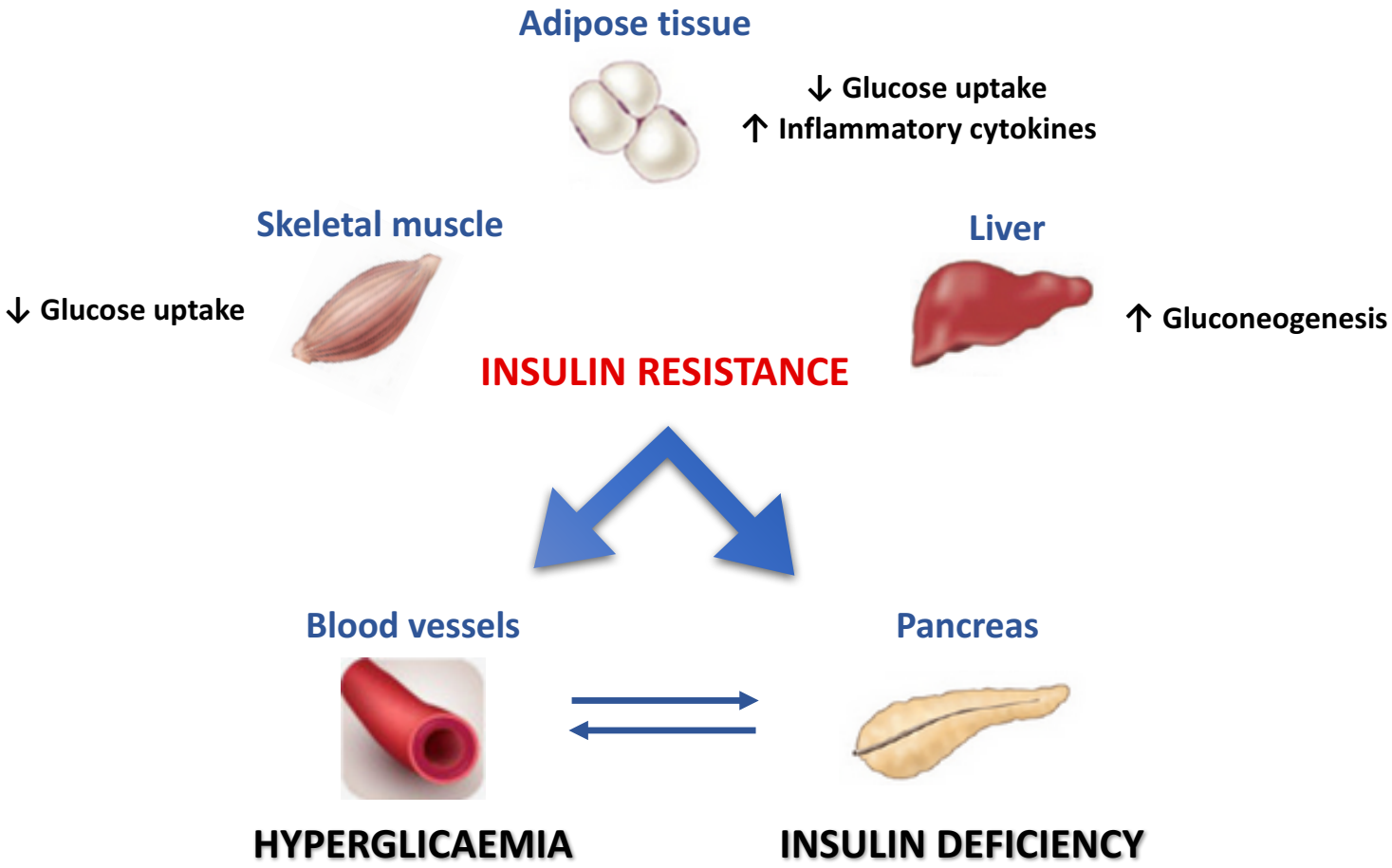
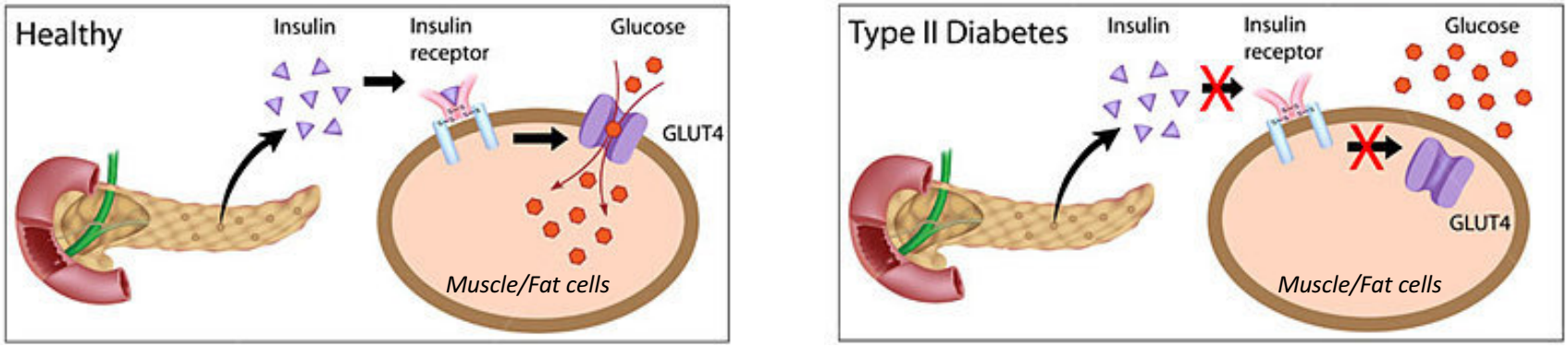
Pathogenesis of type 2 diabetes

- Type 2 diabetes is caused by peripheral **insulin resistance** and dysfunctional insulin secretion.



Molecular mechanism of insulin resistance

- Impairment of insulin-stimulated **GLUT4 translocation** in skeletal muscle and adipose tissue

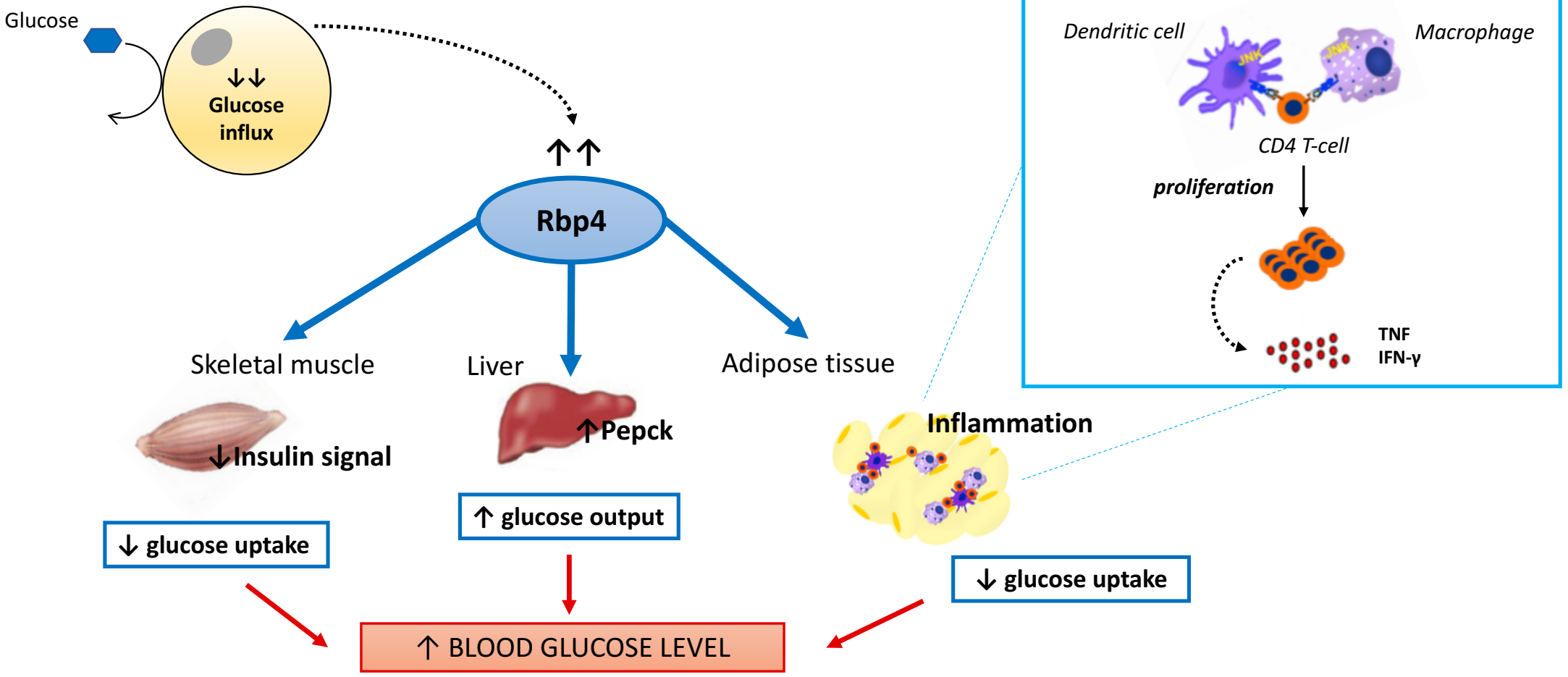


- Decreased **GLUT4 expression** in adipose tissue

Adipose-specific GLUT4 -/- mice show systemic insulin resistance[(1)]

High serum Rbp4 levels contribute to insulin resistance[1-2-3]

- **GLUT4 -/- adipocyte**



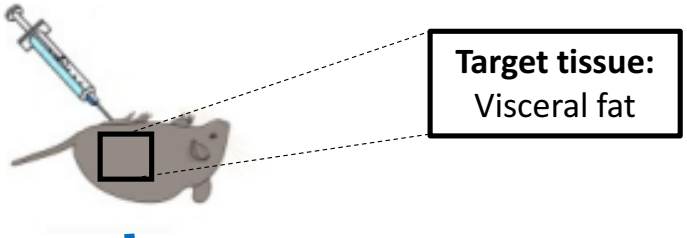
OUR GOAL

Restore insulin sensitivity and glucose tolerance by reducing Rbp4 expression in adipocytes

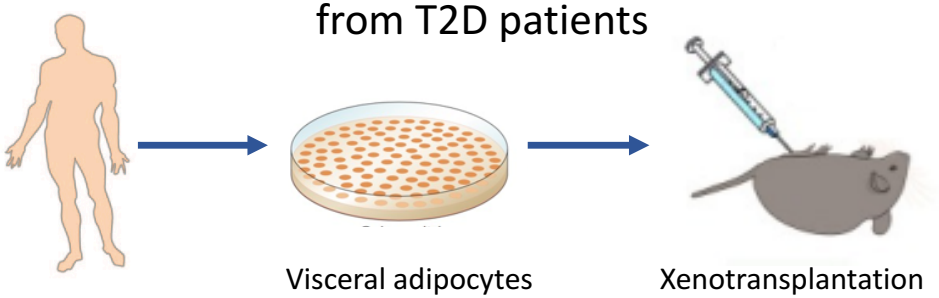
How to reach our goal?

- ***Tool*: CRISPR interference**
- ***Delivery system*: AAVRec2**
- ***Therapy administration*:**

1. **IN VIVO →** intraperitoneal injection in adipose-specific GLUT4 -/- mice

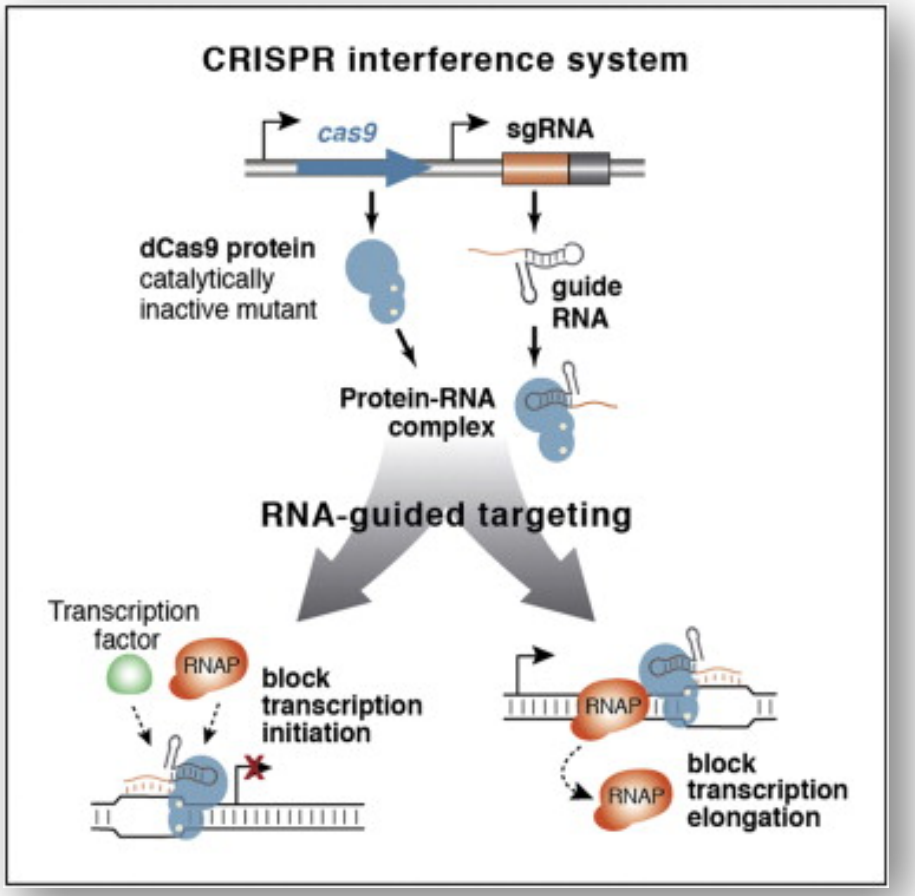


2. **EX VIVO →** treatment of adipocytes derived from T2D patients



Post-therapy analysis

Why CRISPR Interference?[4]



✓ It can *efficiently* repress expression of targeted genes

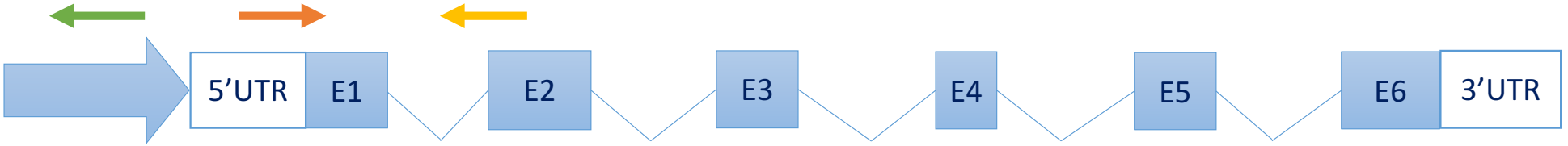
✓ Its effects are *reversibile*: no genetically alteration of targeted sequences

✓ It can be adapted for gene repression in *mammalian cells*

Qi L.S. et al. *Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression*. Cell. (2013)

1 *In silico* design of sgRNAs

Rbp4 gene
Mus musculus



5' TATTTTCTCAGCGCCAATGGCCGAGT 3' ⋯⋯▸ sgRNA1

5' CCGATGGATCCTCTGGGCTGGAGAGT 3' ⋯⋯▸ sgRNA2

5' TCTGCCCTGCGGGACAGGCGCAGGGT 3' ⋯⋯▸ sgRNA3

PAM targeting sequence

➢ The sgRNAs were designed using **CHOPCHOP** web tool

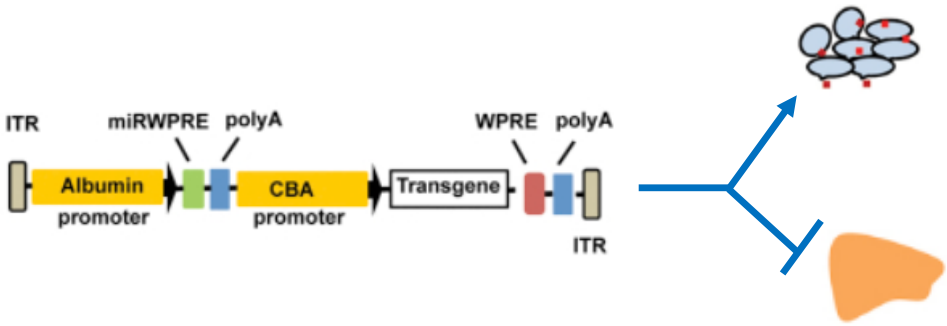
2 *Choice of delivery system*: AAVRec2[5]

- Recombinant adeno-associated virus capsid serotype

- ✓ Non-pathogenic and low immunogenic
- ✓ Transduce dividing and non-dividing cells
- ✓ Long-term transgene expression in animal models and humans
- ✓ ~**4.5 kb** packaging limit
- ✓ TROPISM: **visceral fat (VAT) and liver**

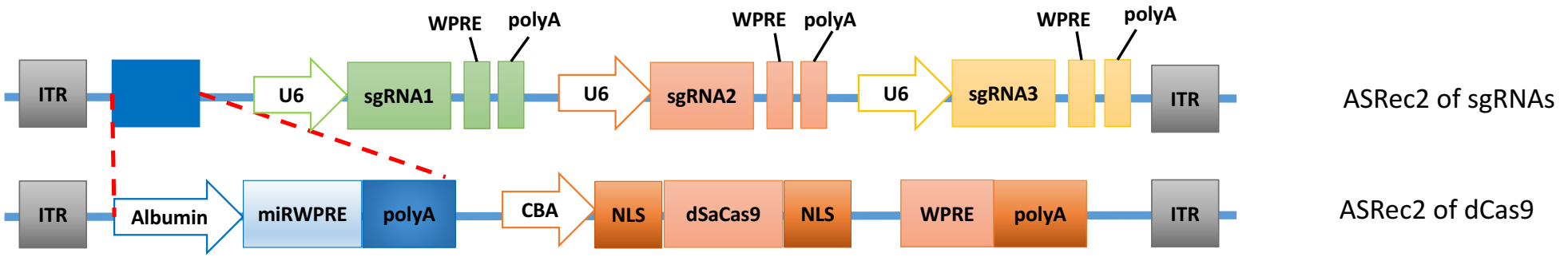
How to prevent transgene expression in the liver?

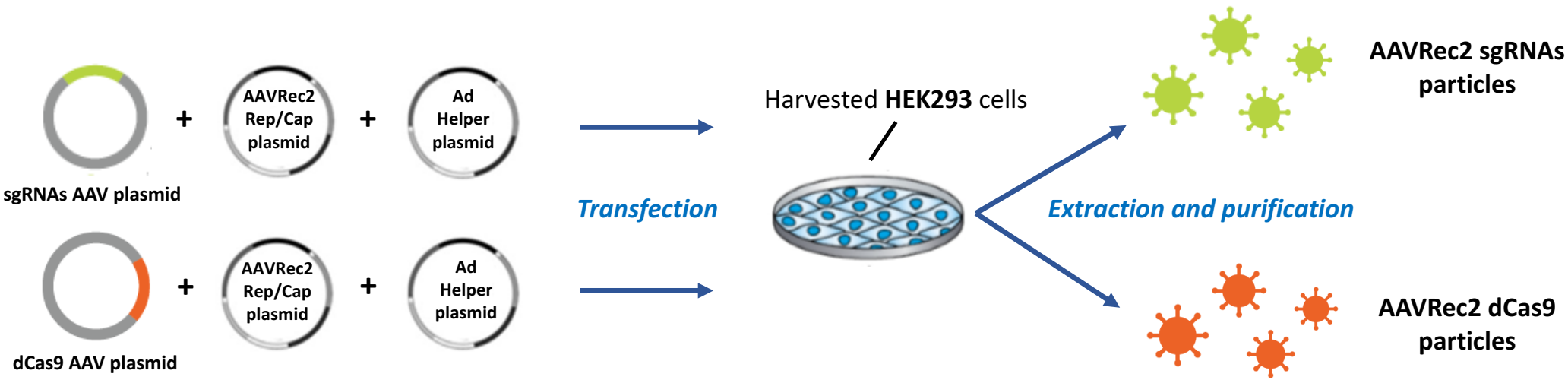
AS/Rec2→ Liver-restricted Dual-cassette vector[6]



Huang W. et al. *Targeting Visceral Fat by Intraperitoneal Delivery of Novel AAV Serotype Vector Restricting Off-Target Transduction in Liver.* Mol Ther Methods Clin Dev. (2017)

3 rAAV vectors *construction and packaging*





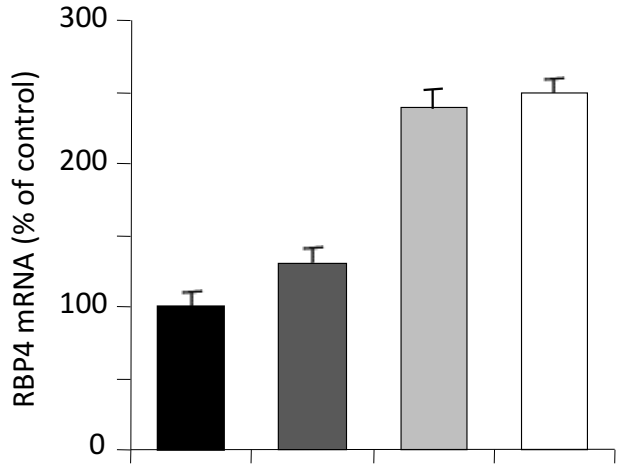
4 *Treatments* in MOUSE

Therapy administration	Control 1	Control 2	Control 3
AAVRec2 sgRNAs + AAVRec2 dCas9	AAVRec2 dCas9	NO Treatment	
*Intraperitoneal injection**	*Intraperitoneal injection**		
			
Adipose-specific GLUT4 -/- mice	**Adipose-specific GLUT4 -/- mice**	**Adipose-specific GLUT4 -/- mice**	**WT mice**

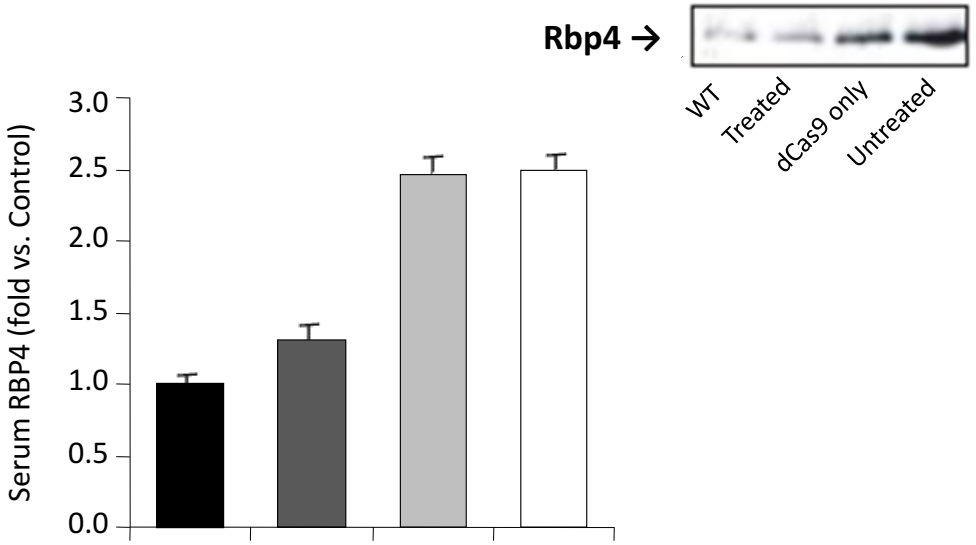
*in 150 µL of AAV dilution buffer at a dose of 4 x 10^{10} vg per mouse

Is Rbp4 downregulated?

- RT-PCR quantification of **Rbp4 mRNA** levels in mouse adipocytes:



- **Serum RBP4** measurment by Western blotting:



WT mice

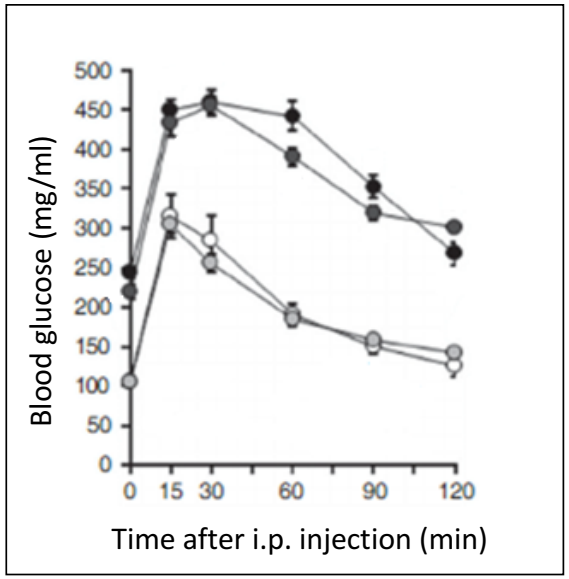
GLUT4 -/- mice treated with the therapy

GLUT4 -/- mice treated only with AAV dCas9

GLUT4 -/- mice untreated

Is insulin sensitivity restored?

Glucose tolerance test



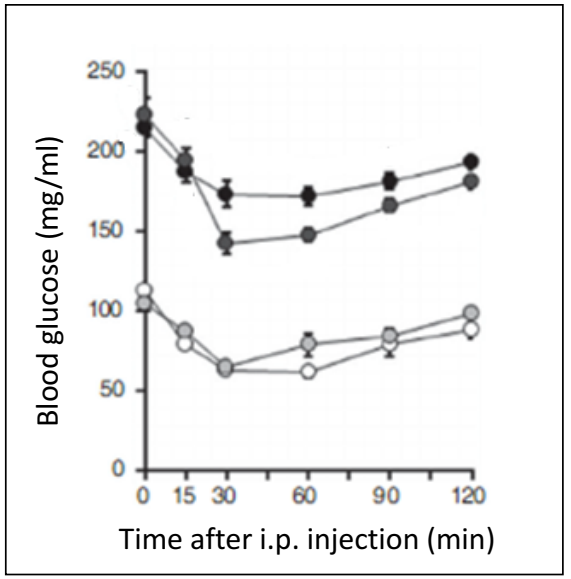
GLUT4 -/- mice untreated

GLUT4 -/- mice treated only with AAV dCas9

GLUT4 -/- mice treated with the therapy

WT mice

Insulin tolerance test

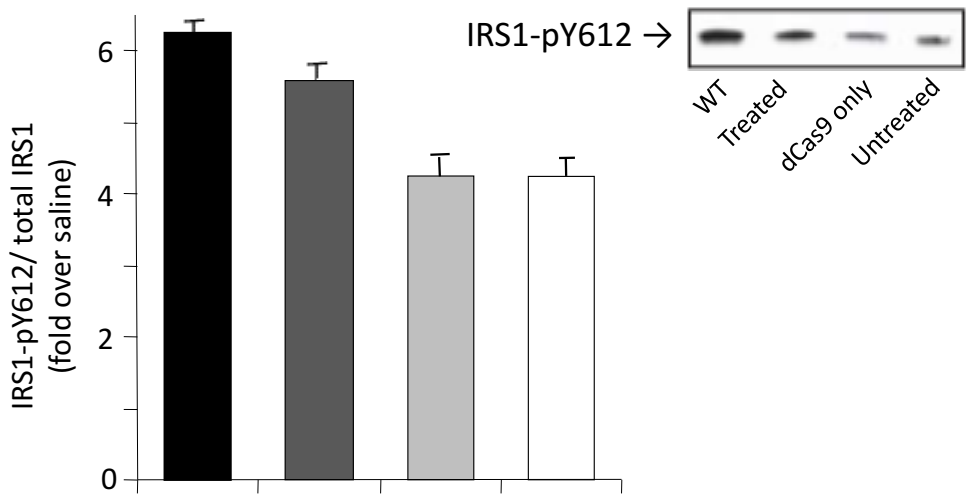


- Glucose tolerance test (**GTT**) was performed by i.p. injection of glucose (*1 g kg^{-1} body weight*) after overnight fasting.

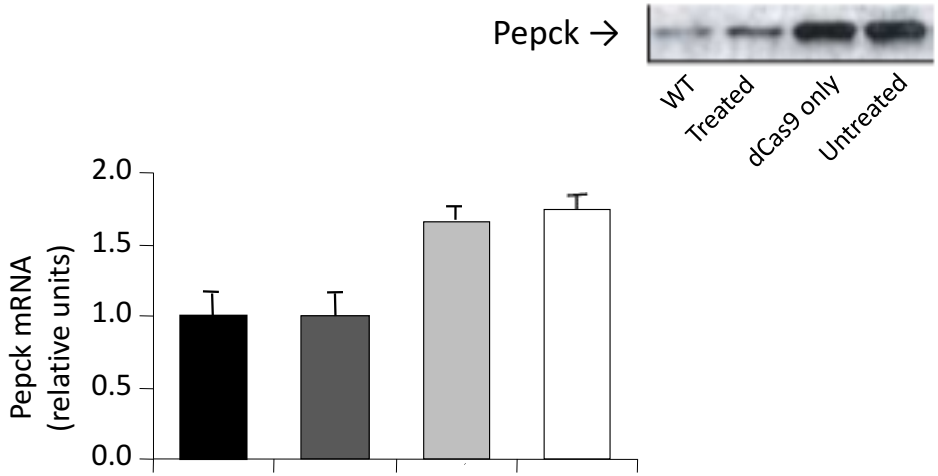
- Insulin tolerance test (**ITT**) was performed by i.p. injection of recombinant regular human insulin (*0.9 U kg^{-1}*) 3-4 hours after food removal.

Is insulin response in muscle and liver rescued?

- Western blot analysis of insulin-stimulated tyrosine-**phosphorylation of IRS1** in muscle:



- Northern blot quantification of hepatic **Pepck mRNA**:

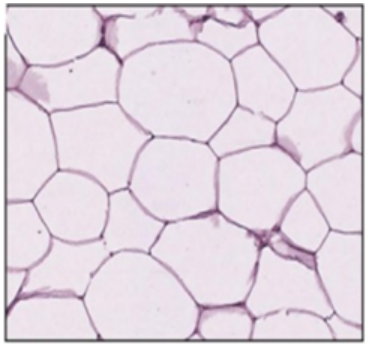


WT mice | GLUT4 -/- mice treated with the therapy | GLUT4 -/- mice treated only with AAV dCas9 | GLUT4 -/- mice untreated

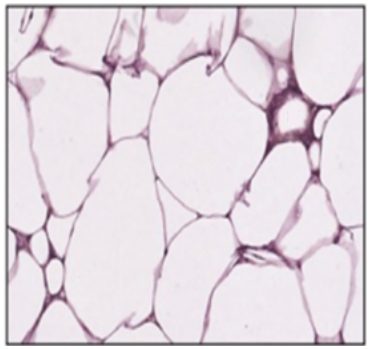
Mice were fasted for 16–18h, injected intravenously with saline or insulin (*10 U kg^{-1} body weight*) and killed 3min after injection.

Is adipose tissue inflammation decreased?

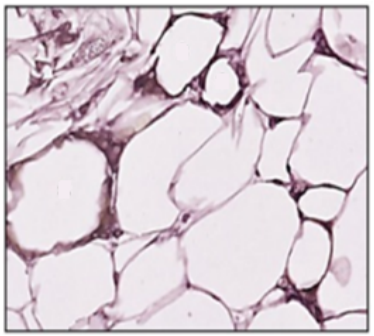
- Immunohistochemical analysis of **macrophages** in visceral fat using staining for F4/80:



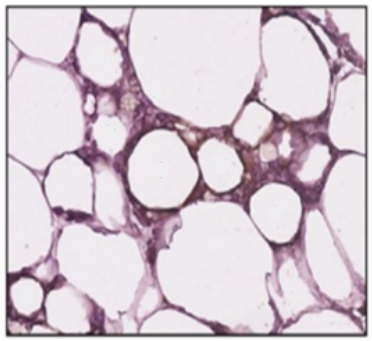
WT mice



GLUT4 -/- mice treated with the therapy

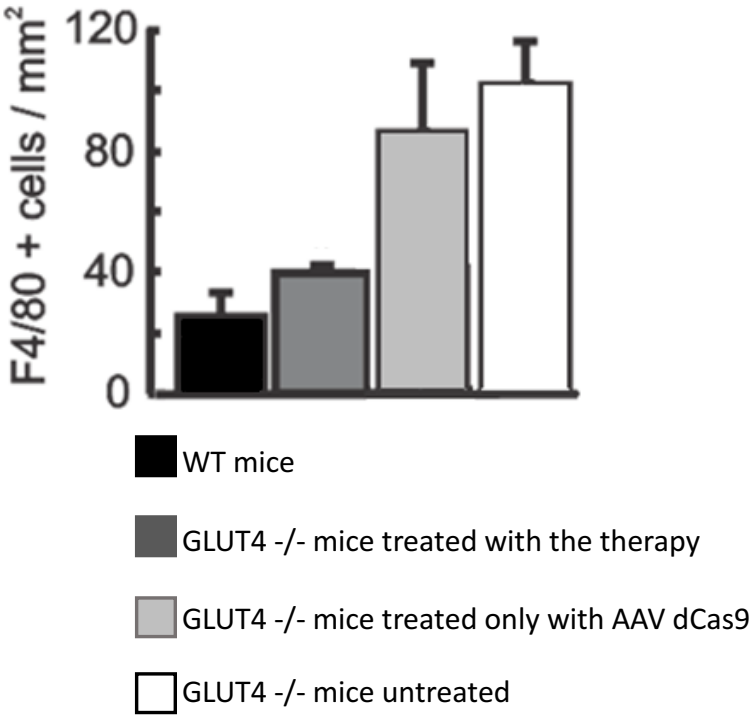


GLUT4 -/- mice treated only with AAV dCas9

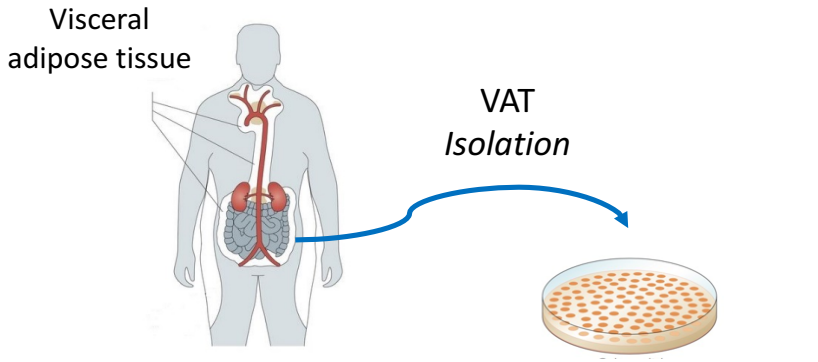


GLUT4 -/- mice untreated

- Flow cytometric analysis of **macrophages** in the stromal vascular fractions of adipose tissue



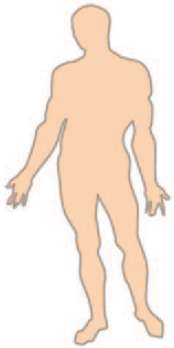
5 *…and in* HUMAN?

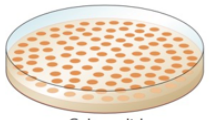


Therapy administration	Control 1	Control 2	Control 3
AAVRec2 sgRNAs* + **AAVRec2 dCas9** → Patient's VAT → *Xenotransplantation* into the visceral cavity[7]	**NO transfection** → Patient's VAT → *Xenotransplantation* into the visceral cavity	**NO Treatment**	
***Immunodeficient* GLUT4 -/- mice**	***Immunodeficient* GLUT4 -/- mice**	**GLUT4 -/- mice**	**WT mice**

*sgRNAs targeting ***human*** RBP4 gene designed through CHOP CHOP web tool

Future perspectives





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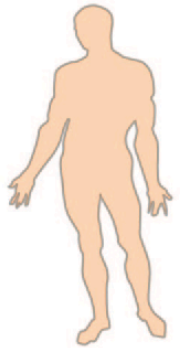

Is the therapy functional in human adipocytes?

validation of Rbp4 knock down with RT-PCR

What are the systemic effects of treated human adipocytes in xenografted mice?

Validation of insulin sensitivity amelioration (GTT, ITT, peripheral insulin signalling)

…Is it possible to start a clinical trial?

Possible pitfalls

- **Inefficient knock down of Rbp4**

- **The therapy alone is not sufficient to reverse insulin resistant states**

- **CRISPR/dCas9 causes off-target repressions**

Solutions

✓ Use of dCas9 fused with a chromatin repressor (KRAB domain of Kox1)[8]

✓ Combinatorial approach: our therapy + therapies toward others adipokines involved in metabolic disorders[9]

✓ Put dCas9 gene under an adipocyte specific promoter (adiponectin promoter)[10]

Cost and time

GLUT4-/- mice (x15) + WT mice (x5) - *The Jackson Laboratory*	**$2595**
Stabulation	**$500/months**
Dual-cassette adipose-specific vector - *A gift from Wei Huang, Department of Cancer Biology and Genetics, The Ohio State University, Columbus, Ohio, USA*	**/**
Sa-dCas9-NLS-3xFLAG/pcDNA3.1 - *Addgene*	**$65**
Invitrogen TrueGuide™ Rbp4 sgRNA (x3) - *Thermo Fisher Scientific*	**$813**
pLX-sgRNA - *Addgene*	**$65**
Service of AAVRec2 production at small scales (0.75ml to 1ml), ddPCR titration and Endotoxin assay – *Pen Vector Core*	**$2048**
TaqMan Gene Expression RT-PCR Master Mix 5mL - *Applied Biosystems*	**$505**
Rbp4 polyclonal antibody 100µg - *Invitrogen*	**$316**
IRS-1 phosphorylated (Tyr612) Polyclonal Antibody 0,1mL - *BioSource*	**$681**
F4/80 monoclonal antibody 250µg - *Invitrogen*	**$566**
32P-labeled PEPCK cDNA - *A gift from R.W. Hanson, Case Western University school of medicine, Cleveland, Ohio, USA*	**/**
RNA extraction Trizol reagent (Life Technologies) - *Thermo Fisher Scientific*	**$349**
One Touch Profile glucometer - *Lifescan Inc.*	**$20**
Human Adipocyte Maintenance Medium 250 ml – *Sigma Aldrich*	**$90**

Time of the project:
2-3 years

Cost per year:
$ 15 000

+ Additional costs from basic lab manteinance and materials

Bibliography

(1) Yang, Qin, et al. *"Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes."* Nature (2005).

(2) Moraes-Vieira, Pedro M., et al. *"RBP4 activates antigen-presenting cells, leading to adipose tissue inflammation and systemic insulin resistance."* Cell metabolism (2014).

(3) Tamori, Yoshikazu, Hiroshi Sakaue, and Masato Kasuga. *"RBP4, an unexpected adipokine."* Nature medicine (2006).

(4) Qi, Lei S., et al. "*Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression."* Cell (2013).

(5) Charbel Issa P, De Silva SR, Lipinski DM, Singh MS, Mouravlev A, You Q, et al. *"Assessment of tropism and effectiveness of new primate-derived hybrid recombinant AAV serotypes in the mouse and primate retina"*. PloS one. (2013)

(6) Huang, Wei, et al. "*Targeting Visceral Fat by Intraperitoneal Delivery of Novel AAV Serotype Vector Restricting Off-Target Transduction in Liver.*" Molecular Therapy-Methods & Clinical Development (2017).

(7) Ablamunits, Vitaly, et al. *"Functional human to mouse adipose tissue xenotransplantation."* Journal of Endocrinology (2012).

(8) Thakore, Pratiksha I., et al. "*RNA-guided transcriptional silencing in vivo with S. aureus CRISPR-Cas9 repressors*." Nature communications (2018).

(9) Andrade-Oliveira, Vinícius, Niels OS Câmara, and Pedro M. Moraes-Vieira. *"Adipokines as drug targets in diabetes and underlying disturbances."* Journal of diabetes research (2015).

(10) Wang, Zhao V., et al. *"Identification and characterization of a promoter cassette conferring adipocyte-specific gene expression."* Endocrinology (2010).