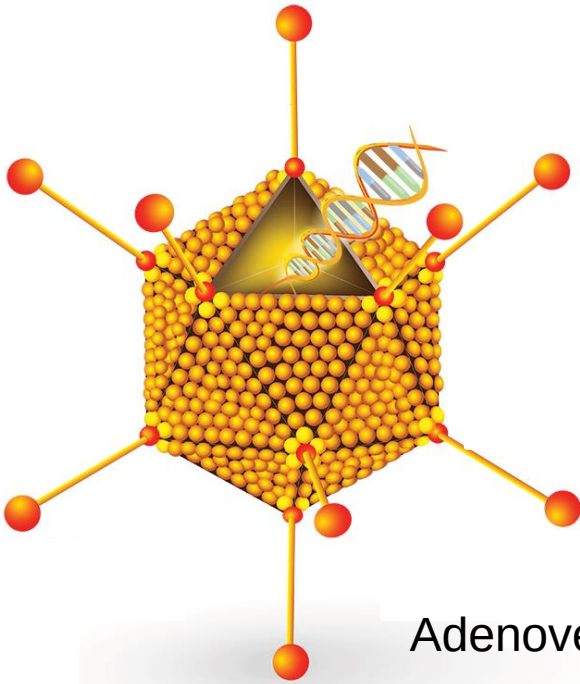
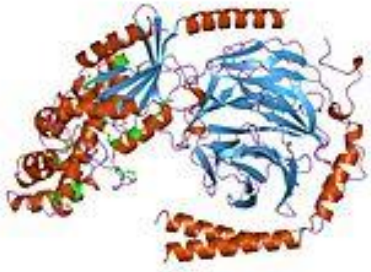
2015/2016 – Group 2:
Justine Habault, Federica Caldarelli, Silvia Gasparini and Walter Di Donato

GENE THERAPY PROJECT:
A NOVEL STRATEGY FOR THE TREATMENT OF FIBROUS DYSPLASIA



Adenovector



Gsα receptor

Professor:
Isabella Saggio
Tutors:
Mattia LaTorre, Romina Burla

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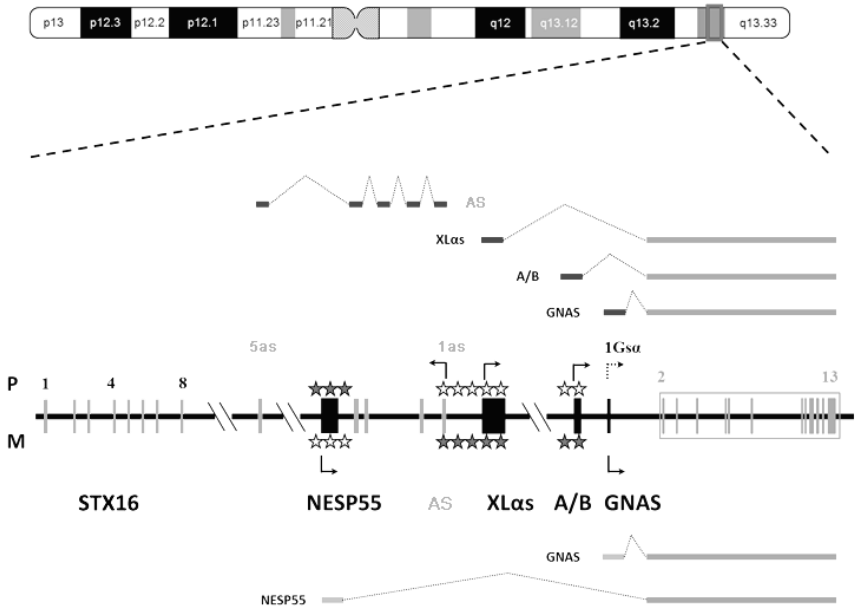
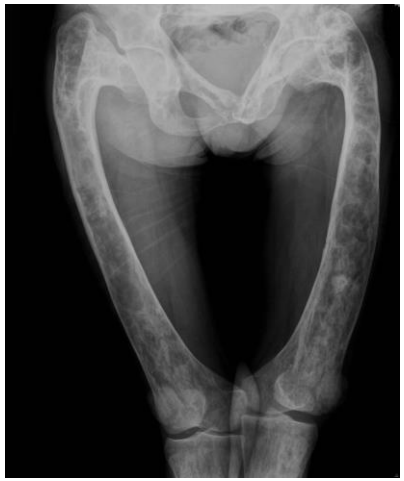
Fibrous Dysplasia (FD)

- Genetic, noninherited disease
- Missense mutation in the GNAS complex locus (R201C and R201H) encoding G protein's α subunit ($G\alpha_s$)
- The disease-causing mutations occur post-zygotically (somatic mosaic disorder)
- Affects skeletal stem cells causing dysfunctional osteoblasts
- May affect one (monostotic form) or several bones (polyostotic form)



Atlas of Genetics and Cytogenetics in Oncology and Hematology



→ No existing cure
→ Severe manifestations, pain
→ Can evolve into sarcoma

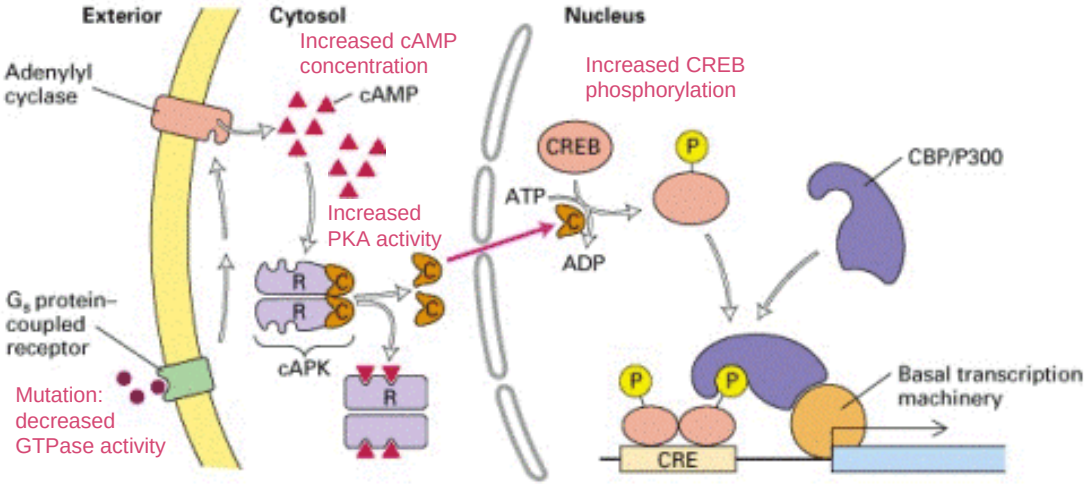
Gsα-receptor signaling pathway

Wt Gsα: normal GTPase activity

Physiological cAMP level

Normal PKA activity

Normal bone formation



Gsα mutated pathway

VS

Mutated Gsα:
⇩ GTPase activity:
constitutively active Gsα

⇧ cAMP level

⇧ PKA activity

Abnormally differentiated osteoblasts

Higher PKA activity

- **Lack of mineral deposition**
- **Upregulation of osteogenic markers (ALP, c-fos…) + IL-6**

Potential therapeutic targets:

- **GNAS mutations: single-nucleotide**

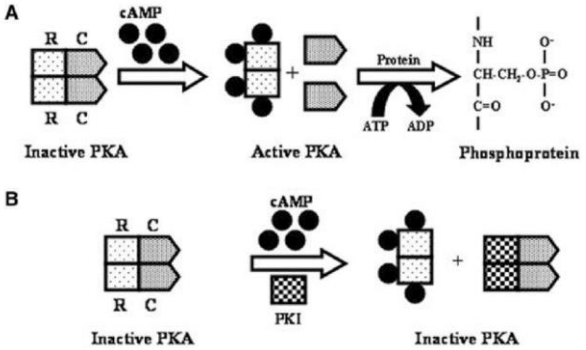
→ Gene-correction should be very specific (high risk of off-target)

- **cAMP/PKA pathway: universal pathway**

→ Necessity for a very specific therapeutic strategy (high risk of side effects)

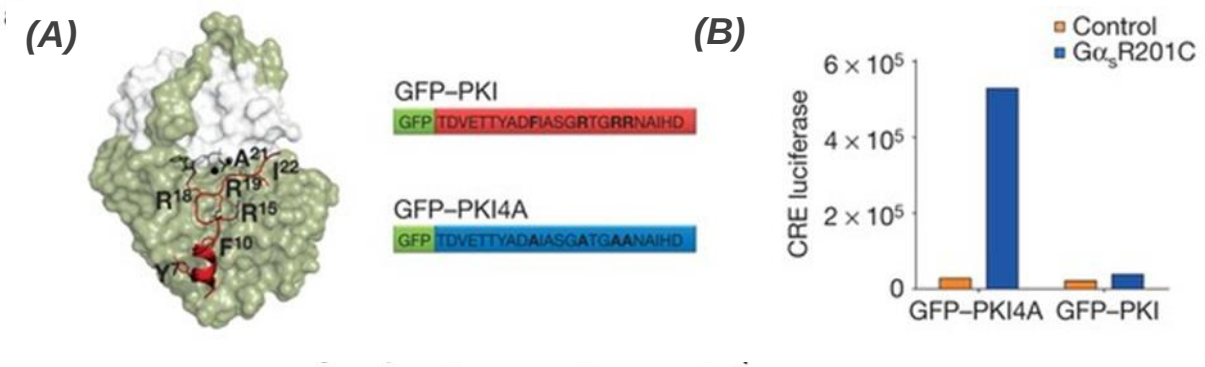
Our choice: PKIγ, a specific PKA C-subunit inhibitor

- **PKI: Protein Kinase Inhibitor**
- 3 isoforms (PKIα, β and γ)



Dalton G. D., Dewey W. L. (2006).

- **PKIγ**
- Short peptide (7,7kDa)
- Specifically inhibits PKA C-subunit (catalytic activity)
- Endogenously expressed in BMSCs (low imunogenicity)
- PKIγ persistent overexpression leads to downregulation of osteogenic markers
- No evidence that it is involved in FD (Its pattern of expression is not altered by PKA signaling)



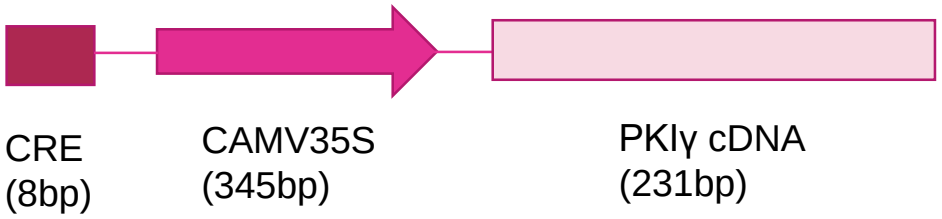
Structure of PKI and its inactive mutant PKI4A (A) and their effect on CRE-luciferase activity (B)
Iglesia-Bartolome and al, (2015)

Our proposal

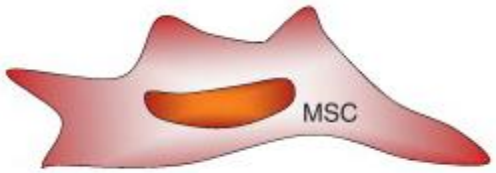
→ *Restore PKA normal catalytic activity by using PKIγ under the control of a CRE: restore a normal osteogenesis*

- **CRE: cAMP Response Element**
→ Enhancer that directly responds to cAMP endogenous level

- **CAMV 35S: minimal promoter**
→Not strong enough to induce the transgene expression itself
→Expression directly dependent to the CRE

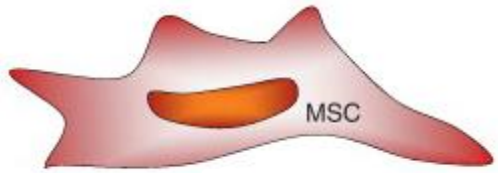


Total size of the construct = 584pb



Normal BMSC :
Physiological level of cAMP

→ *Low expression of the CRE-induced PKI*



Diseased BMSC:
Higher level of cAMP

→ *High expression of the CRE-induced PKI at first*
→ *When PKA activity is sufficiently decreased, lower expression of CRE-induced PKI*

What system of delivery ?

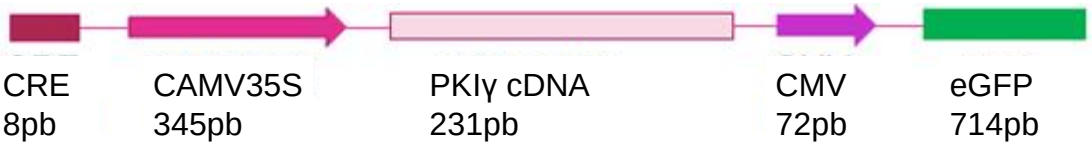
- **The Adeasy system combined with an ameliorated PiggyBac system**

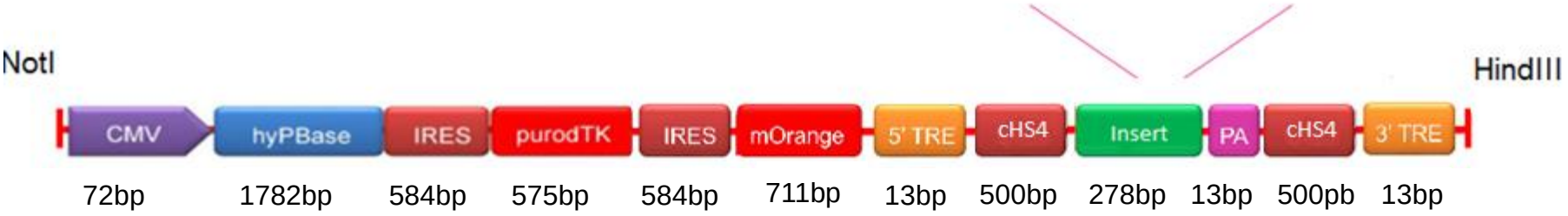
→ Combines the advantages of :

- **Adenovectors**: safe and well-tolerated, high efficiency of Ad cell/ nucleus entry process, transduction of both dividing and non-dividing cells
- **Adeasy system**: Rapid and efficient, preparation of high stock of purified viruses, insert size up to 7,5kb
- **PiggyBac system**: Stability of transgene expression, robust and highly efficient transposition

Specificity of the system:
→Transposase and transposon delivered in the same plasmid
→ Self-inactivation of the transposase induced by the lost of pA signal (shared with the GOI)
→ HSV-tk/ganciclovir based negative selection to eliminate the rare transposase-expressing cells

Total size insert 6,905bp

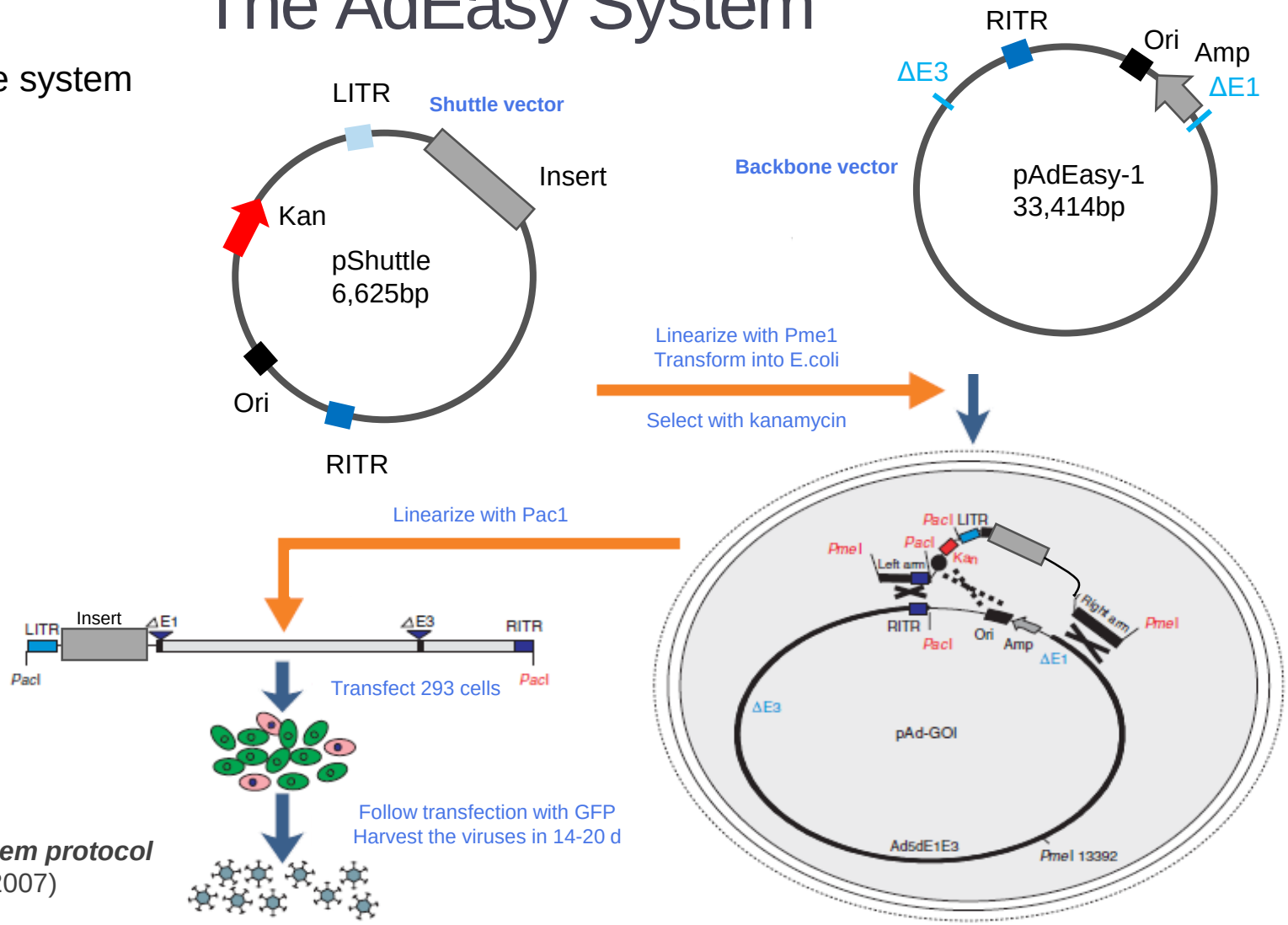




Adapted from
Chakraborty and al. (2015)

The AdEasy System

- Overview of the system



AdEasy system protocol
Luo and al. (2007)

- Minimum of enzymatic manipulations, employing homologous recombination in bacteria cells
- Very fast and efficient generation of recombinant adenoviruses

The AdEasy System

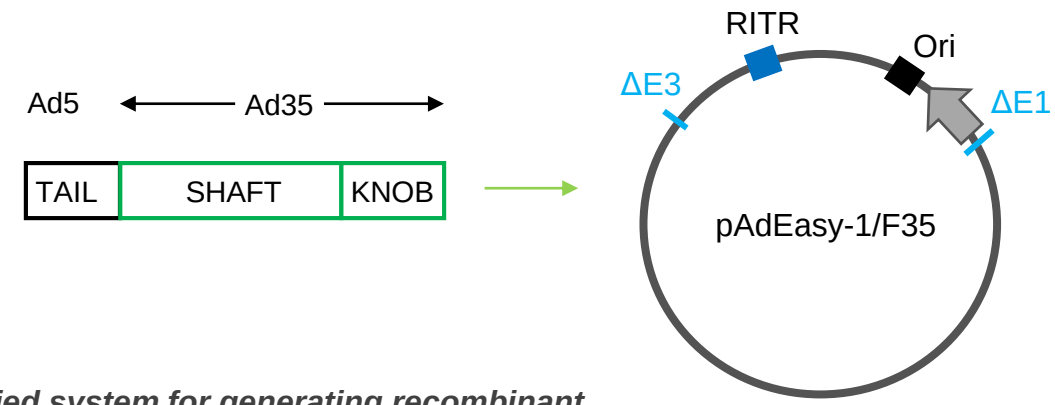
- **Cloning of the construct into a shuttle vector :**

→ 4 different conditions for our experiments :

1) Mock
2) Vector GFP: AdShuttle (GFP alone)
3) Vector PKI4A: AdShuttle-PKI4A (GFP+ CAMV35S-CRE-PKI4A construct → mutant version of PKI)
4) Vector PKIγ: AdShuttle-PKIγ (GFP + CAMV35S-CRE-PKIγ construct)

- **Modify the Ad fiber domain on the backbone vector :**

→ Ad5 fiber gene will be replaced by a chimeric Ad5 fiber tail domain and Ad35 fiber shaft and knob domains with increased tropism for hMSCs



A simplified system for generating recombinant Adenoviruses *He and al., 1997.*

TIMING

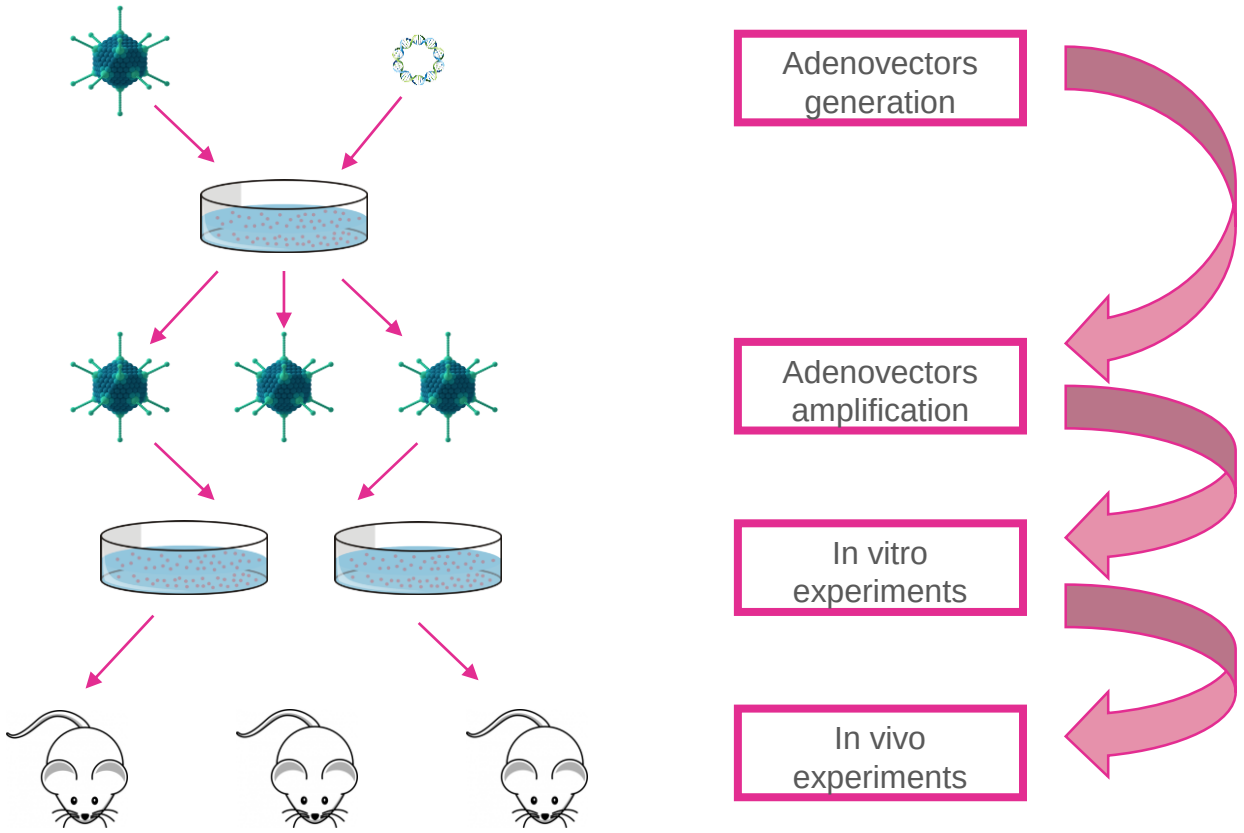
- Step 1 : 6-15 d

→ cloning insert into a shuttle vector
→ modify the fiber domain on Ad genome

- Step 2 : 10-30 d

→ generating the initial stocks of adenoviruses
→ stepwise amplification for high-titer adenoviruses
→ determining viral titers

Experimental setup and objectives of the project

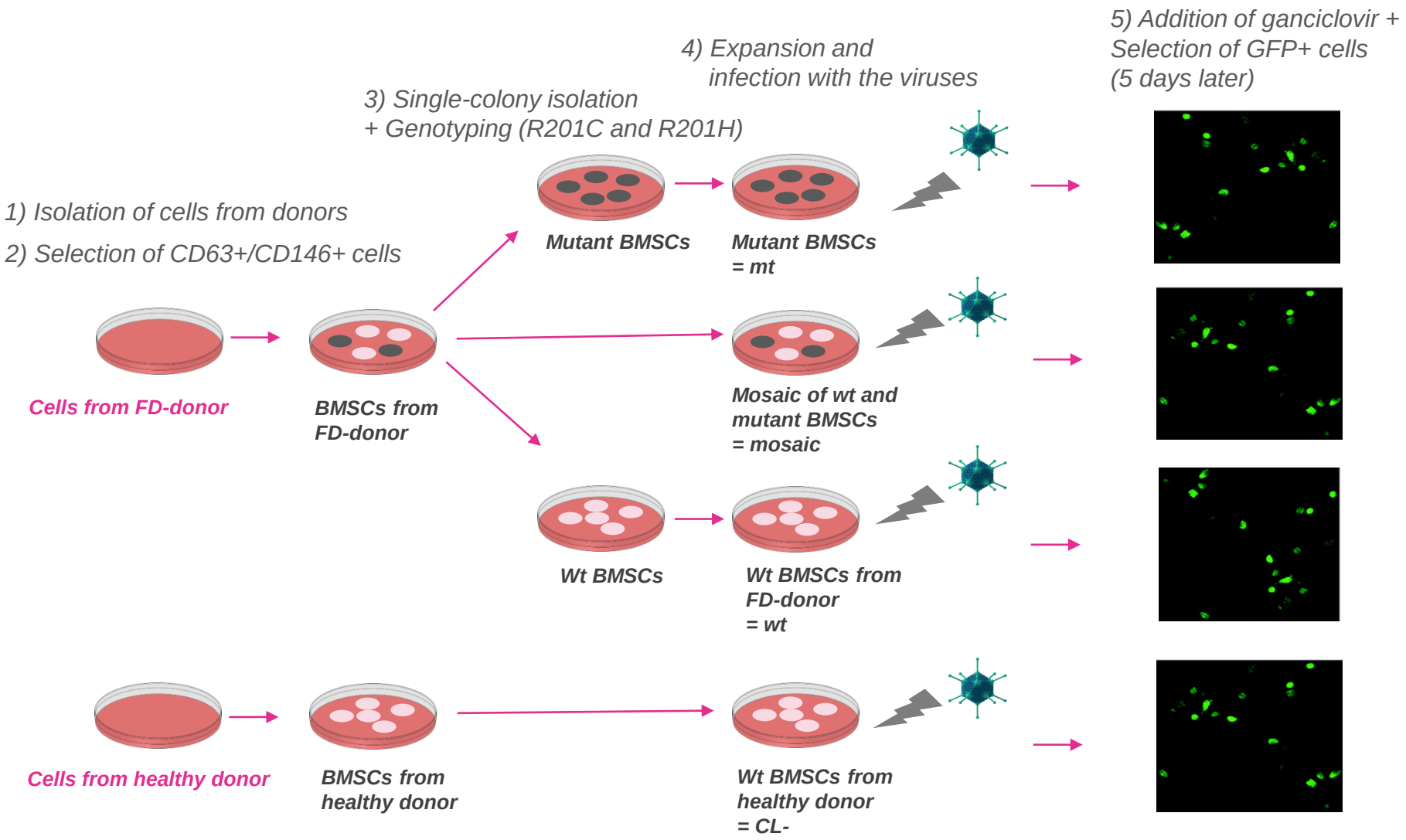


- **Proof of concept that PKI can serve as a therapeutic strategy for FD:**
- In vitro (hMSCs)
- In vivo (mouse model of xenograft)
- **Show that AdPKI-F35 is the right vector to efficiently deliver PKI to target cells**

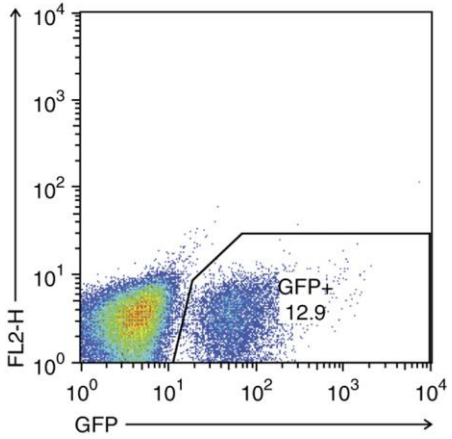
In vitro experiments (hMSCs)

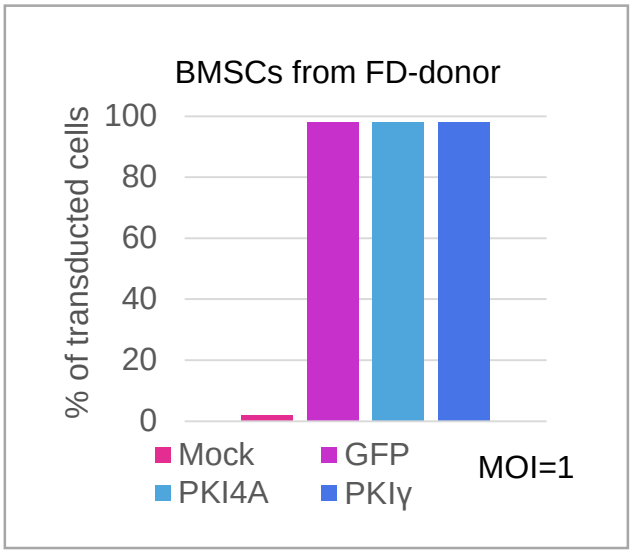
- **Infection of hBMSCs with the different vectors**

→ 4 types of cells:



Is the transduction efficient ?





- Serial MOI trials
- **Transduction** : GFP

  Cell death: PI + Annexin V

→ *The results should be identical whatever the cell type and vector used*

→ *We will choose an optimal MOI for further experiments*

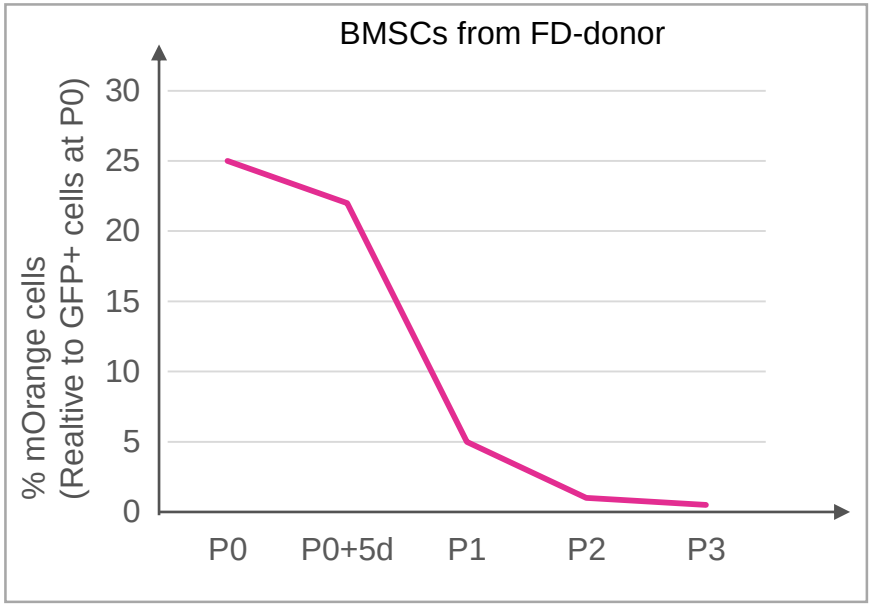
Are the integration and transposase inactivation efficient ?

- **Measurement of number of copy/genome**: qPCR copy number PiggyBac Kit (SBI**)**

→ *We expect to have about one copy/genome*

- **Measurement of residual transposase expression**: mOrange expression as a proxy

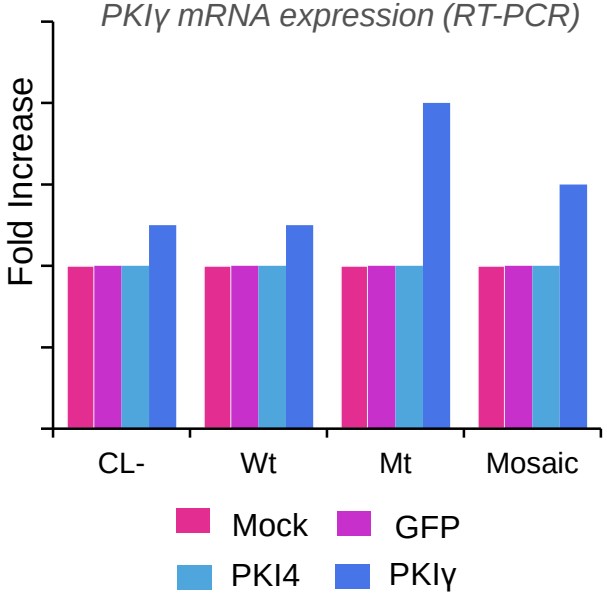
→ *We expect the transposase to have no residual activity*



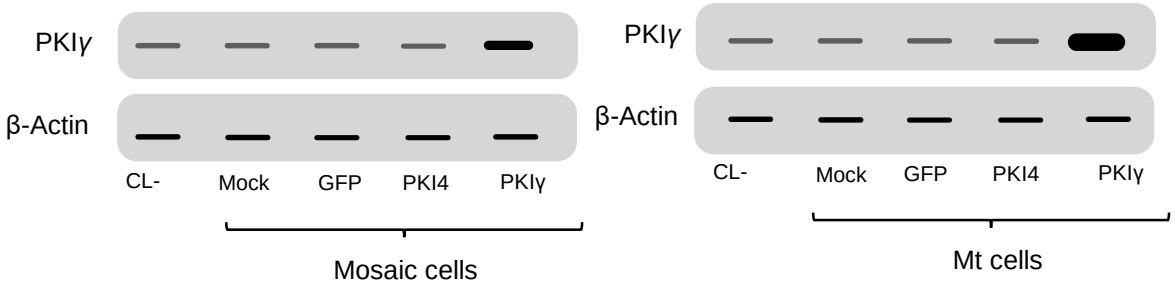
Is the strategy working ?

- **PKIγ expression**

PKIγ mRNA expression (RT-PCR)



PKIγ protein expression (Western Blot)

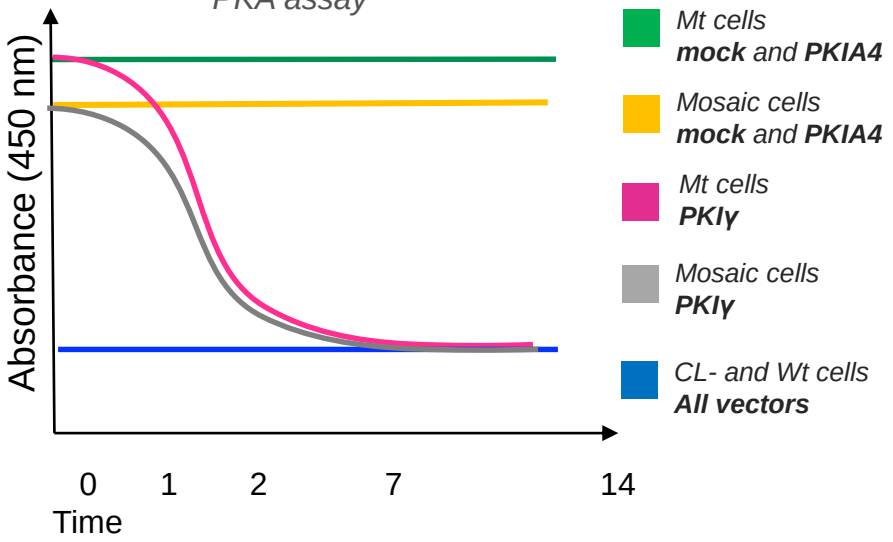


→ *PKIγ expression should increase in PKIγ treated FD cells*
→ *This global increase should be higher in mutant pure cultures*

- **Functional assay: PKA activity**

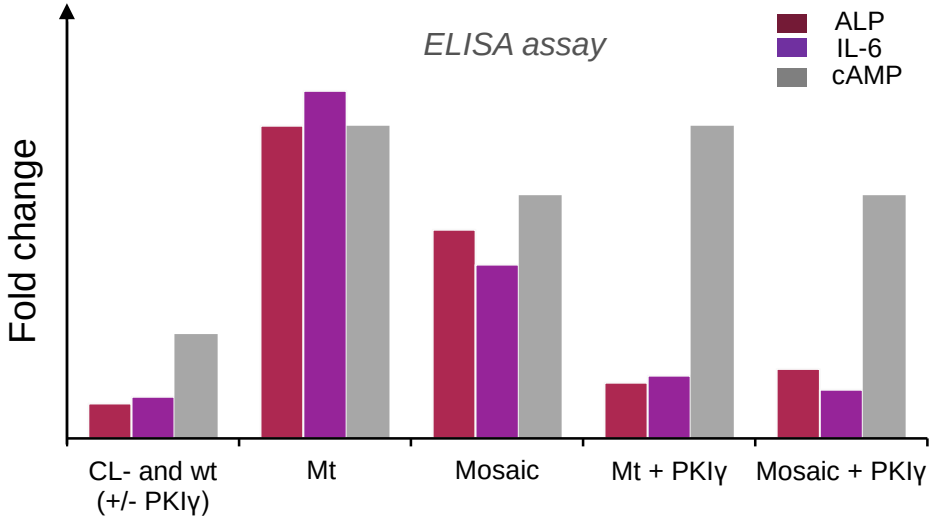
→ *PKA activity should be normalized in FD cells*
→ *There should no effect or only a slight decrease in normal cells*

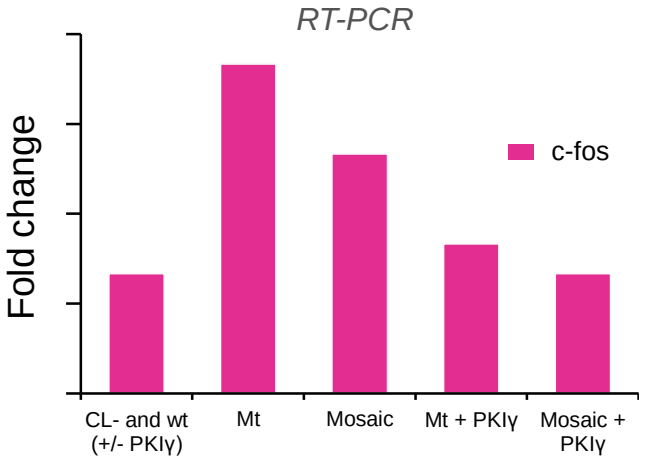
PKA assay



Is the strategy working ?

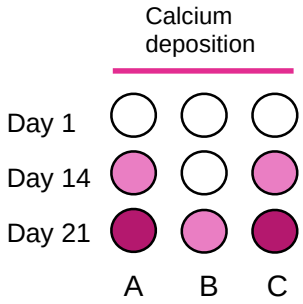
- **Effect on FD markers levels**





→ *We expect a normalization of ALP, c-fos and IL-6 levels*
→ *We expect no change in cAMP level*

- **Effect on in vitro differentiation**



A: Normal BMSCs
B: FD BMSCs
C: FD BMSCs + PKIγ

Cultured in osteogenic medium

→ *PKIγ-transduced FD cells should normally differentiate*

Is the system safe ?

- **Control of insertion sites by Splinkerette-PCR**
- **Cytotoxicity measurement by FACS analysis**

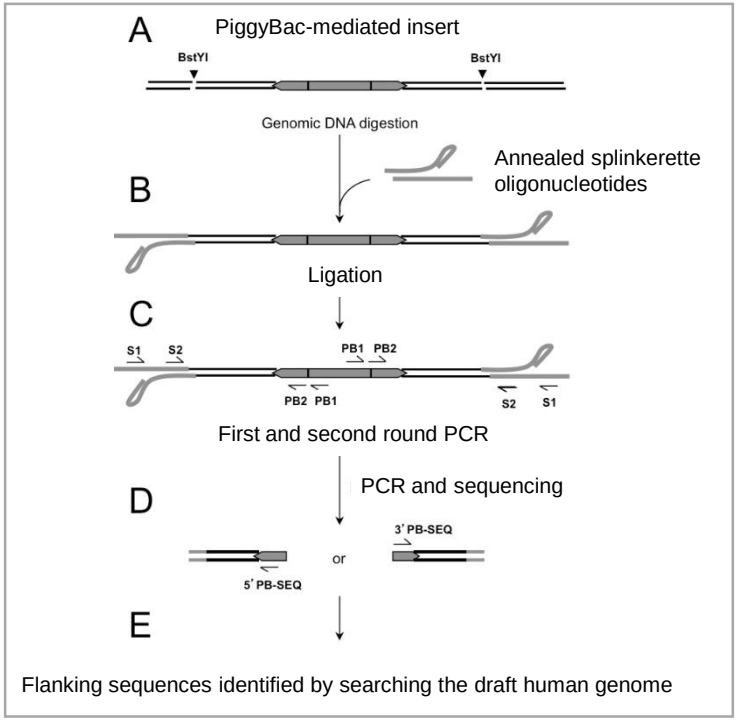
→ *There should be no effect on cell death*

- **Proliferation assay**

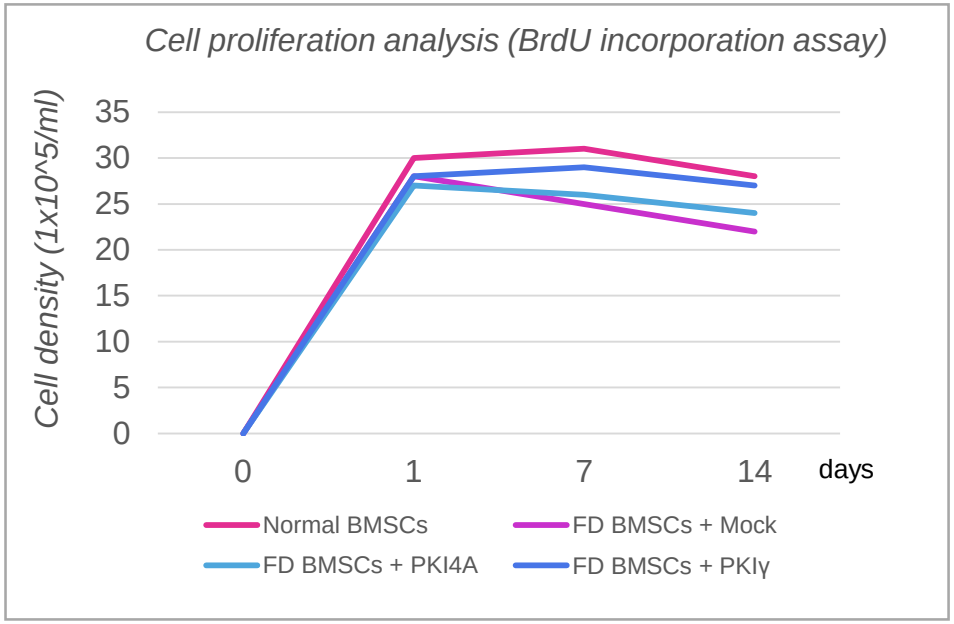
→ *There should be no effect on cell proliferation*

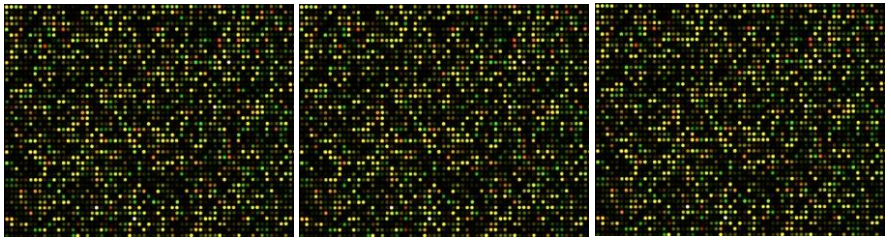
- **Effect on other essential cellular pathways**

→ *There should be no dysregulation of other signaling pathways*



Splinkerette-PCR





hBMSCs from healthy donor

hBMSCs from healthy donor + PKIγ

hBMSCs from FD donor + PKIγ

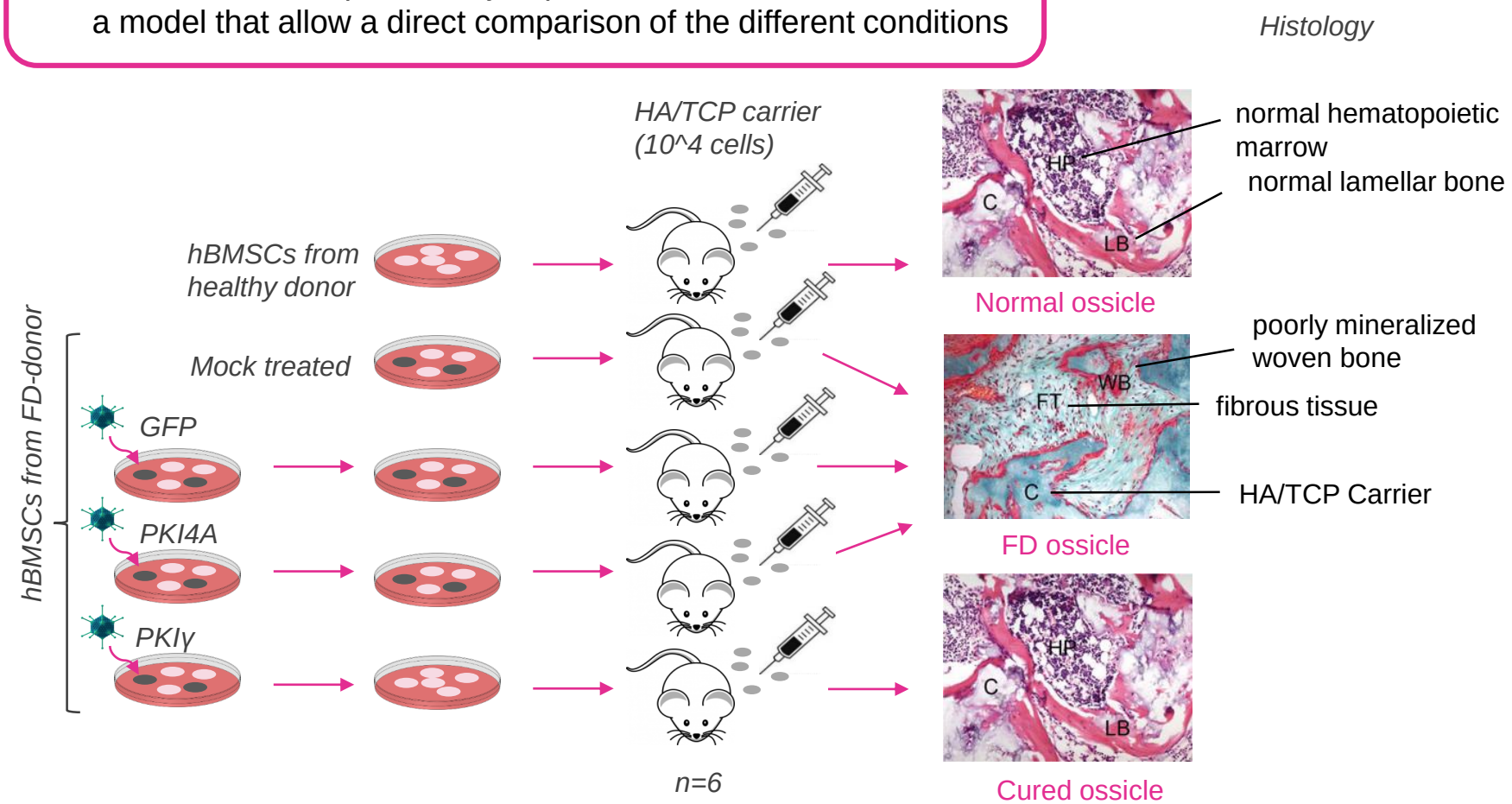
Microarray analysis

Xenograft model of SCID mice

- Xenograft of hBMSCs in immunocompromised mice to evaluate AdShuttle-PKIγ potential to regenerate a complete ectopic ossicle

Why using this model ?

- In vivo: Adenoviruses too immunogenic (IL-6 response)
- Ex vivo: Need for preliminary experiments on mice cells, need for a model that allow a direct comparison of the different conditions



Future experiments

Mouse model characterization for PKA activity

Use 3rd generation adenoviruses

In vivo experiments

Only if ex vivo is not efficient

Repeat the experiments in mouse cells (GsαR201C and PKA mouse models)

Ex vivo experiments

Clinical trial

Potential pitfalls and solutions

- **Trouble to isolate or expand single-clones**
- Try other growth media
- Try BMSC-LV-Gs201C cell model

- **Trouble to efficiently transduce BMSCs**
- Try another adenovector with RGD-modified domain

- **GFP toxic effect**
- Use another reporter gene (HrGFP)

- **Insertional mutagenesis**
- Use of a suicide gene

- **PKI efficiency issues**
- Increase the number of CRE copies
- Try another isoform (PKIα →affinity=6-fold higher)
- Try a strong promoter (such as CRE)

- **PKI toxicity issues (it causes cell death, or trigger too strong effects) or interfers with other pathways**
- Try a synthetic PKI

- **cAMP accumulation in the cell due to lower PKA activity**
- Try to combine our system with PDE4 to degrade cAMP

Timescale, materials and cost of the project

- NOD-SCID Mouse (one male, one female): 188€
- MicroArray Analysis: 695€
- cAMP assay (c-AMP-Glo™ assay): 299€
- Alizarin Red S staining: 65€
- Annexin V-FITC Apoptosis Detection Kit: 505€
- RT-qPCR analysis QuantiTect SYBR Green PCR kit: 417€
- BrdU Cell Proliferation Assay Kit: 339€
- Alkaline Phosphatase ELISA Kit: 680€
- PKIγ antibody: about 300€
- AdEasy Adenoviral Vector Systems: 357€
- Functional Assay of PKA activity kit: 567€
- IL-6 ELISA Kit: 450€
- Super piggyBac transposase expression vector: 350€
- PiggyBac™ splinkerette PCR Kit: 399€
- PiggyBac copy number Kit: 522€
- Cell culture: 2 000€
- Animal facility: 5 000€

- http://www.taconic.com
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- https://www.promega.com
- http://www.sigmaaldrich.com
- http://www.abcam.com
- http://www.qiagen.com
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- http://www.transposagenbio.com
- http://www.integratedsci.com
- http://www.transposagenbio.com

This project has a timescale of 2 years and a total cost of 30.000 €

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SBI System Biosciences · QIAGEN · MERCK MILLIPORE · ThermoFisher SCIENTIFIC · addgene The nonprofit plasmid repository

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Thank you for your attention