Fibrous dysplasia: new approaches

Poster 2019/2020

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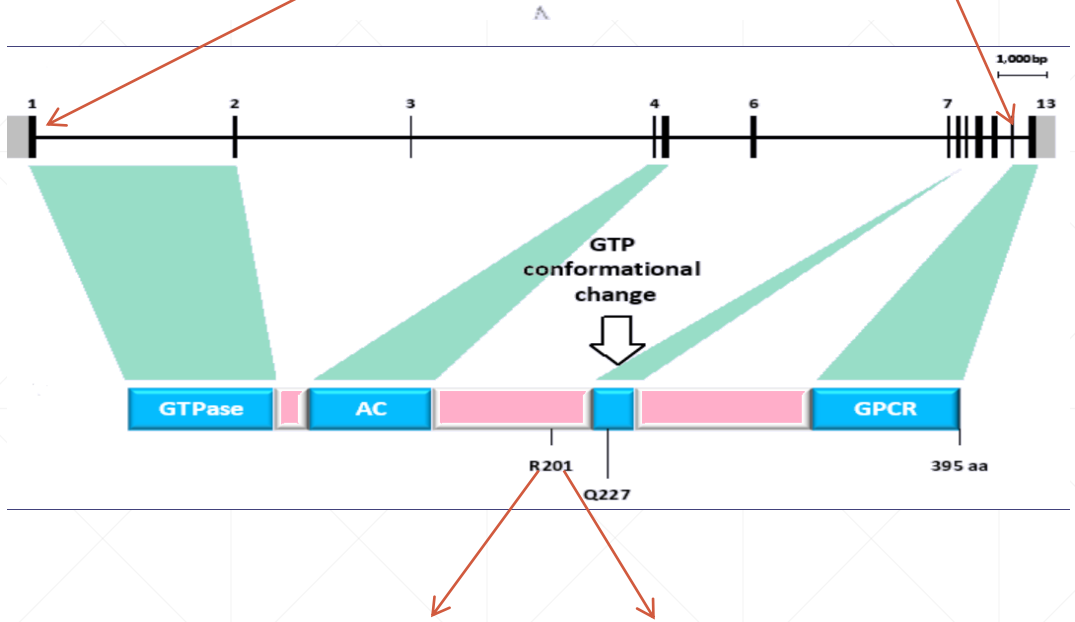
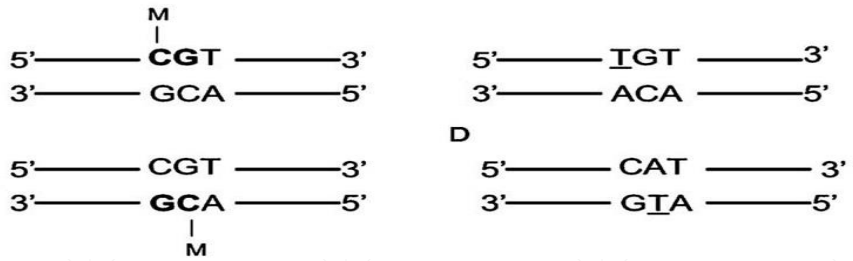
Organization and imprinting of the GNAS complex locus

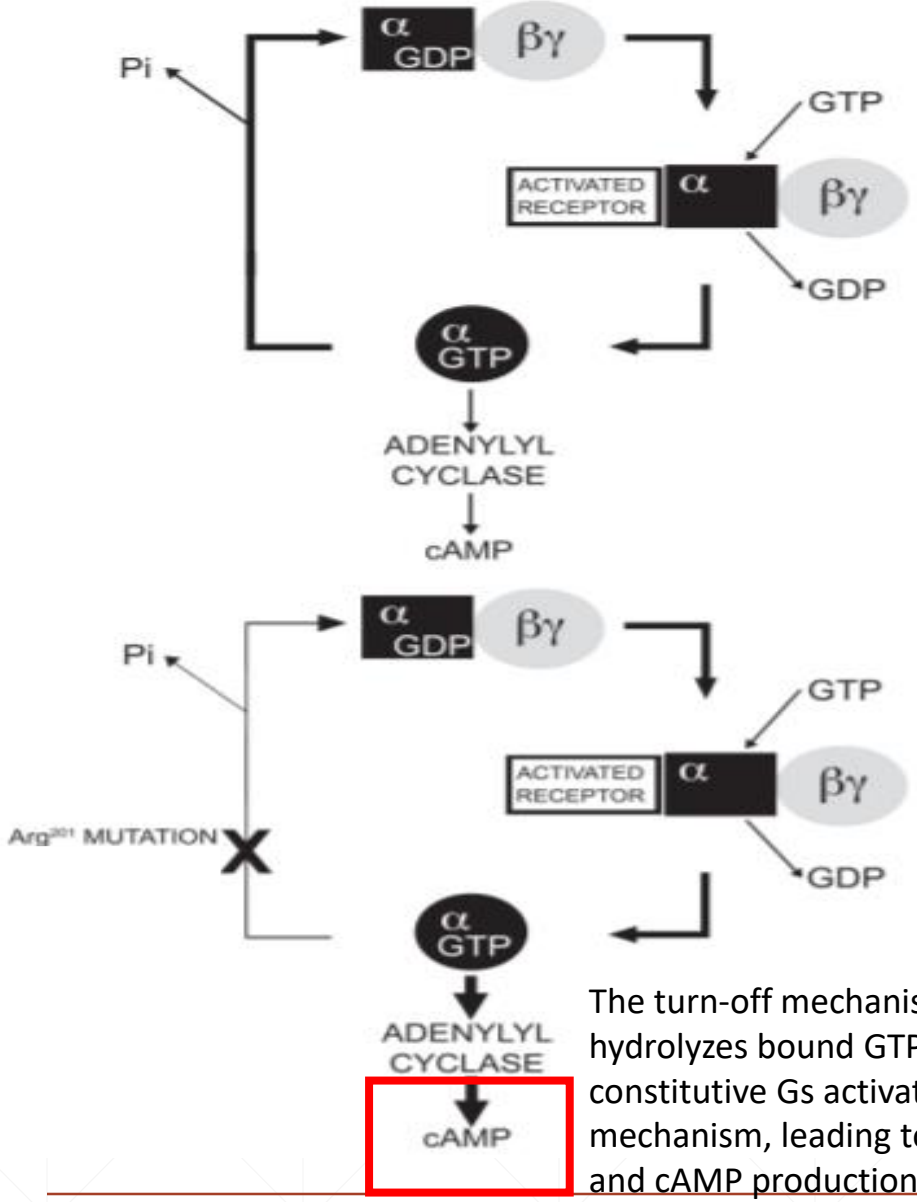


Schematic representation of GNAS gene and Gsa protein

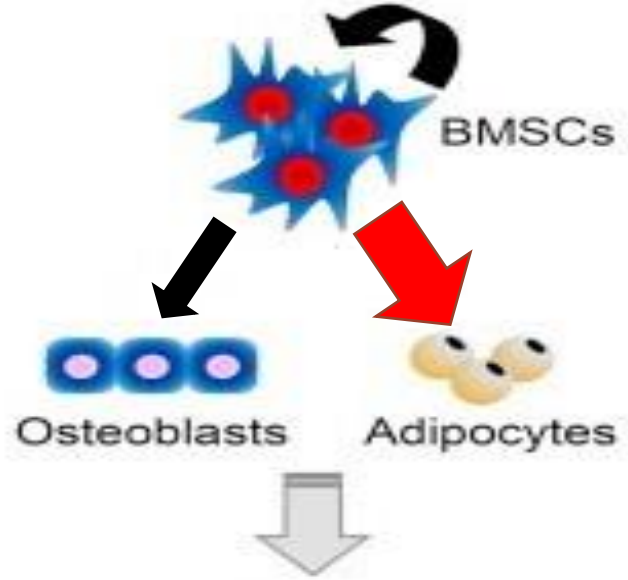


R201

FD is caused by a mutation in GNAS gene

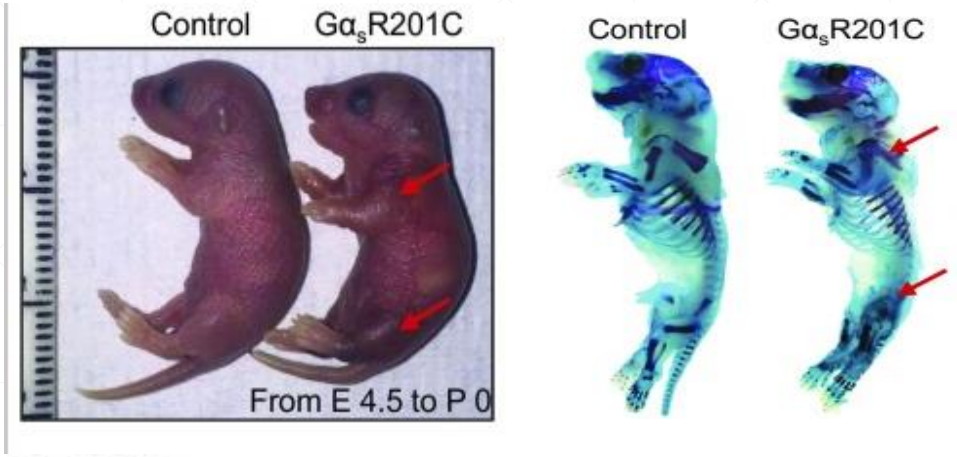


The turn-off mechanism is an intrinsic GTPase activity that hydrolyzes bound GTP to GDP. Mutation of Arg201 lead to constitutive Gs activation by disrupting the GTPase turn-off mechanism, leading to excess adenylyl cyclase activation and cAMP production.

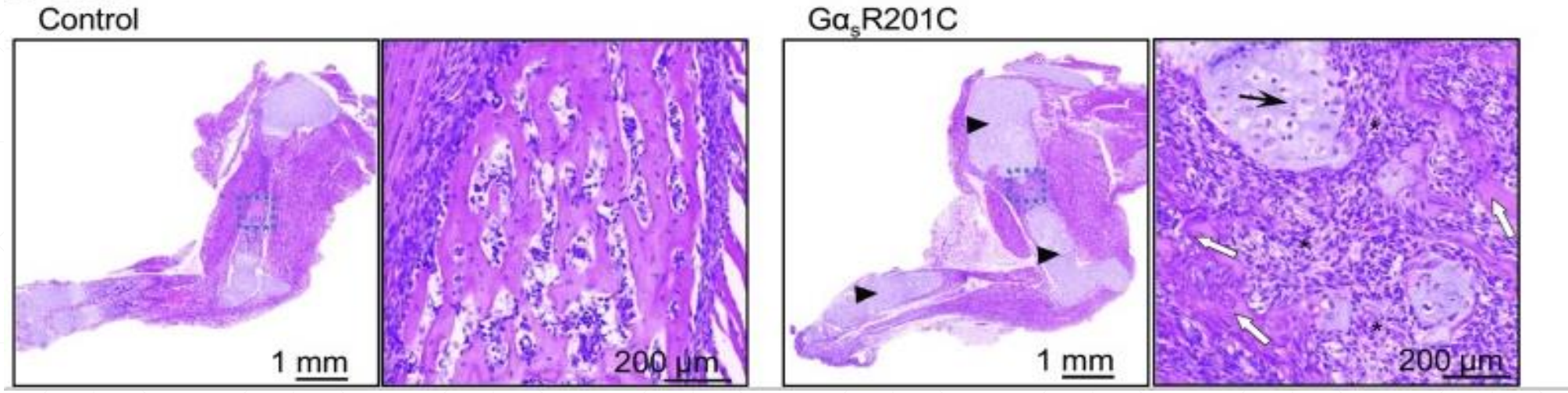


FD lesions

Mutation consequences



Rapresentative image of the expended limbs of $G\alpha_s^{R201C}$ mice



H&E sections present limb bone deformity of $G\alpha_s^{R201C}$ mice with predominance of chondroid matrix (arrowheads) and reduced endochondral ossification

PRESENT SITUATION:

Drugs are just a palliative

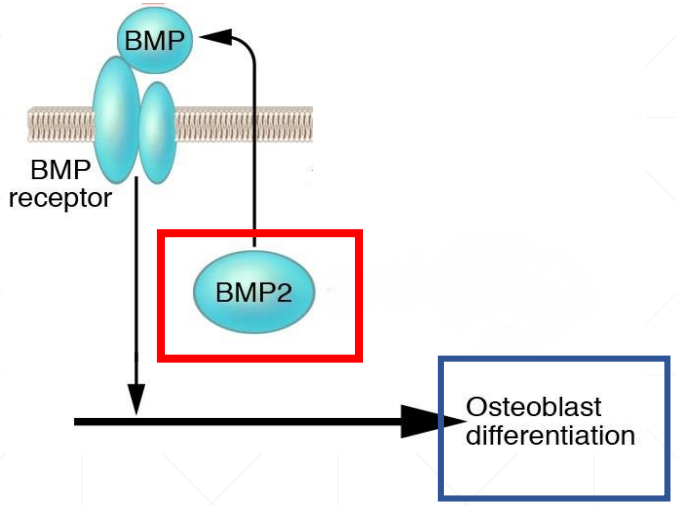
Osteotomy is a solution, but too invasive

We need new ideas for treatments

Our goals and what we are going to do

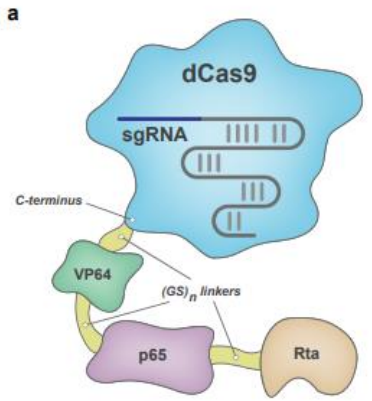
Goals:

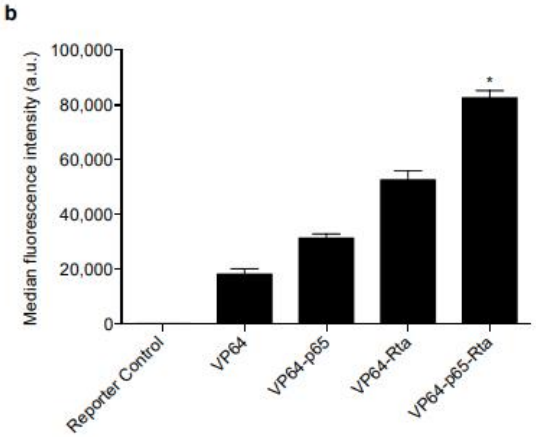
Increase the proliferation of healty cells to restore a wild type condition



Strategy:

Produce iPS and use dCas9 to overexpress BMP-2





Experimental plan

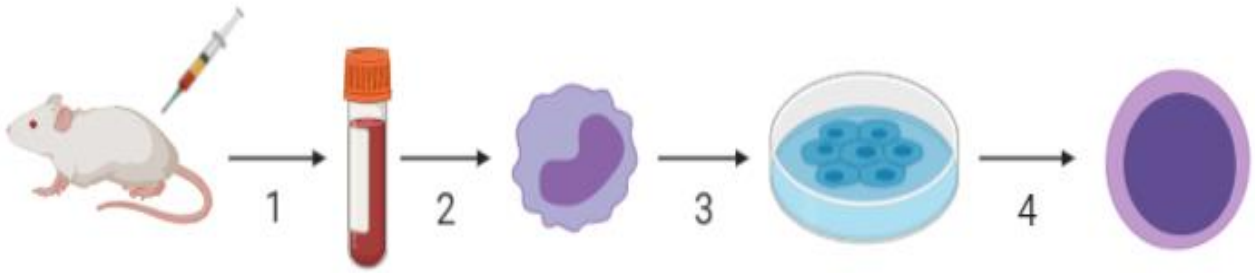
In vitro

- Explant healthy cells from mice peripheral blood
- Inducing PSCs
- Insertion of the dCas9 gene into the produced iPS's genome
- Promotion of osteoblast differentiation
- In-plate control

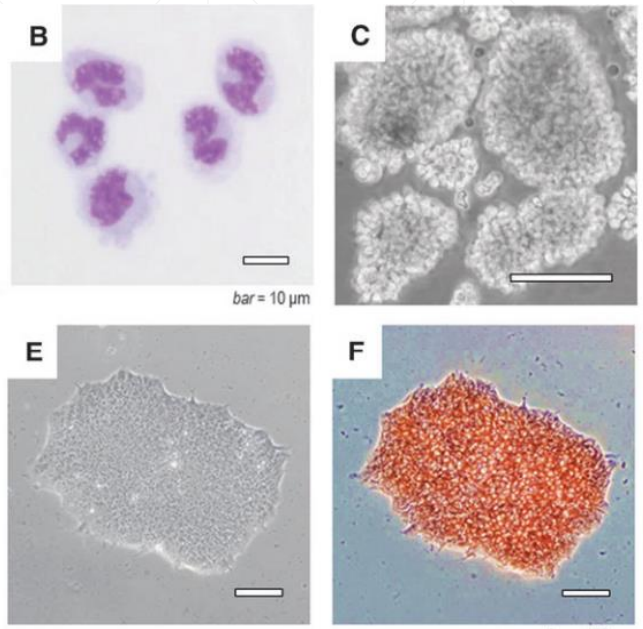
In vivo

- Re-implant in mice
- Control and results

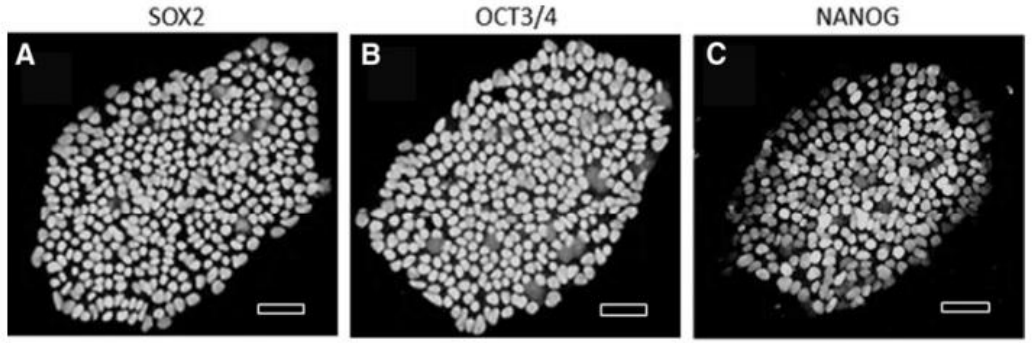
iPS production

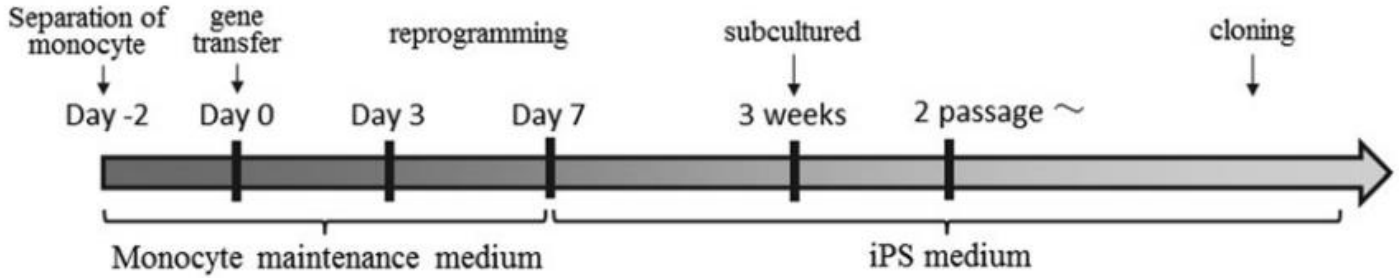


1. Take a blood sample from mouse
2. Isolate **monocites**
3. Growth on BIOTARGET™
4. Induce **iPS** using a vector based on Sendai virus (following Fusaki et al., 2009, protocol)
5. Growth iPS on NutriStem®



- B: Purified monocytes
- C: Monocytes in culture medium
- E: iPS
- F: Alkaline phosphatase assay (all cells resulted positive)
- Down: Immunostaining against three iPS marker proteins





(Timing, adapted from Isogai et al., 2018)

About iPS production

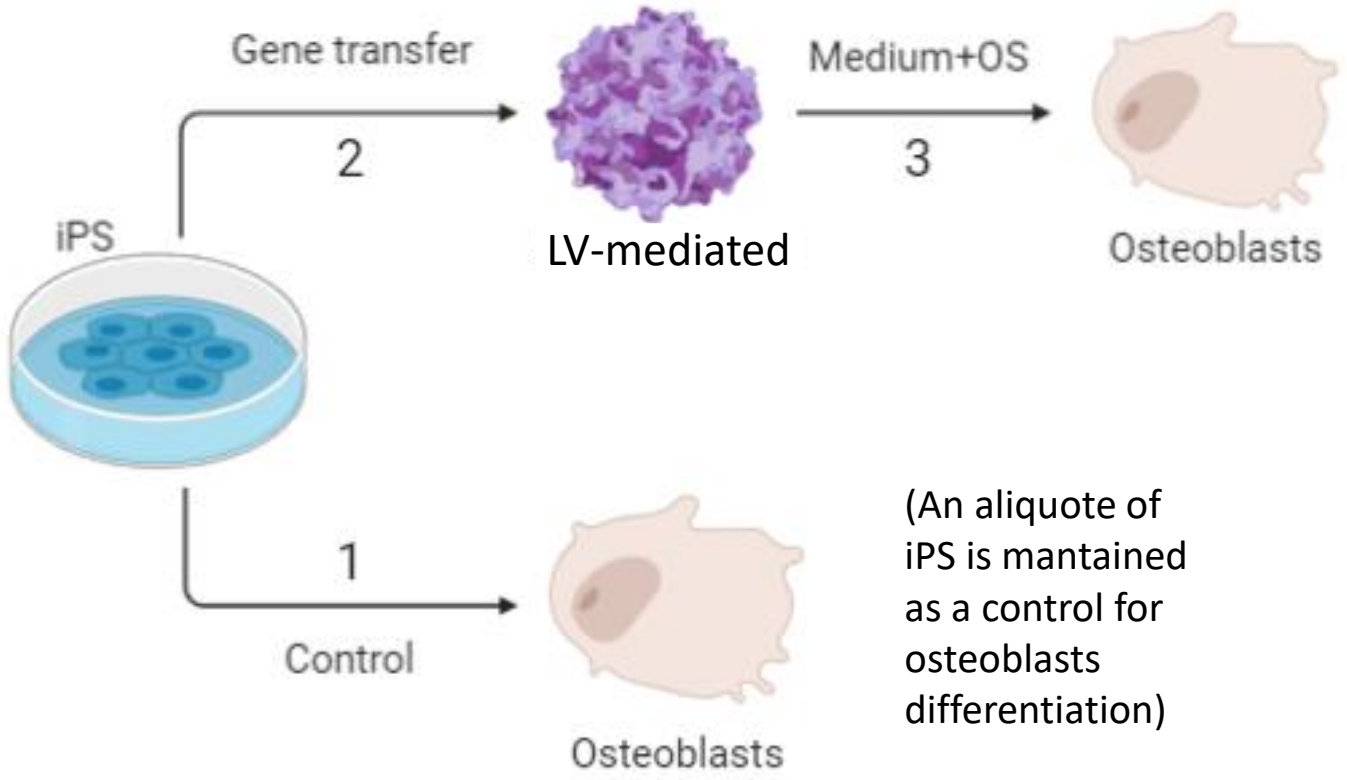
Why not:

- Monocytes barely proliferates in vitro
- Monocytes are very difficult to maintain in vitro

Why yes:

- Sendai virus based vectors can’t cause tumors (there’s no gene integration)
- Sendai virus based vectors are the only one capable of inducing reprogramming pattern into monocytes
- Monocytes are not fully differentiated cells: reprogramming should be easier
- Taking peripheral blood from patient is the less invasive possibility of explant.

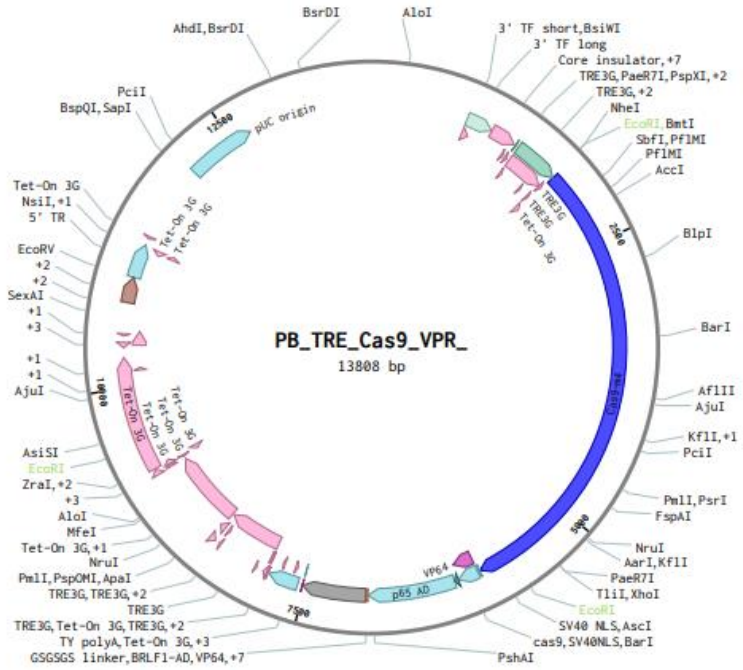
The iPS fate, an overview



(An aliquote of iPS is mantained as a control for osteoblasts differentiation)

1. Control is induced to differentiate into osteoblasts in order to confirm differences between transformated and WT cells
2. The rest of iPS undergoes the LV-mediated gene transfer, in order to over-express BMP-2 (using dCas)
3. And is then induced to differentiate

How to increase BMP2 expression?



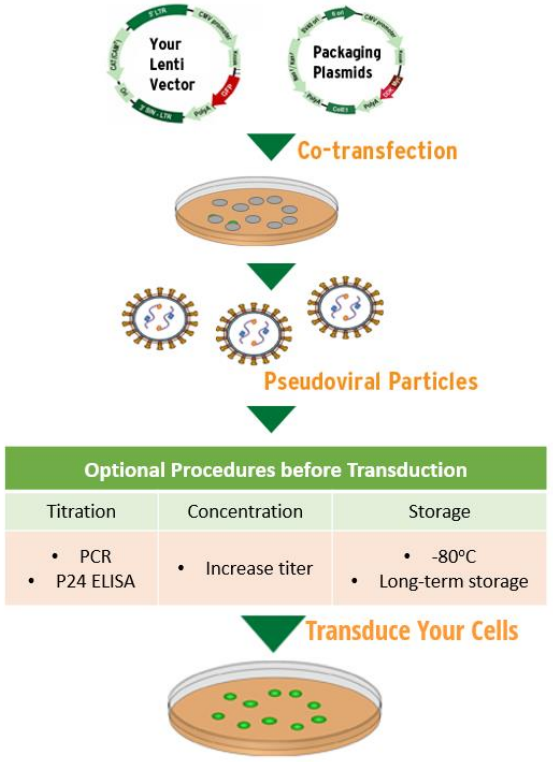
gRNA:

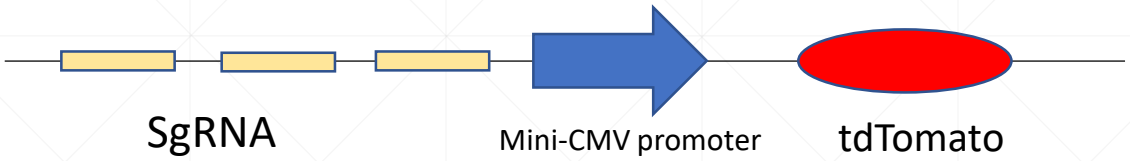
gcggcgacggcggcggcggccg tgg

agcgcggcggggcgaggactccgg cgg

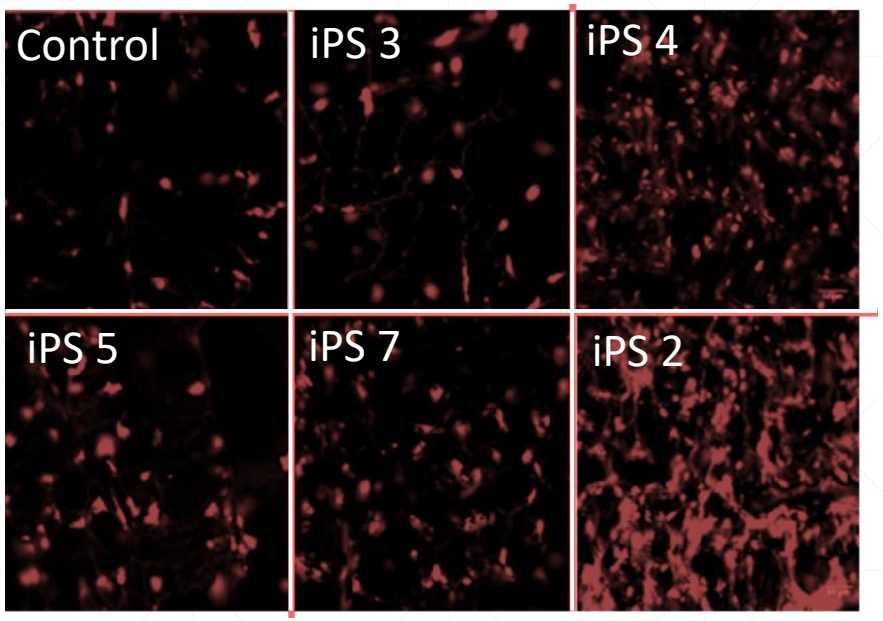
gcgcagcgcgagccgggcgcagcg cgg

Gibson Assembly Cloning kit

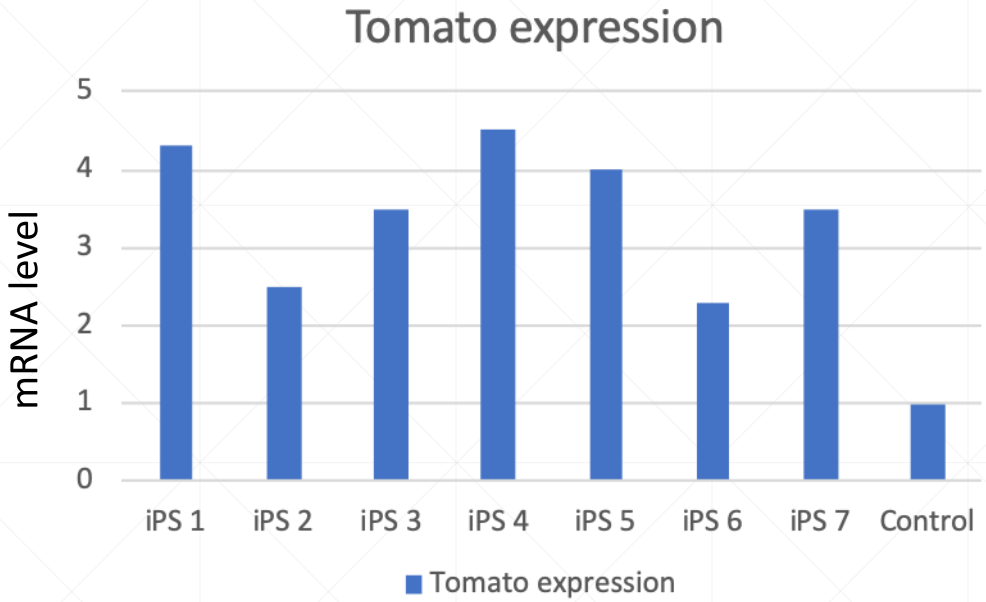


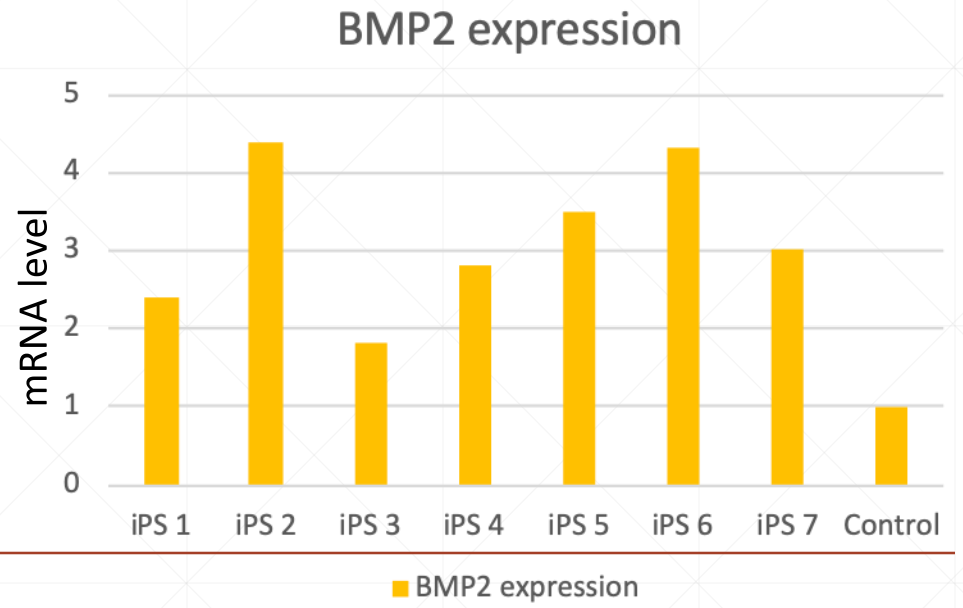


How to check your modified cells?



We obtained different cell lines and performed confocal analysis to check the expression of our construct and qPCR on Tomato and BMP2 expression

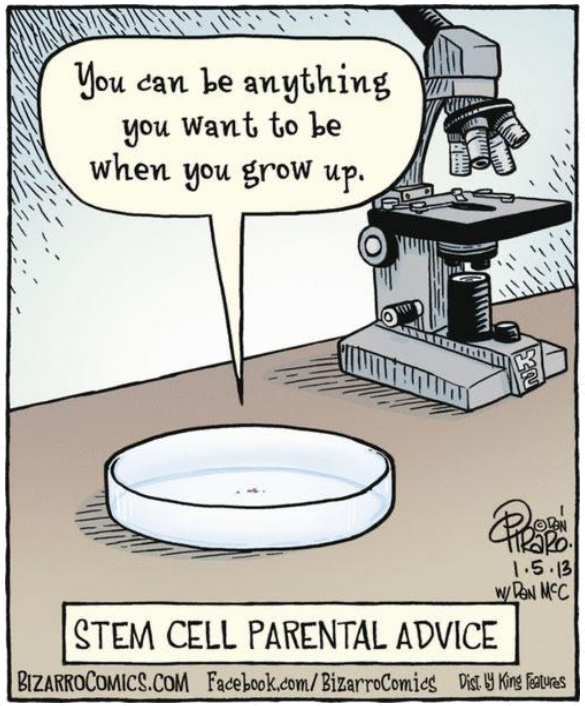




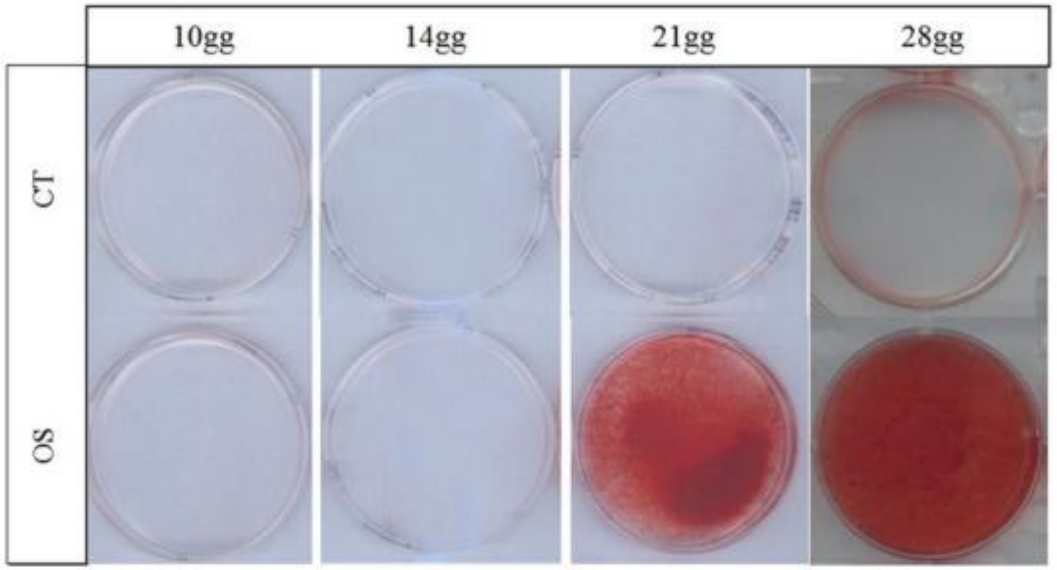
How to induce differentiation?

In order to obtain the differentiation of MSCs, 4000 cells / cm2 are grown *in vitro* in the presence of fetal bovine serum (FBS). In the presence of the following osteogenic supplements:

1. **Dexamethasone:**
   - RUNX2 (Run- related transcription factor 2)
   - OSX (Osterix)
   - Bone matrix proteins
2. **β-glycerolphosphate:**
   - Phosphate groups donor
   - Increasing type I collagen secretion
3. **Ascorbic acid:**
   - Collagene maturation and deposition
   - Induces alkaline phosphatase activity



How to determine a correct differentiation?



Histological staining:

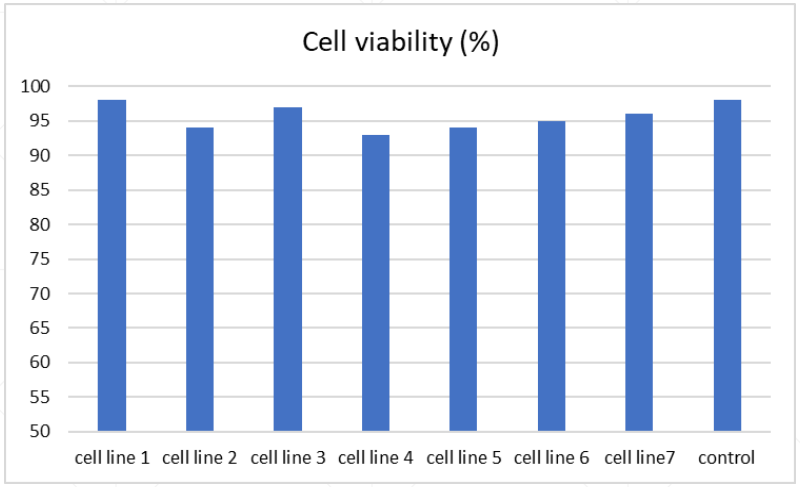
- Von Kossa;
- **Alizarin red S.**

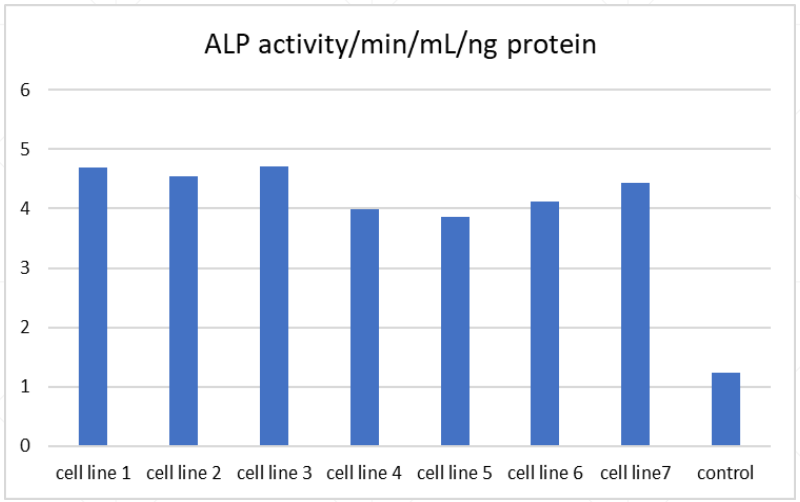
Alkaline phosphatase activity assay

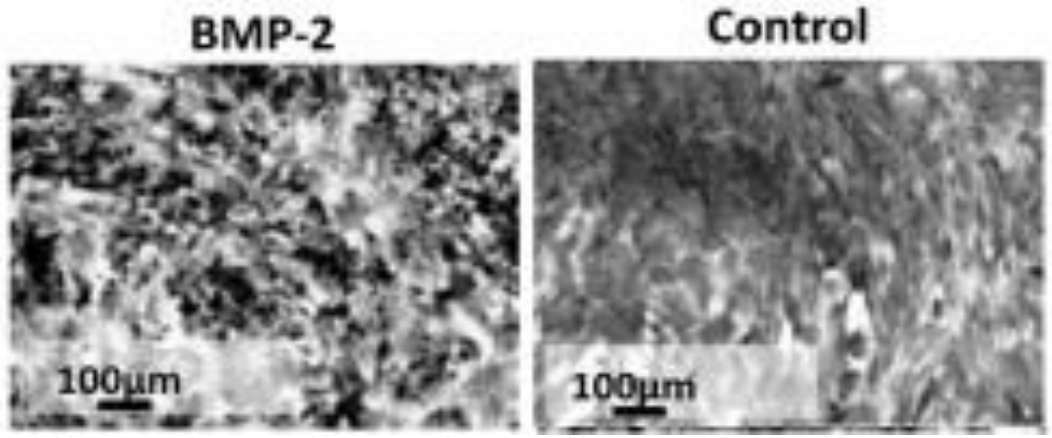
Expression of surface markers

- Osteocalcin;
- Osteopontin;
- Type I collagen.

In-plate results analysis

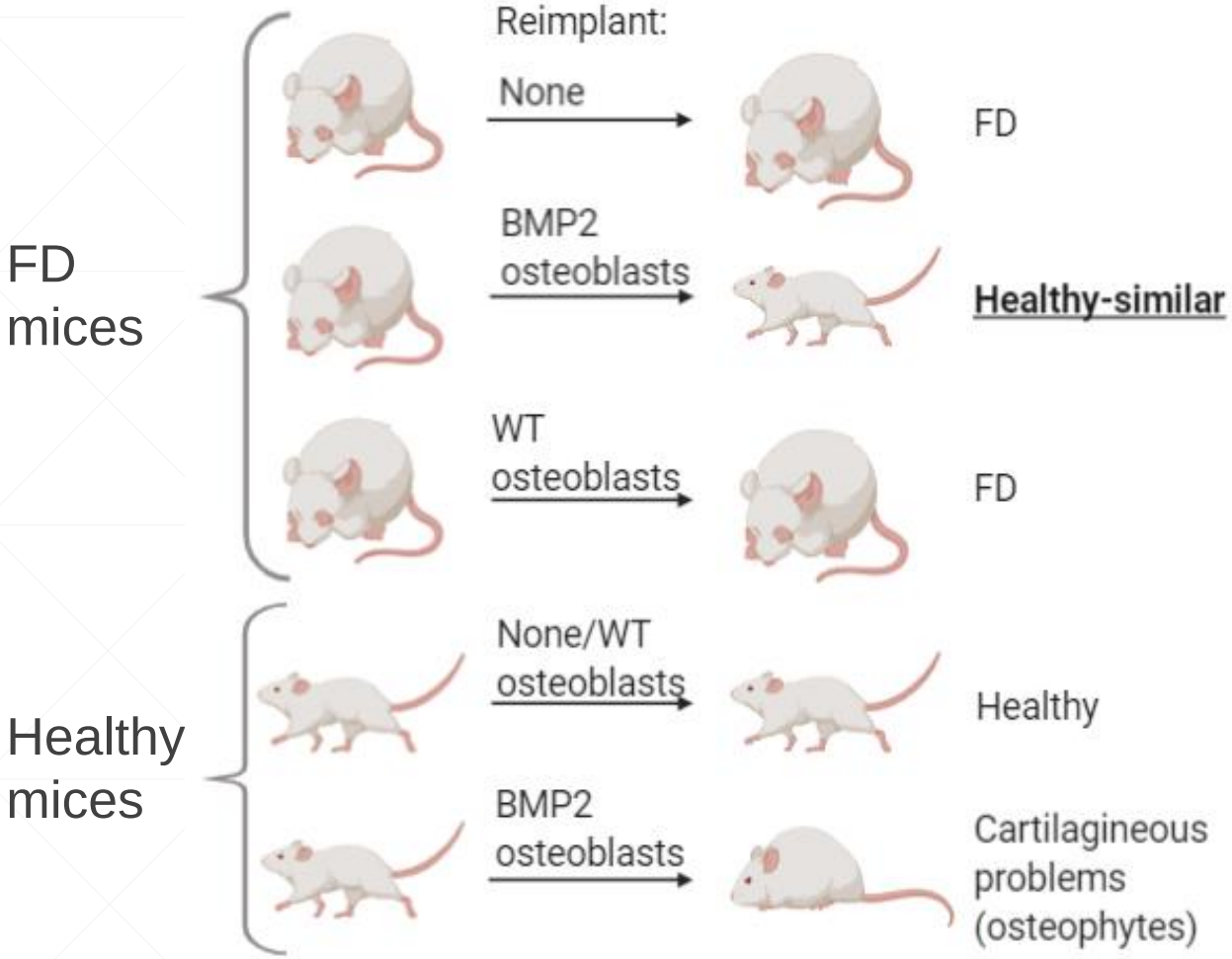


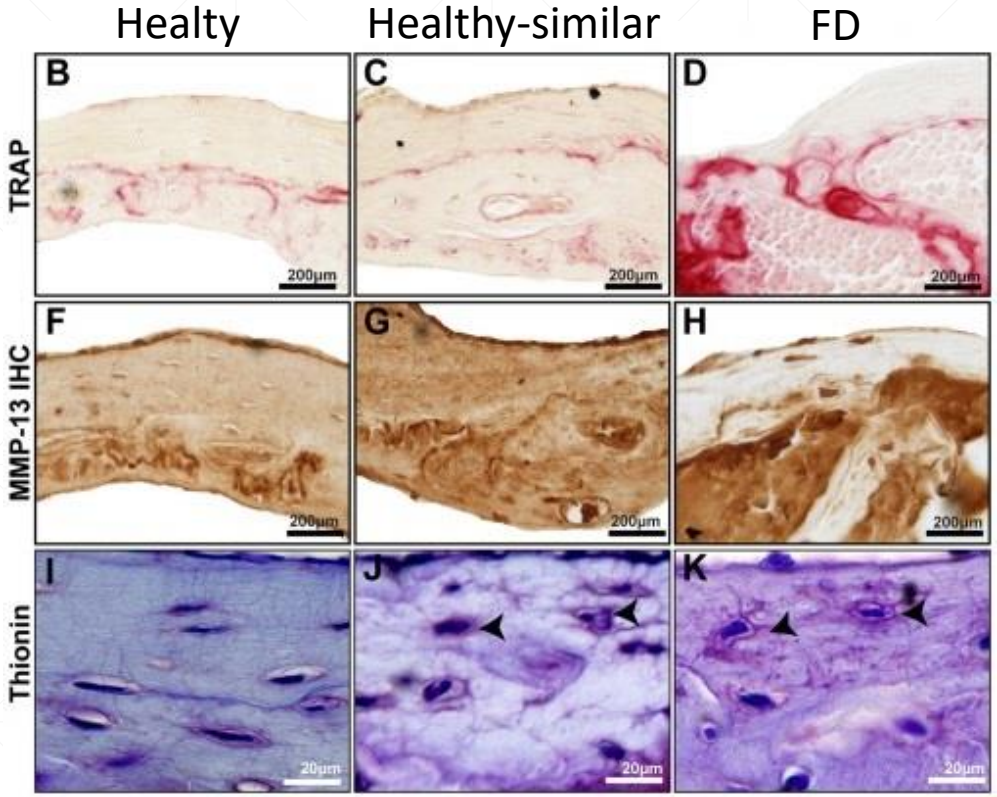




- Viability seemed not to be affected by transfection
- ALP activity assay showed a great increase in ALP activity in all transfected cell lines
- Von Kossa staining showed a greater calcification of transfected osteoblasts
- Treated cells were bigger than control
- Results displayed confirm hypotesis and allow next step: in vivo reimplant.

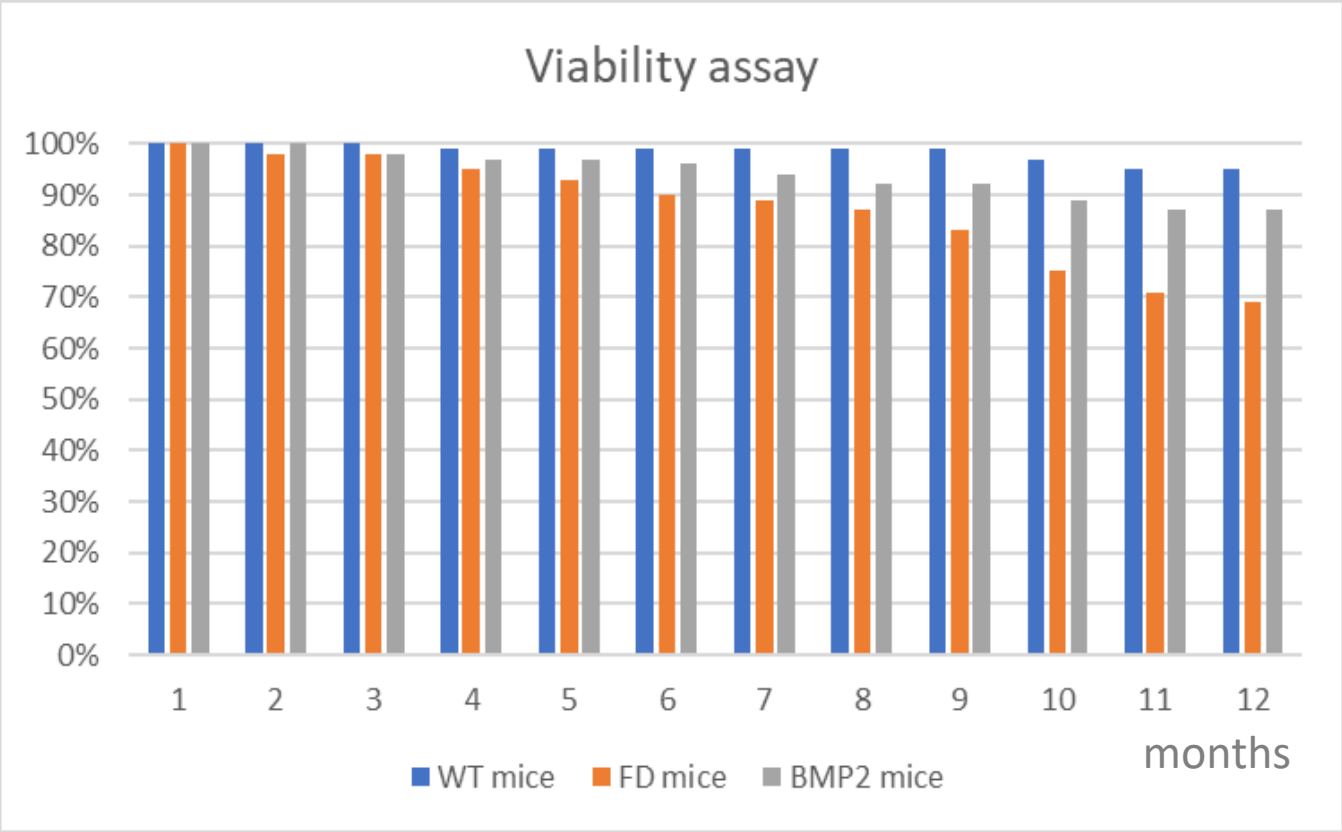
In vivo schematic results

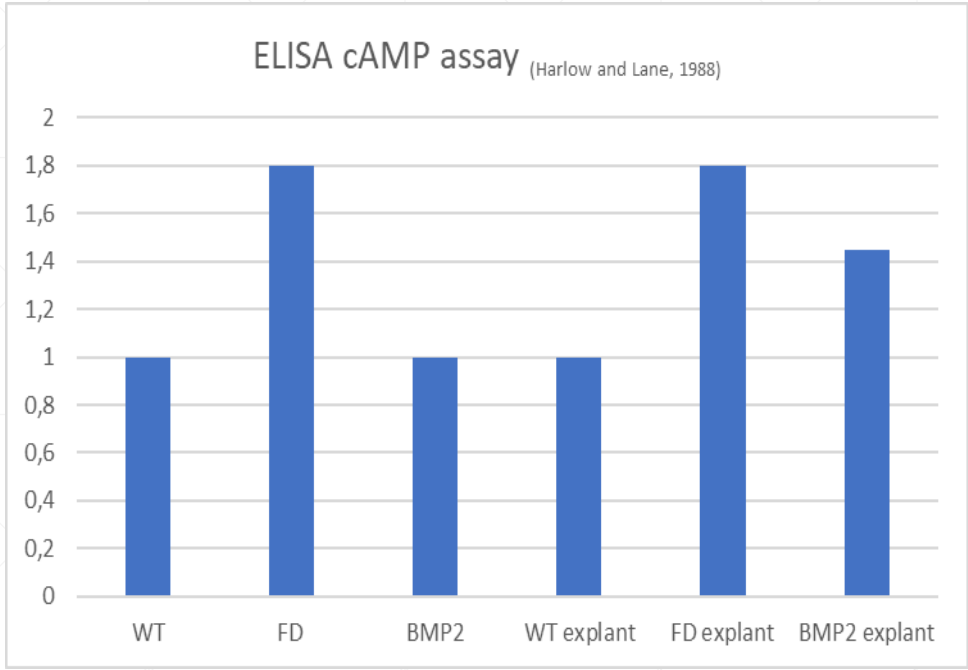




Adapted from Akil et al., 2014

In vivo schematic results





Pitfalls and solutions

Pitfalls

1. Reimplant seems to be deleterious in healty mices.
2. Therapy can’t rescue hormonal WT pattern.

Solution

1. By now, therapy has to start when symptoms show up. A correct timing can avoid collateral problems.
2. A pharmacological treatment can be combined with this therapy, in order to treat McCune-Albright syndrome. This therapy is particularly efficient against skeletal problems and related.

Future perspectives

- BMP6 seems to be more efficient in bone formation then BMP2 when overexpressed but we hypotesized that it could cause an excess of ossification, specifically in the younger patients’ cartilages (yet to test).

Materials and costs

✓ 20 x C57BL/6 (WT)	€ 480
✓ 10 x C57BL/6 (FD induced)	€ -
✓ Stabulation Costs (monthly)	€ 1000
✓ Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc)	€ 1214
✓ Plasmid	€ 2455
✓ Lentivirus	€ 490
✓ cDNA synthesis Kit	€ 552
✓ RT primers	€ 660
✓ Von Kossa S staining Kit	€ 171
✓ Alizarin red S staining Kit	€ 67
✓ Cloning Kit	€ 172
✓ Ficoll-Paque	€ 209
✓ E.Coli DH5α	€ 322
✓ β – glycerolphosphate	€ 290
✓ Dexamethasone	€ 95
✓ Ascorbic Acid	€ 24
✓ Alkaline Phosphatase Detection Kit	€ 211
✓ ELISA cAMP assay	€ 370
✓ Research team (estimated for 3 years)	€ 450000
TOTAL	**€ 458782**

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