



SAPIENZA
UNIVERSITÀ DI ROMA

G_sα and G-coupled receptor linked diseases: FIBROUS DYSPLASIA

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HUMAN FIBROUS DYSPLASIA (hFD)



General features

Skeletal involvement may be:

- Limited to one bone (monostotic FD): 75%
- Extended to multiple bones (polyostotic) or the entire skeleton (panostotic): 25%

Range:

- 1/100'000 or 1/1'000'000
- in 60% of cases the symptoms occurs before 10 years of age

Symptomatology

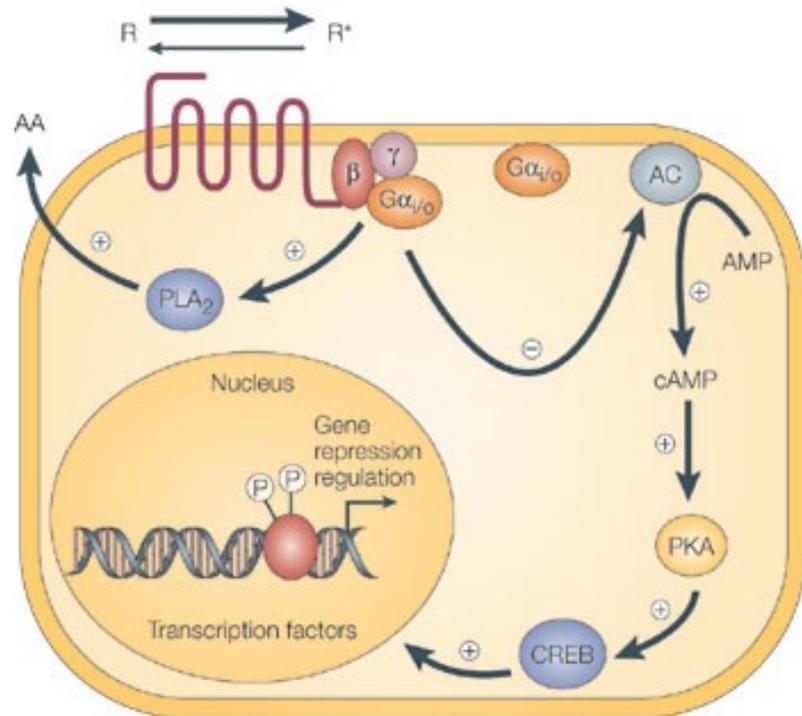
Skeletal

- Pain
- Fracture
- Deformity

Extraskeletal

- Precocious puberty
- Endocrine problems

A $G_s\alpha$ -LINKED DISEASE



$G_s\alpha$ physiological pathway

- Stimulatory G-protein α subunit has a GTPase domain
- Activated $G_s\alpha$ binds GTP and activates Adenylyl Cyclase
- [cAMP] increase and the PKA pathway is launched
- $G_s\alpha$ hydrolyze GTP to GDP and reunites to the $\beta\gamma$ subunit

$G_s\alpha$ in fibrous dysplasia

- The GTPase domain undergoes an **aminoacidic replacement** due to a **missense mutation**.
- The mutant form of $G_s\alpha$ remains **constitutively active** leading to cAMP overproduction.

FD: GENOTYPIC FEATURES

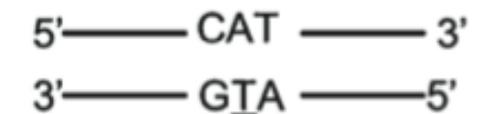
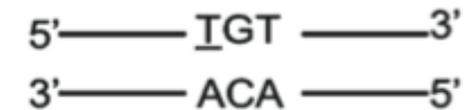
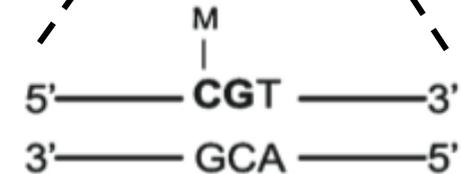


- A CpG dinucleotide in exon 8 (GTPase domain) undergoes methylation
- Replacement of arginine 201 either by cysteine (**R201C**) or histidine (**R201H**).
R201C is the most frequent and studied mutation

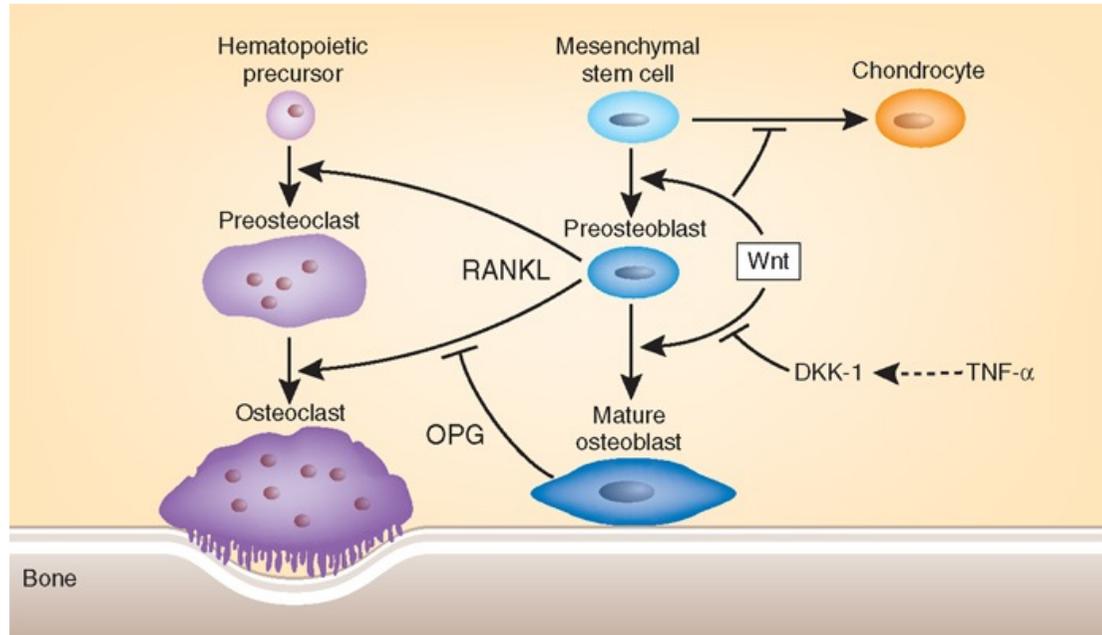


CONSTITUTIVELY ACTIVE $G_s\alpha$

- The mutation occurs postzygotically in **BMSCs**
- Half chromatid
- Dominant
- Non-inherited



FD IS A DISEASE OF SKELETAL STEM CELLS



ACTIVATED MUTATION OF $G_s\alpha$ GENE (GNAS) IN BMSCs

Inappropriate high levels of cAMP

IL-6 overstimulation

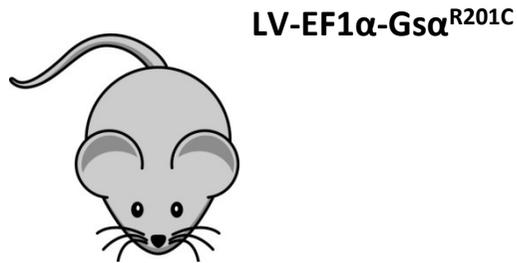
Osteoclasts recruitment

BONE DEGRADATION

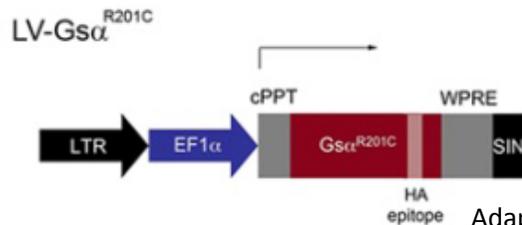
Inhibition of osteoblasts differentiation

ABNORMAL BONE DEVELOPMENT

LV-EF1 α -G α ^{R201C} STRAIN: A DIRECT REPLICATOR OF hFD BONE PATHOLOGY



FD mice were generated by lentiviral transgenesis. Mutated R201C sequence of G α is expressed under control of the EF1 α promoter.



A defined spatial and temporal pattern characterized the onset and progression of lesions across the skeleton:

Adapted from A. Greco, Tesi di dottorato



Early lesions

- excess of abnormal bone trabeculae

Intermediate lesions

- marrow cavity narrowed and distorted
- large, focal structural gaps within an abnormally expanded cortical bone

Late lesions

- fullblown replica of human disease
- bone deformed/fractured
- internal bone architecture effaced

STRATEGY

We invented a brand new construct (GLIMM) based on RNAi strategy delivered by an AAV vector in BMSCs. It is set up on GW182 decay pathway against mutated mRNA



OBJECTIVE:

- Degrade mutated GNAS mRNA in order to bring down constitutively active G-protein concentration

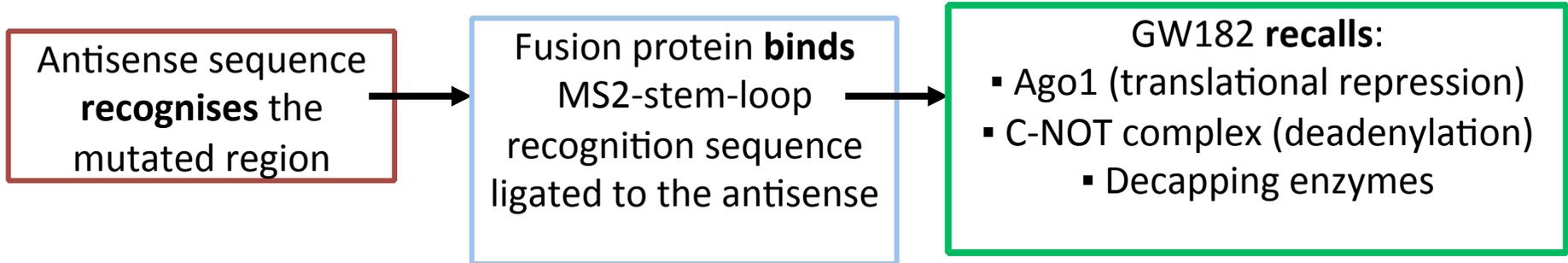
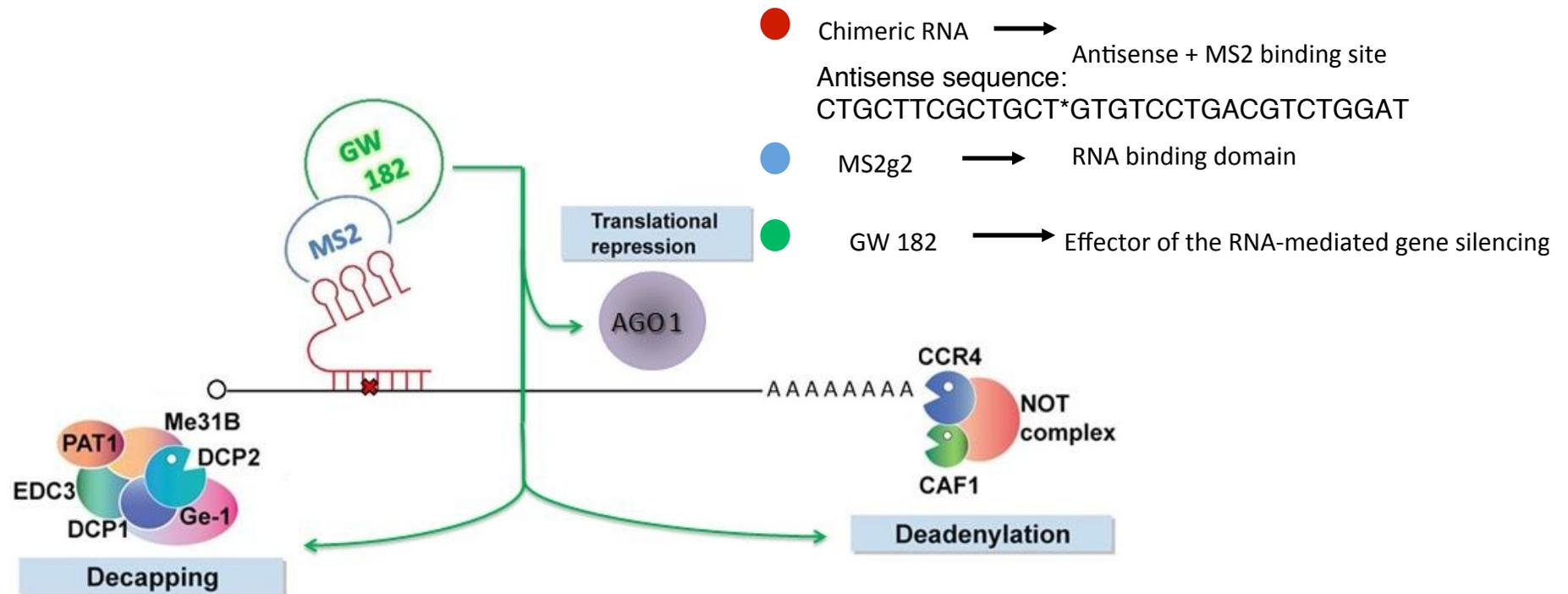
CONSEQUENCES:

- Restore physiological [cAMP]
- Promote osteoblasts differentiation



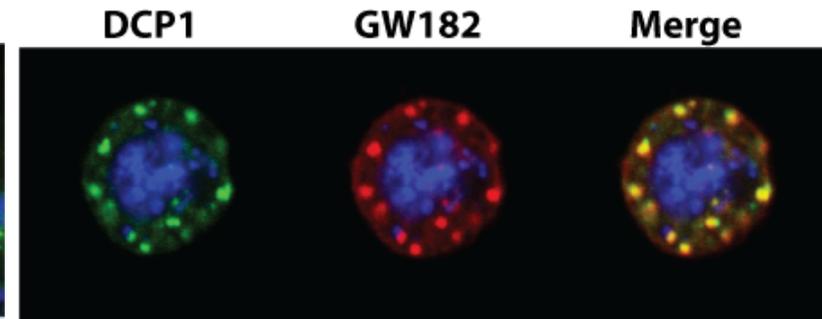
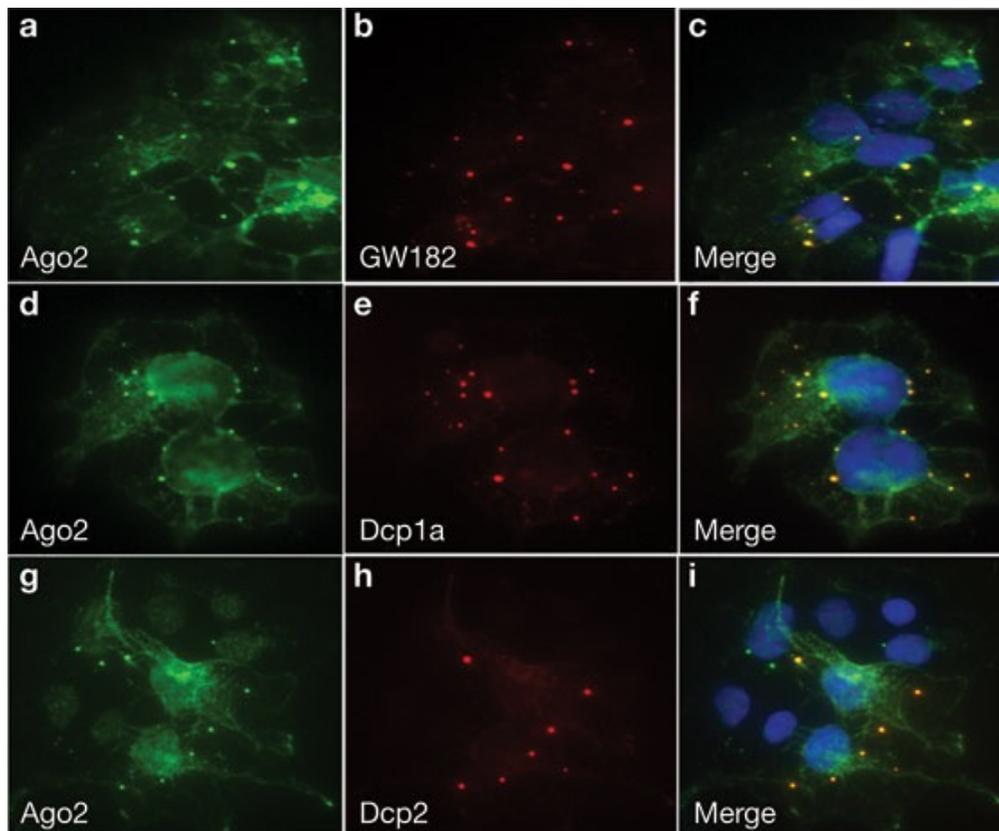
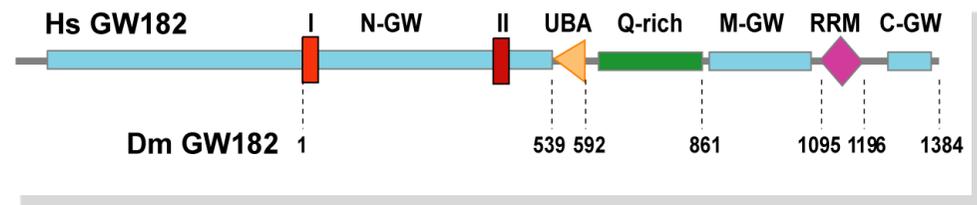
Slowing down FD progression

HOW DOES GLIMM WORKS?



GW 182: A P-BODY MARKER IN METAZOANS

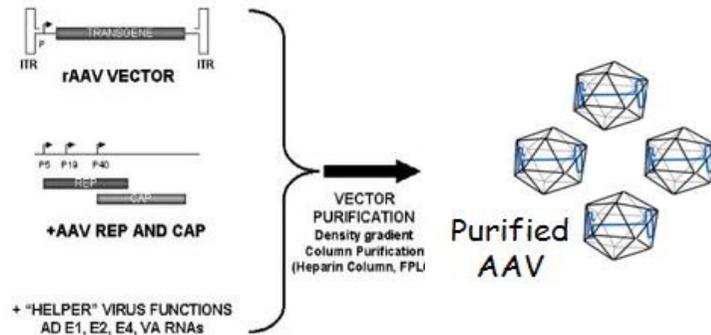
- UBA: ubiquitin associated domain
- RRM: RNA recognition motif
- GW: glycine and tryptophan repeats
- I and II: conserved motifs



GW182 co-localizes with decay enzymes

RECOMBINANT AAV VECTOR (rAAV)

rAAV vector production

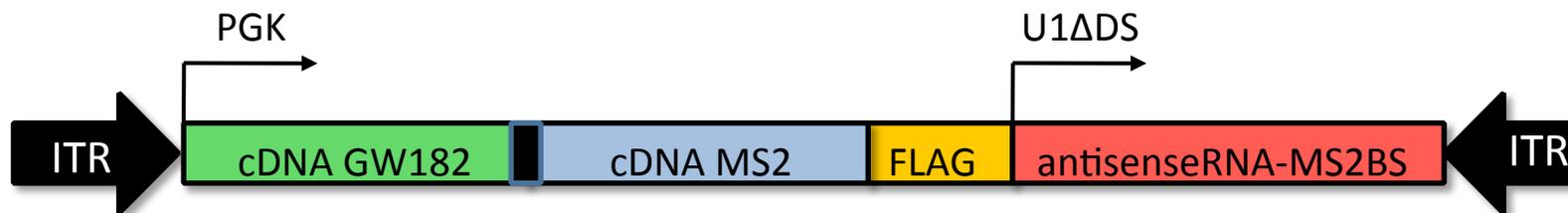


Why rAAV?

- Long-term gene expression of the delivered transgene
- No immunogenicity
- High titers ($10e^{10}$)

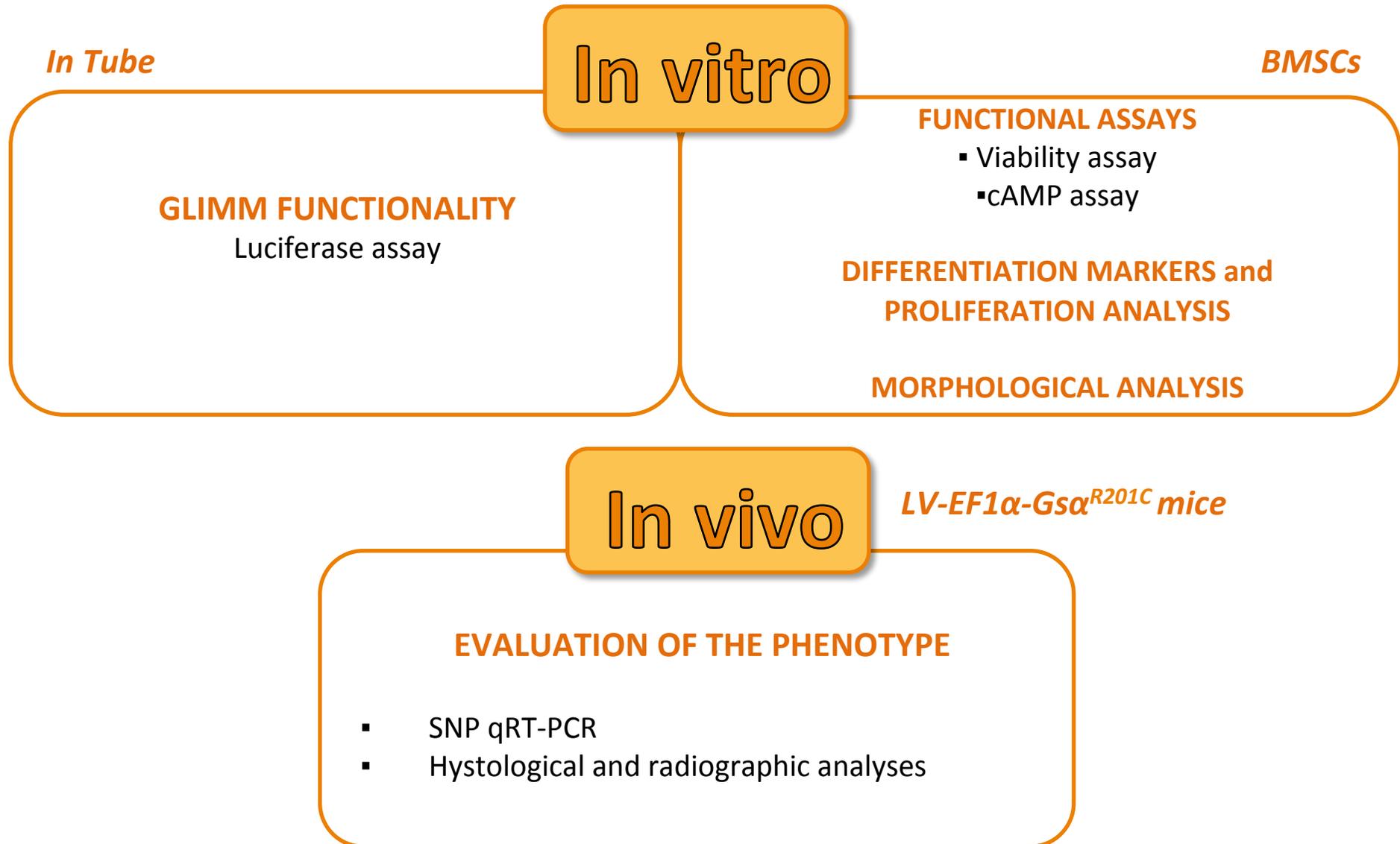
The Ad-helper plasmid assembling E2A, E4 and VA regions (**Ad-helper plasmid**) is cotransfected into the 293 cells, along with plasmids encoding the AAV vector genome (**vector plasmid**) as well as rep and cap genes (**AAV-helper plasmid**)

Our rAAV expression cassette



4,157 Kb

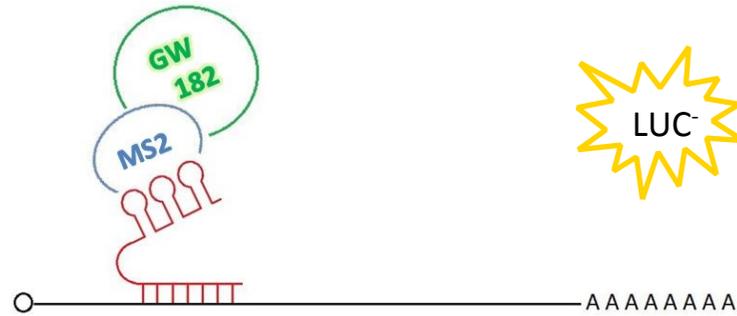
EXPERIMENTAL PLAN



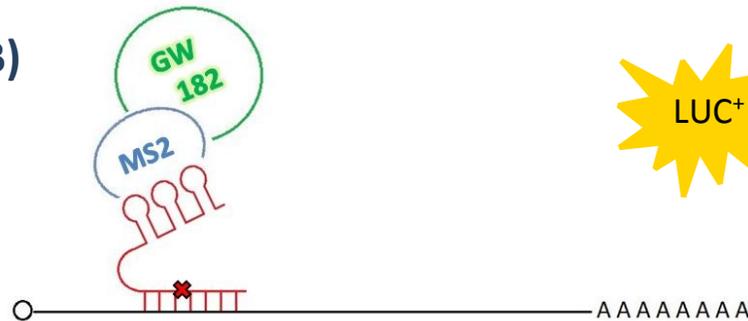
IN VITRO EXPERIMENTS – IN TUBE

Does the construct work?

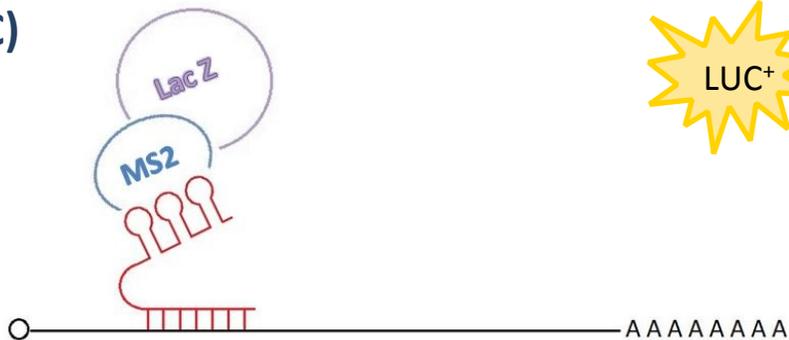
A)



B)



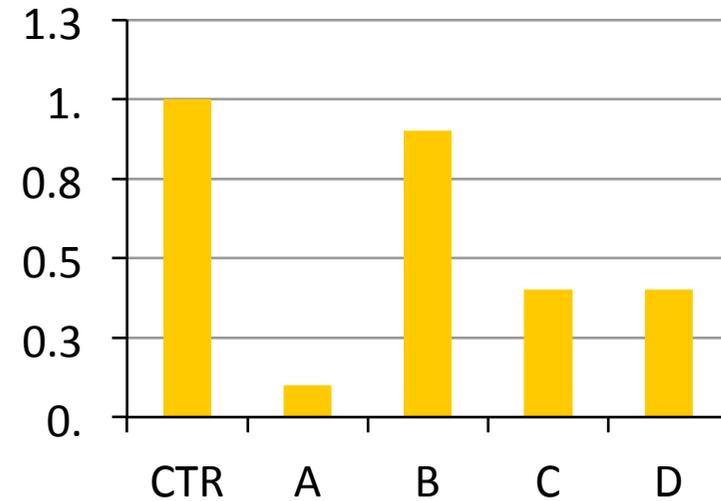
C)



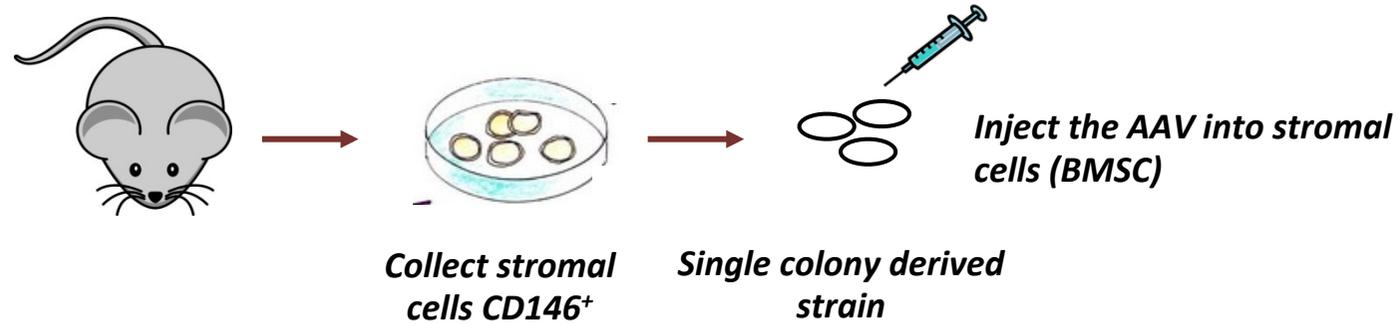
D)



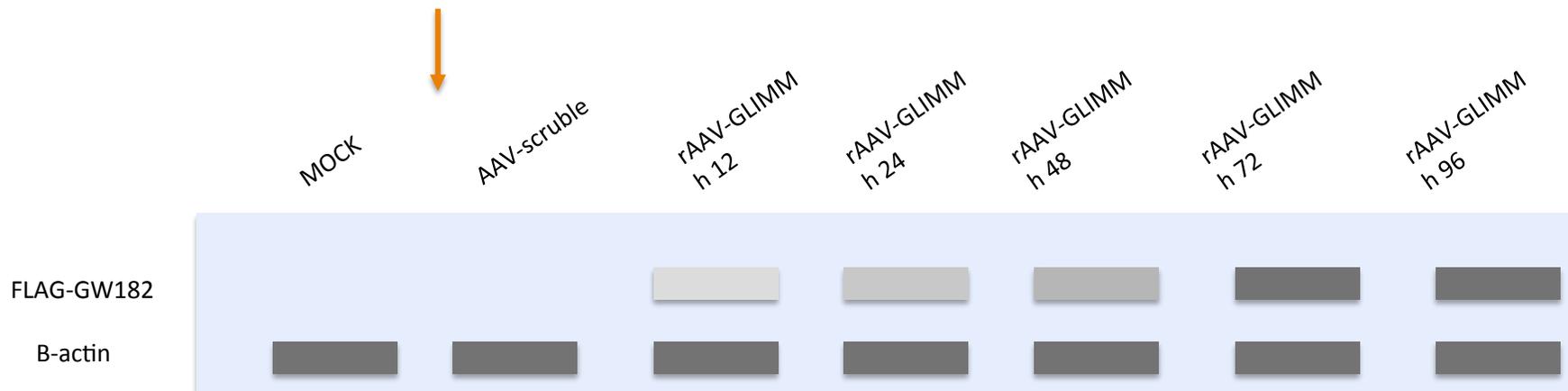
■ Luciferase activity



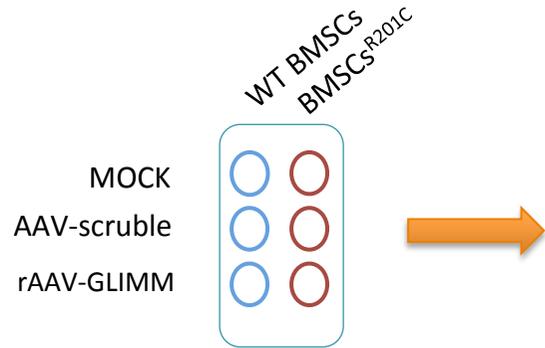
IN VITRO EXPERIMENTS – BMSCs



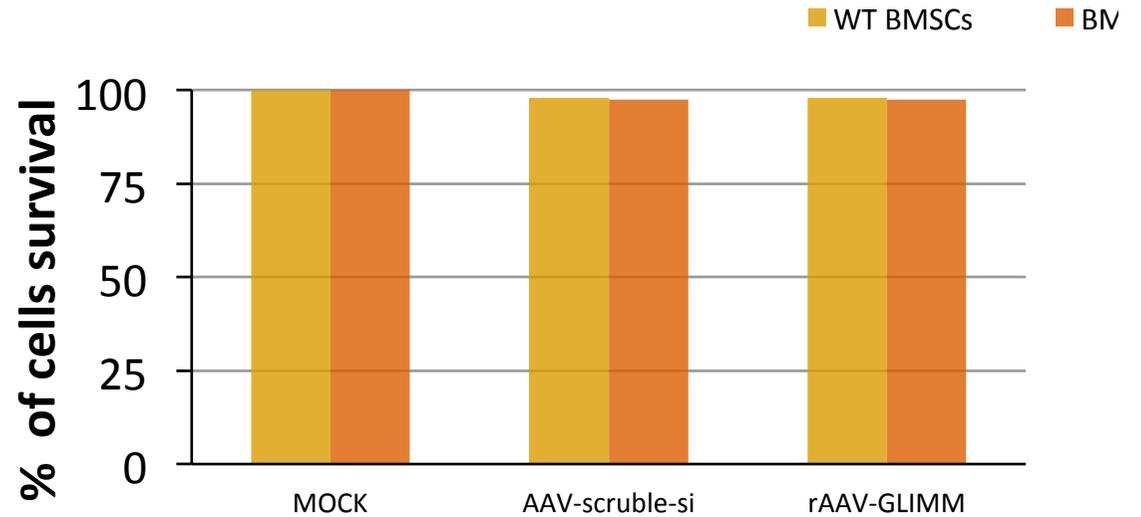
- Mouse BMSCs were trasfected for 2h with the virus at 1×10^5 vg/cell MOI
- The cells were inoculated on a 6-well plate at 1×10^4 cells/well
- Collection after 12, 24, 48, 72 and 96h
- FLAG-GW182 Immunoblot to evaluate GLIMM expression on transduced cells



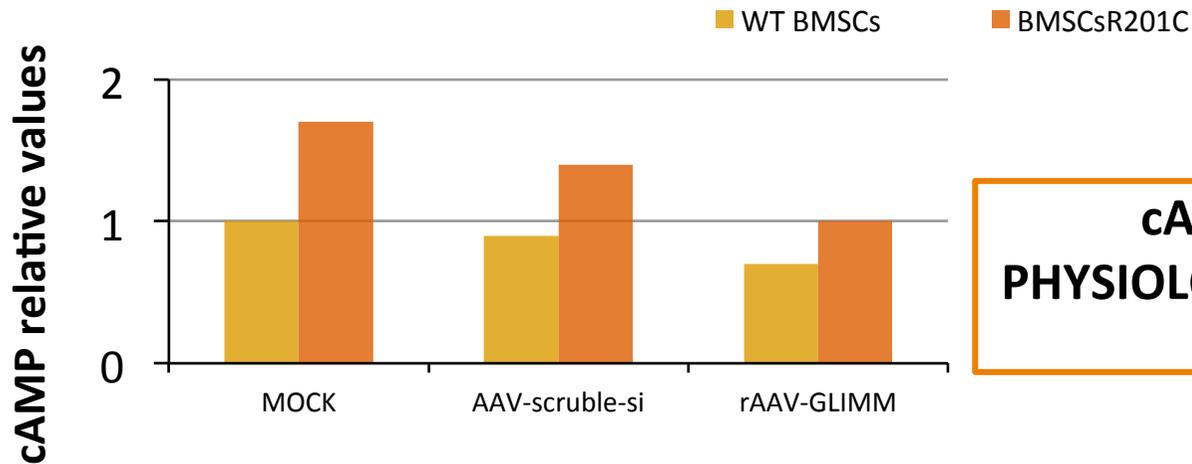
Is GLIMM toxic to BMSCs?



MTT – VIABILITY ASSAY



ELISA cAMP ASSAY



**GW182 OVEREXPRESSION
DOES NOT AFFECT
CELLS VIABILITY**

**cAMP IS RESTORED TO
PHYSIOLOGICAL-LIKE LEVELS AFTER
TRANSDUCTION**

DIFFERENTIATION ANALYSIS

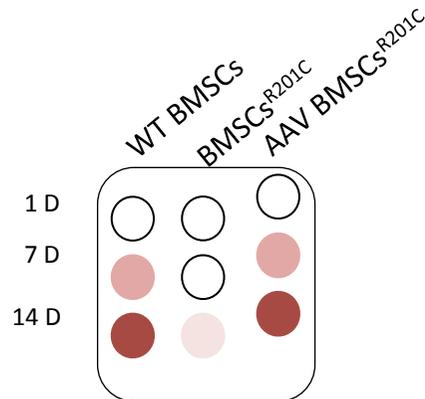
&

CELLS PROLIFERATION

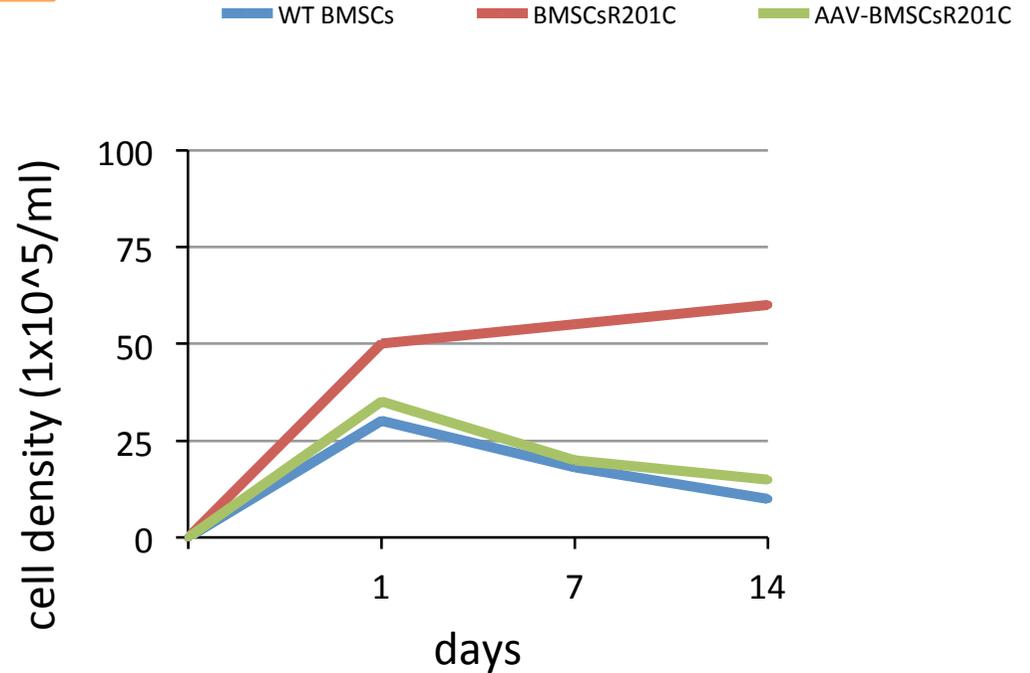
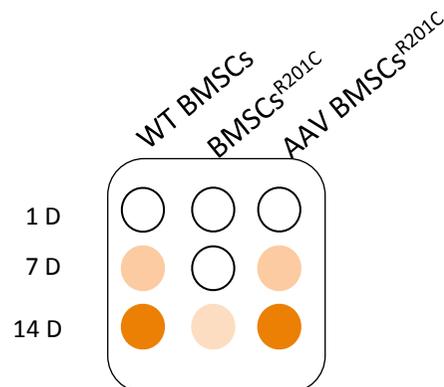
Thymidine incorporation assay

Analysis of bone cell-specific marker like **Alkaline Phosphatase (AP)** or detection of **functional mineralization** is frequently used to evaluate osteoblasts health condition *in vitro*

Detection of Alkaline Phosphatase



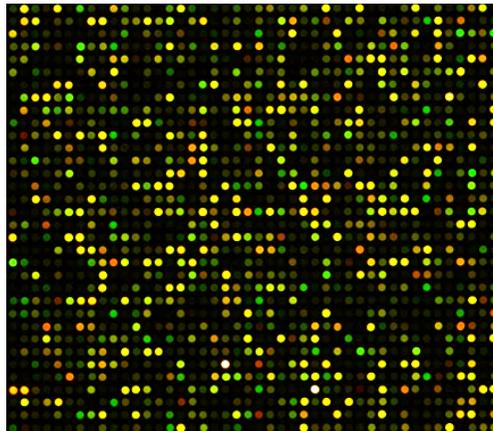
Osteoblast mineralization



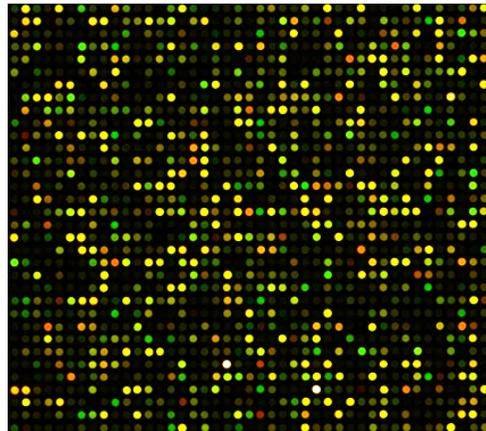
After treatment with rAAV-GLIMM, BMSCs are more differentiated and less proliferating

MICROARRAY ANALYSIS

Gene expression profile of mouse BMSCs was already determined by cDNA microarray analysis.

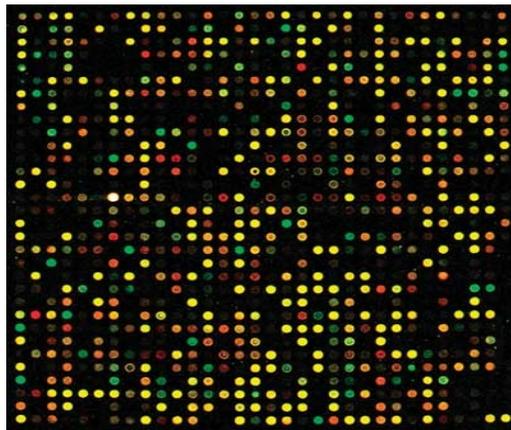


WT BMSCs

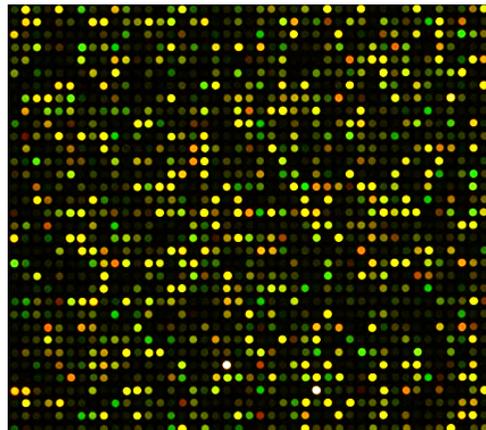


WT BMSCs MS2/GW182

**GW182 OVEREXPRESSION IN
BMSCs DOES NOT AFFECT
OTHER CELLULAR PATHWAYS**



BMSCs^{R201C}

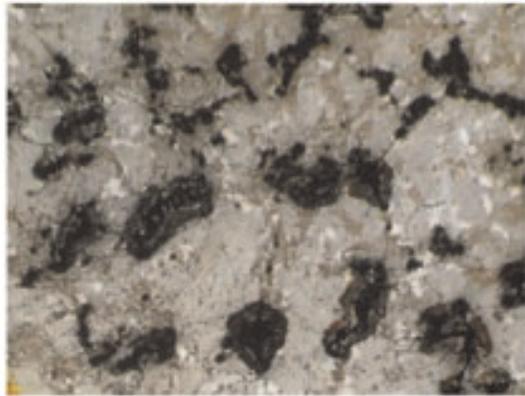


BMSCs^{R201C} MS2/GW182

**GENE EXPRESSION IS
RESTORED AFTER
TRANSDUCTION**

MORPHOLOGICAL ANALYSIS

WT BMSCs



BMSCs^{R201C}



**BMSCs^{R201C}
AAV-MS2/GW182**



Adapted from Piersanti, et al. (2009)

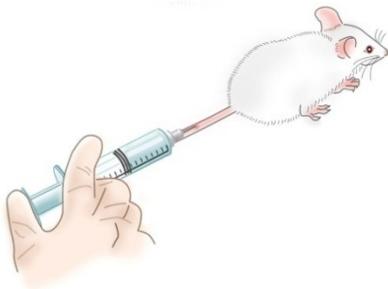
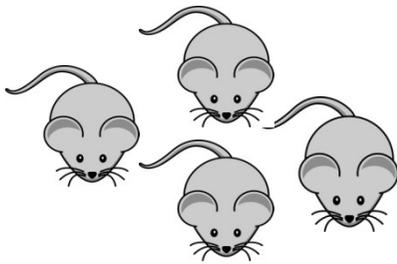
**Restoration of in vitro mineralization in
BMSCs, first trasduced with GLIMM**

IN VIVO EXPERIMENTS

No stable expression of our GLIMM construct

Ex vivo
experiments

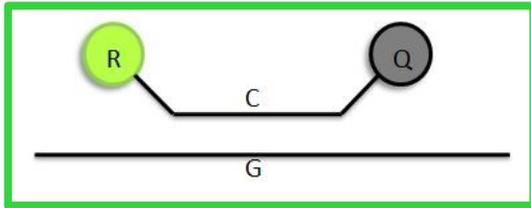
**IN VIVO
EXPERIMENTS**



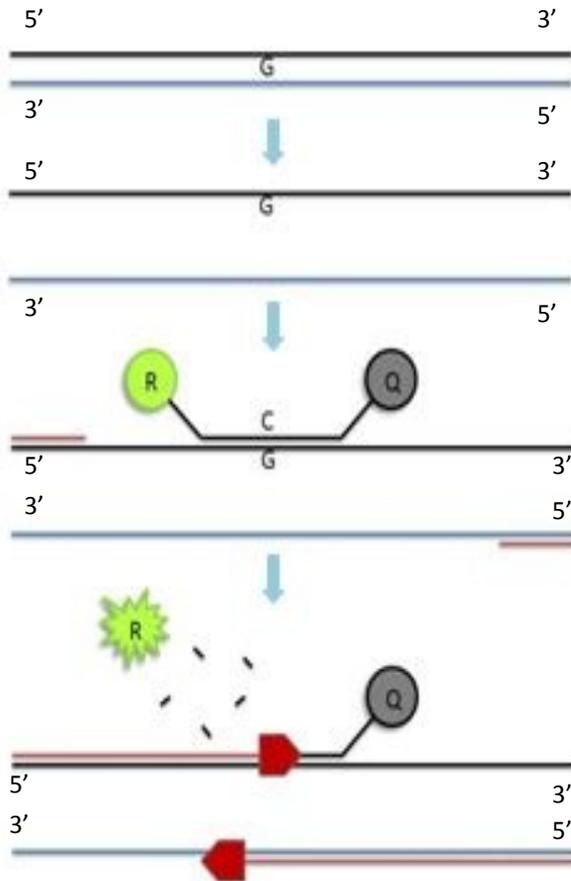
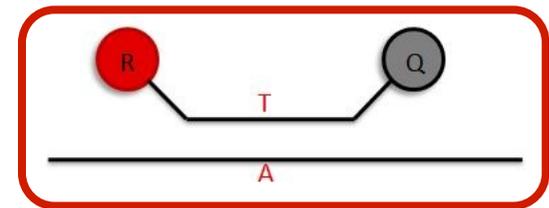
- 2 to 18 months age mice cohort
- Injection in medullary cavity of the affected bone:
 1×10^5 vg/cell MOI
- SNP qRT-PCR
- Radiographic and histological analysis

TaqMan: SNP qRT-PCR

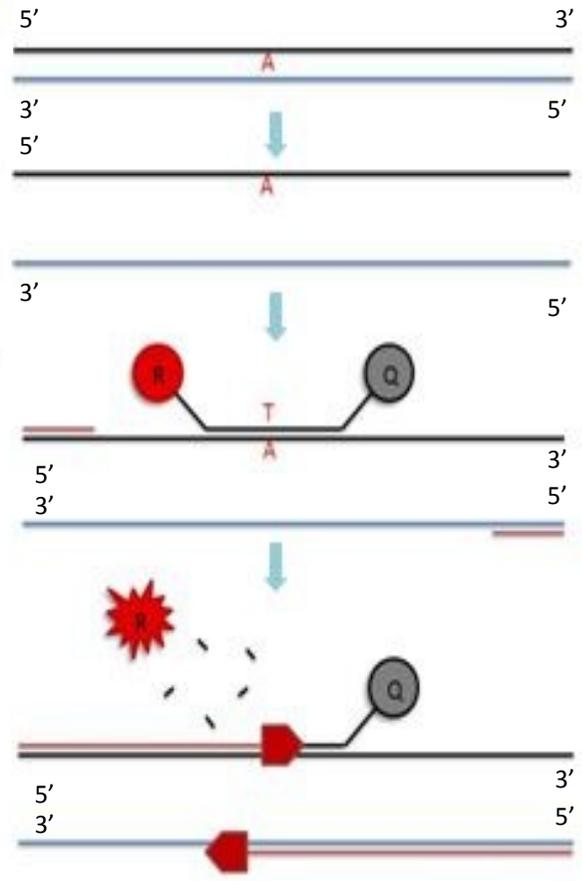
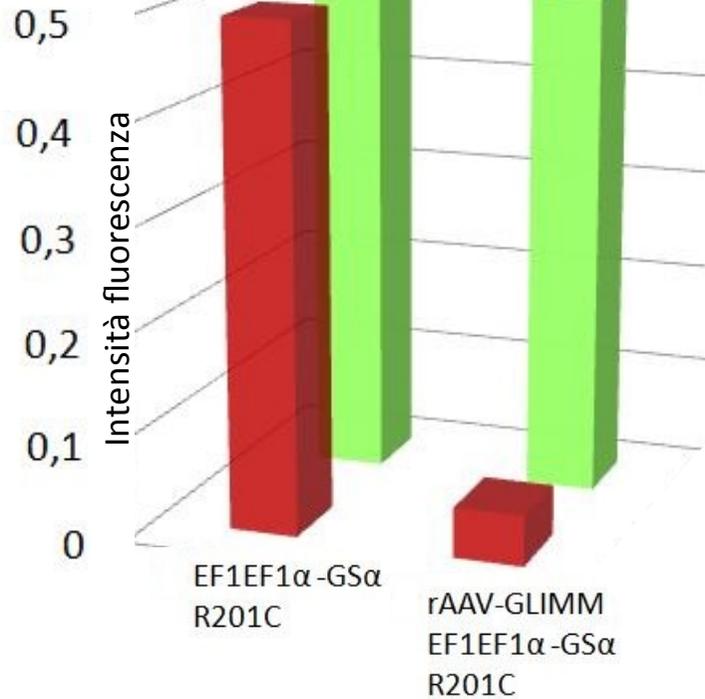
WT TaqMan probe



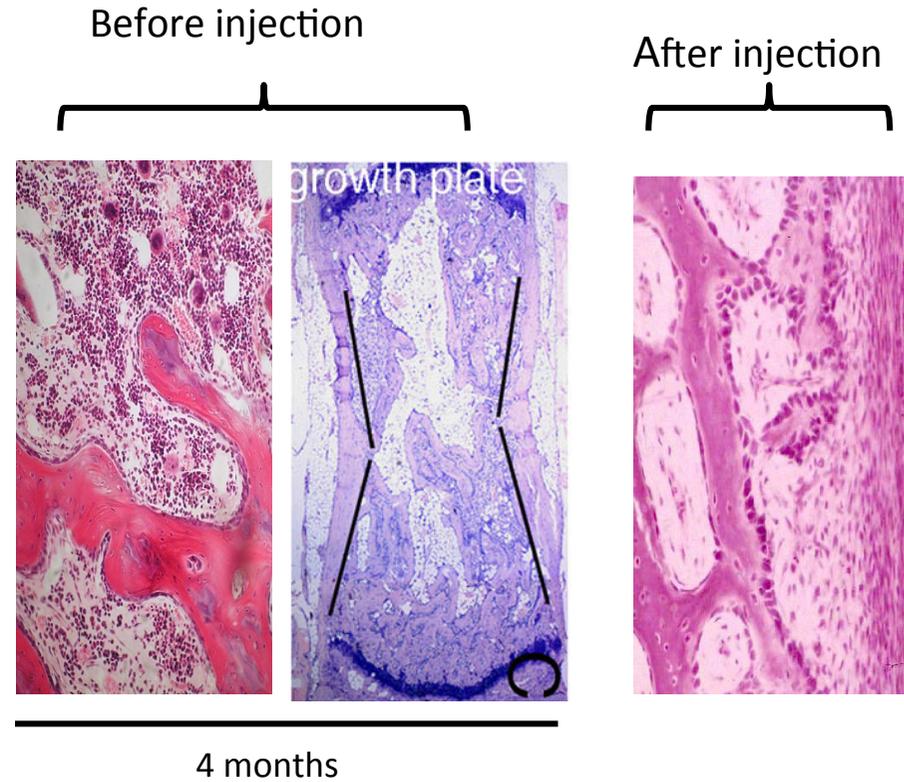
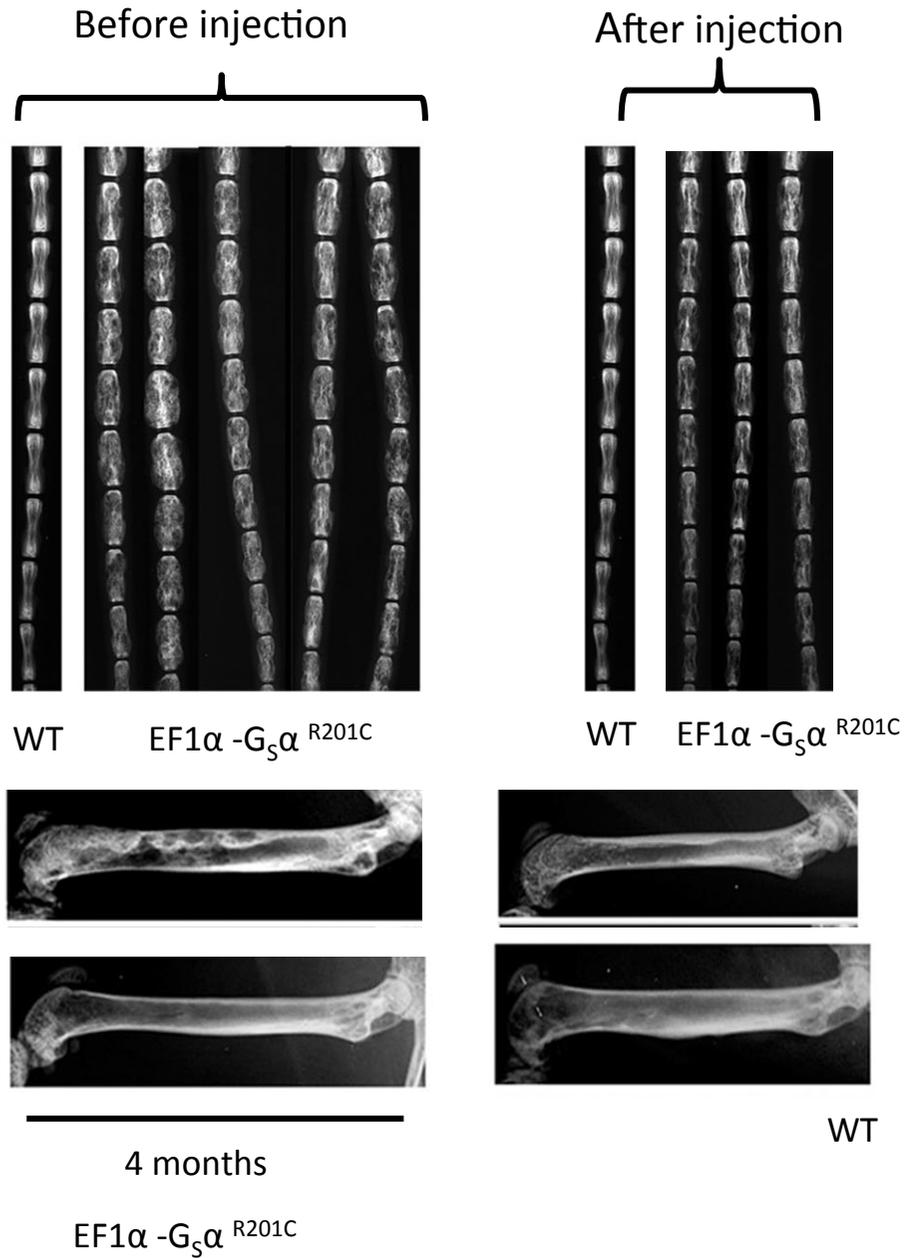
Mut TaqMan probe



■ Wt TaqMan Probe ■ Mut TaqMan Probe



RADIOGRAPHIC AND HISTOLOGICAL ANALYSIS



Adapted from M. Riminucci, et al (2014)

PITFALLS AND SOLUTIONS

Antisense low specificity

- Shift recognition region (containing mutated site)

O-metylation in position 2 to ameliorate affinity

Improve AAV transduction efficacy

- Hybrid transcomplementing constructs

AAV S/T/K mutants in capsid structural proteins VP1,VP2, and VP3 which reduces ubiquitination and proteosomal degradation (increased efficacy~30 fold).

Insertion of RGD polypeptide sequence into the AAV capsid protein VP1

Future perspectives

- Stable expression of GLIMM

Approach FD on other pathways (osteoclasts recruitment)

MAT€RIALS AND CO\$TS

Materials and assays	Product	Cost	Web page
cAMP assay	cAMP-Glo™ assay (300 assays)	299,00 €	https://www.promega.com
Cells	AAV-293 cells	266,00 €	http://www.genomics.agilent.com
Vector	VectorpAAV-IRES-hrREPORTERVector	381,00 €	http://www.genomics.agilent.com
MTT assay: Viability assay	Vybrant® MTT Cell Proliferation Assay Kit (1000 assays)	285,00 €	https://www.lifetechnologies.com/
Luciferase assay	Luciferase Assay System	150,00 €	https://ita.promega.com
Differentiation analysis	Osteoblast differentiation and mineralization	Contact vendor	http://www.promocell.com
Animal model	LV-EF1α-Gsα^{R201C} mouse (x25)	Contact vendor	
Microarray analysis	GeneChip® Mouse Genome 430 2.0 Array	Contact vendor	http://www.affymetrix.com
SNP RT-qPCR analysis	TaqMan® based SNP genotyping technology	278,00 €	http://www.lifetechnologies.com
Cell proliferation assay	Thymidine Incorporation Assay	Contact vendor	http://www.lifetechnologies.com
Alkaline Phosphatase detection assay	Alkaline Phosphatase Activity Assay, 500 tests	\$307.00	http://www.sciencellonline.com
Osteoblast mineralization	OsteoImage™ Bone Mineralization Assay	Contact distributor	http://www.lonza.com
GW182 Immunoblot	ANTI-FLAG Polyclonal	368,00 €	http://www.piercenet.com/

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