Gene Therapy:

Gsa receptor linked diseases and AAV vectors

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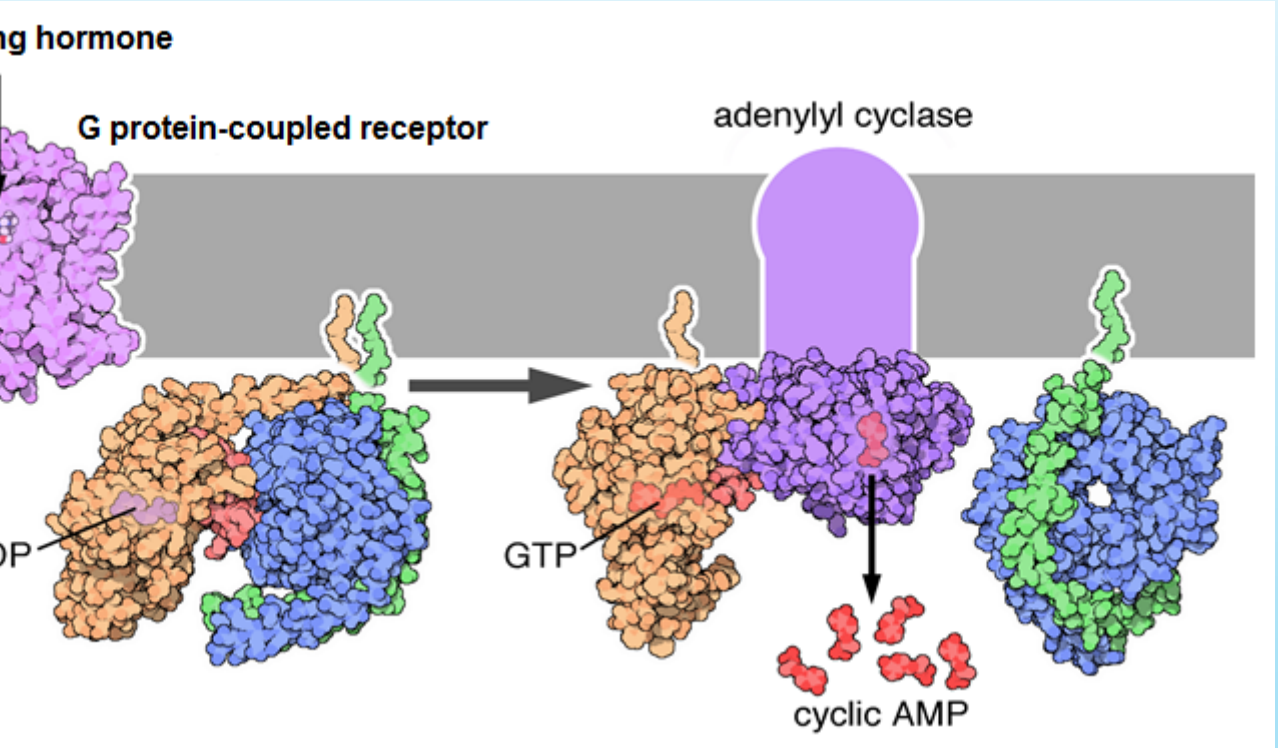
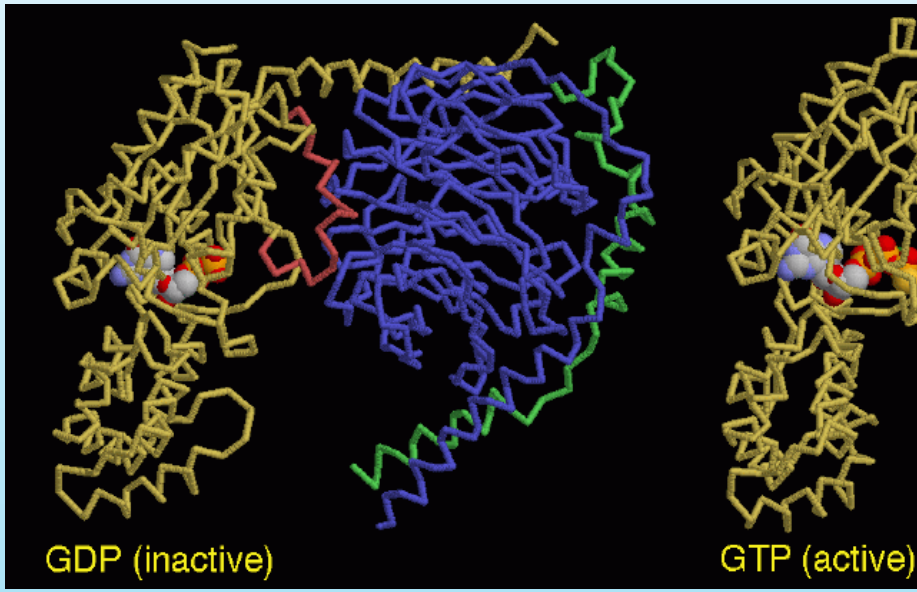
A.A. 2014/2015

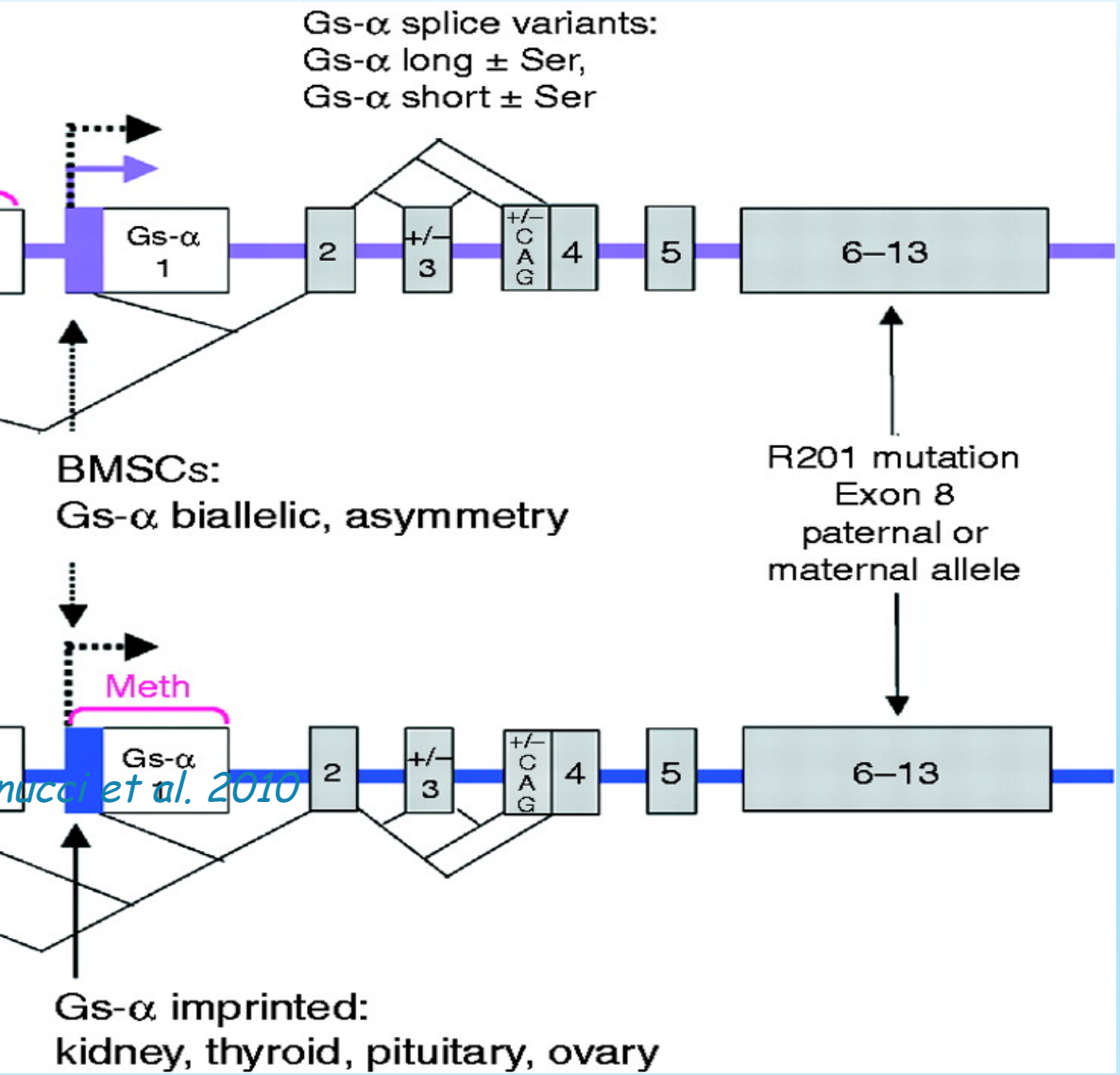
Gsα subunit activity





Adapted from RCSB Protein Data Bank David Goodsell 2004

GNAS complex locus



nucci et al. 2010

- Alternative promoters
- Alternative splicing
- Epigenetic regulation
- Both maternal and paternal imprinting

GS-a TRANSCRIPTS: gs-a-1, gs-a-2, gs-a-3, gs-a-4, bial in most tissues

1A TRANSCRIPT: expressed only from the paternal alle

XLAS TRANSCRIPT: expressed only from the paternal allele

NON-CODING ANTISENSE TRANSCRIPT expressed from the paternal allele (not shown in the picture)

NESP55 TRANSCRIPT: expressed only from the matern allele

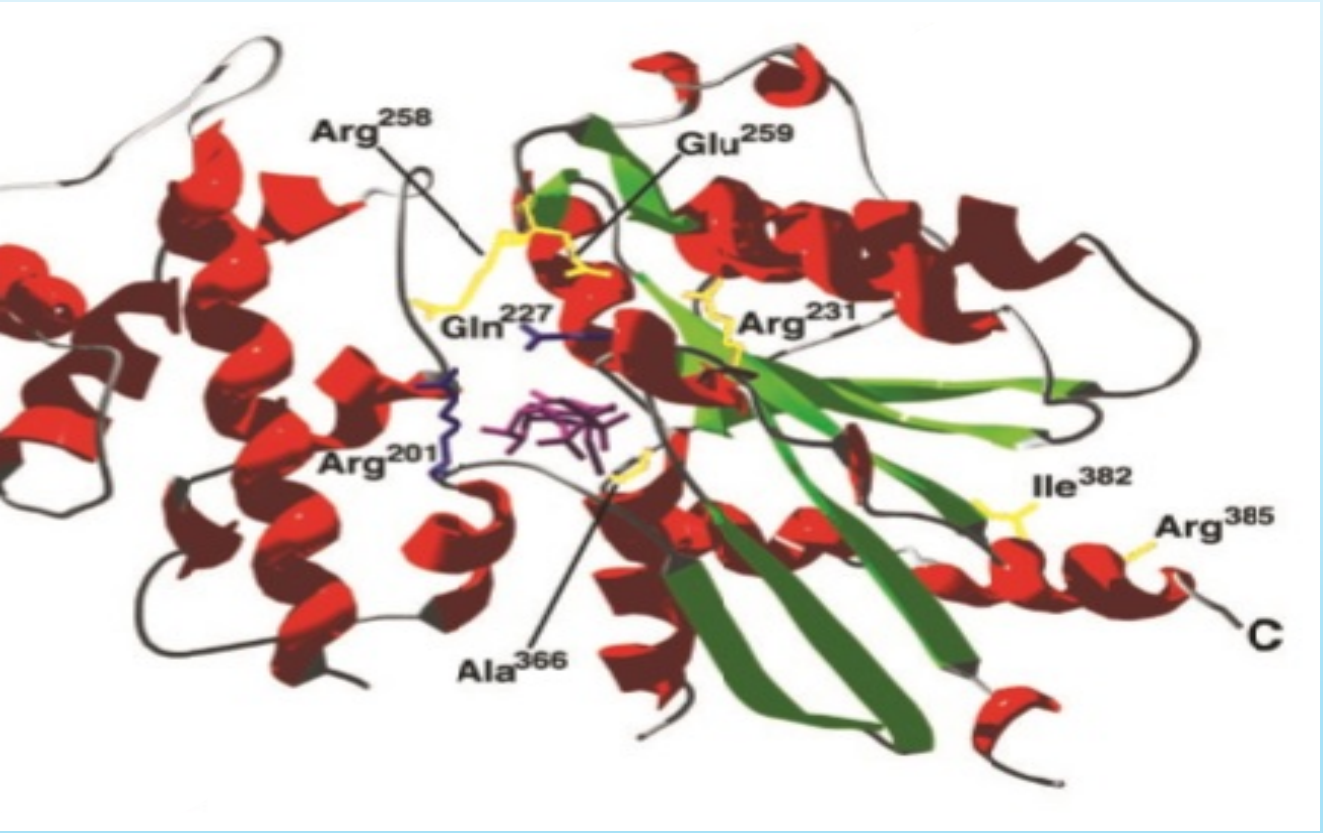
GNAS mutations and related diseases

-α subunit	MUTATIONS	DISEASES	INHERITANCE	cAMP LEVEL
active	In-del, missense, premature stop codons, epigenetic mutations	• Pseudohypoparathyroidism type IA (PHPIA) • Pseudopseudohypoparathyroidism (PPHP)	• Mother (autosomal dominant) • Father (autosomal dominant)	LOW
stitutively active	Post-zygotic mutation: R201C or R201H	• Endocrine tumors • McCune-Albright syndrome (MAS) • Fibrous dysplasia of bone (FD)	NOT INHERITED: MOSAICISM	HIGH
perature sensitive: ive at body perature and tituvely active in testis	A366S	• PHPIA and gonadotropin-independent precocious puberty in males (testoxicosis)	Autosomal dominant (affects only males)	LOW (BODY) HIGH (TESTIS)

OMIM entry 139320

Weinstein et al,20

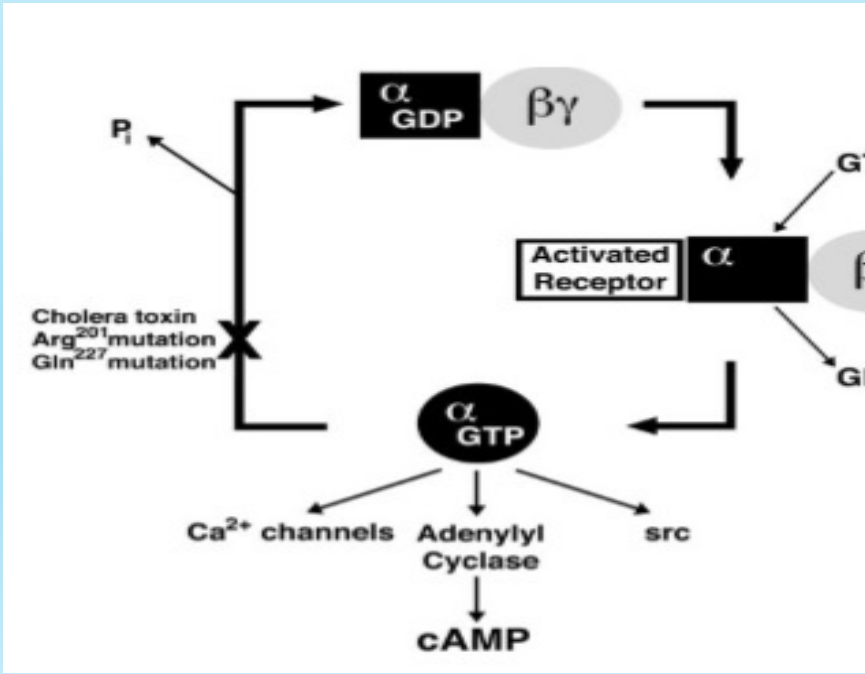
Constitutively active Gsa subunit



Possible mutations:

Arg201 → cys
Arg201 → hys

Gln227 → a
(lethal)
Gln227 → l



Fibrous dysplasia (FD)

haracteristics

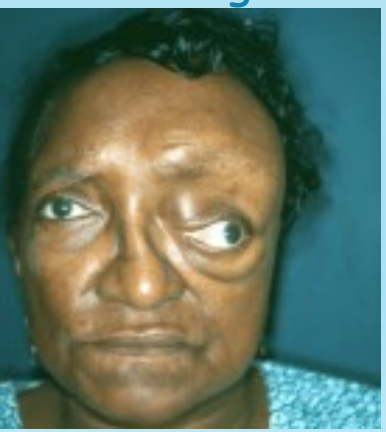
t-zygotic mutation, late detenction

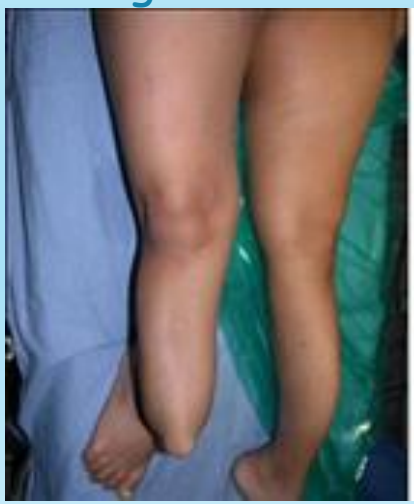
terozygous mutation

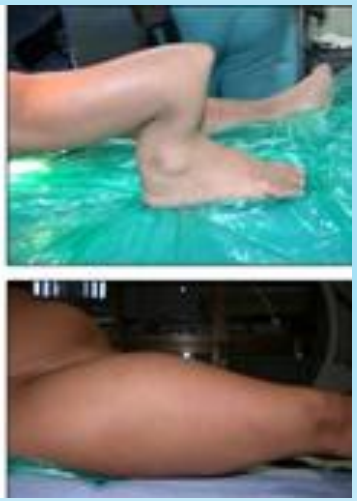
is a lesion composed mainly of fibrous tissue
t originates in the medullary cavity and expands
centrically outward into the surrounding cortical
e.

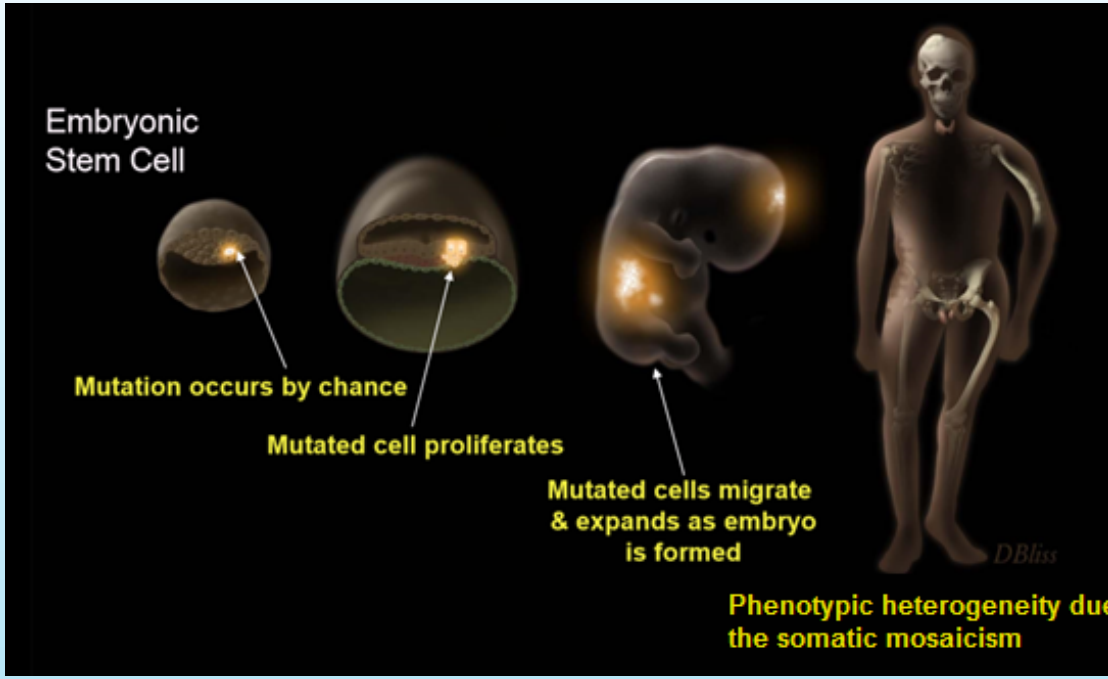
riable phenotypes: Monostotic in 70/80% cases,
ostotic in 20/30%, McCune Albright 3% cases.

ferent diagnosis depending on the severity









Robey et al,2007

Why FD?

- Lack of efficient treatments
- Severe disease

Chapurlat,

Fibrous Dysplasia pathophysiology

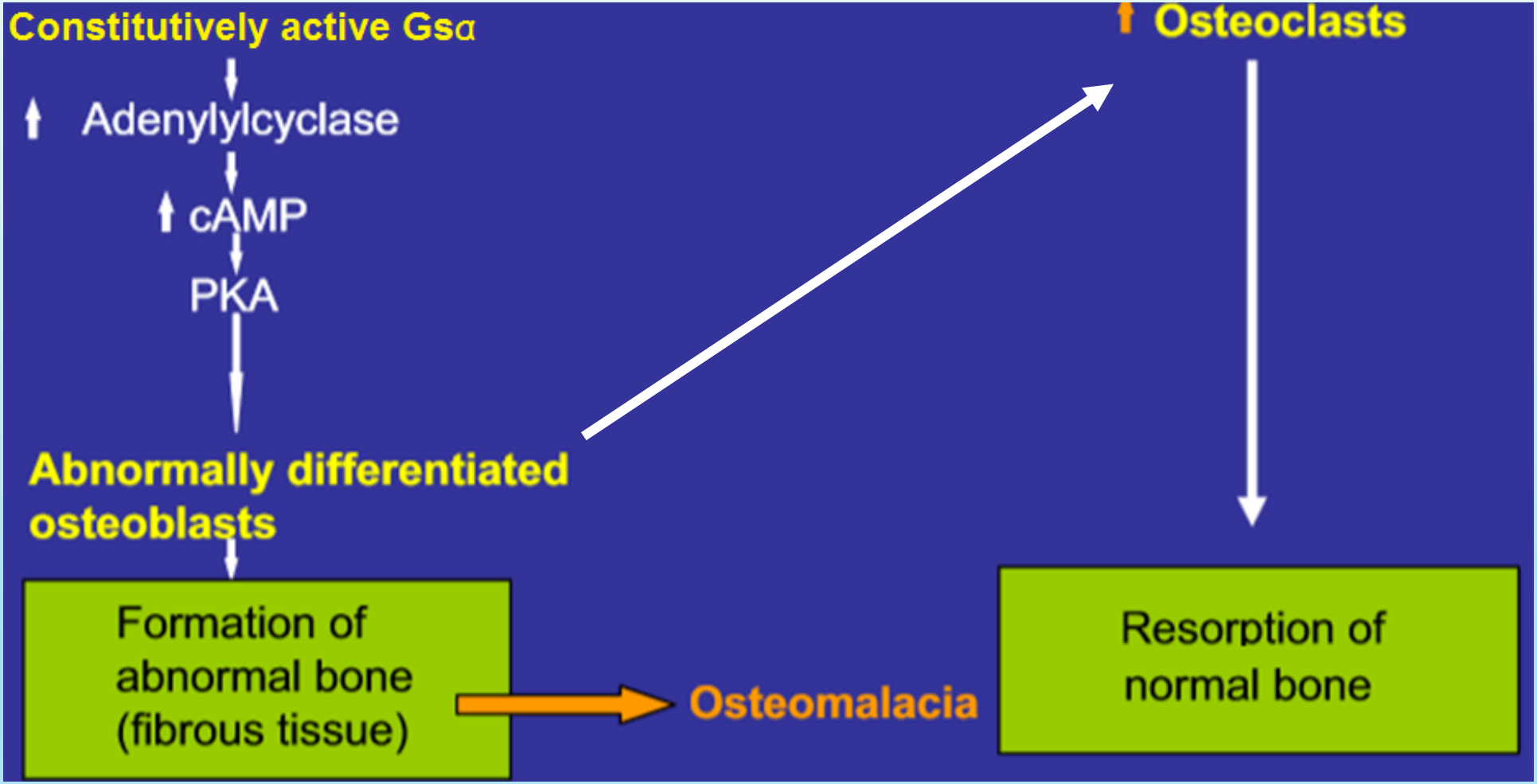
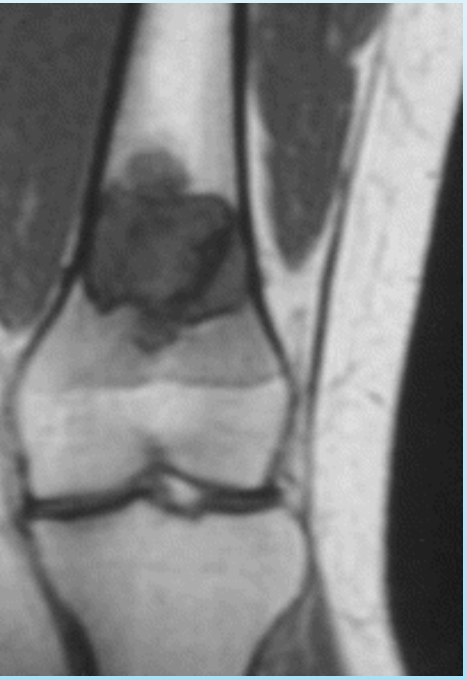
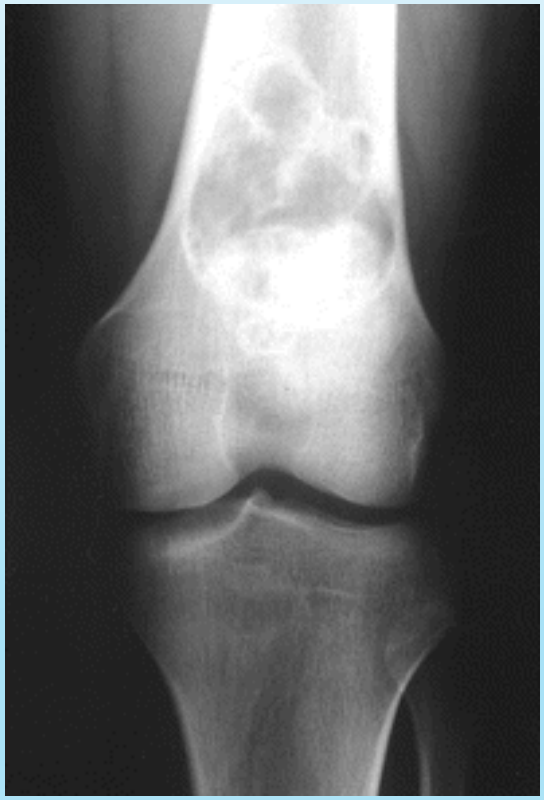


Image adapted from Chapurlat et Orcel, 2008

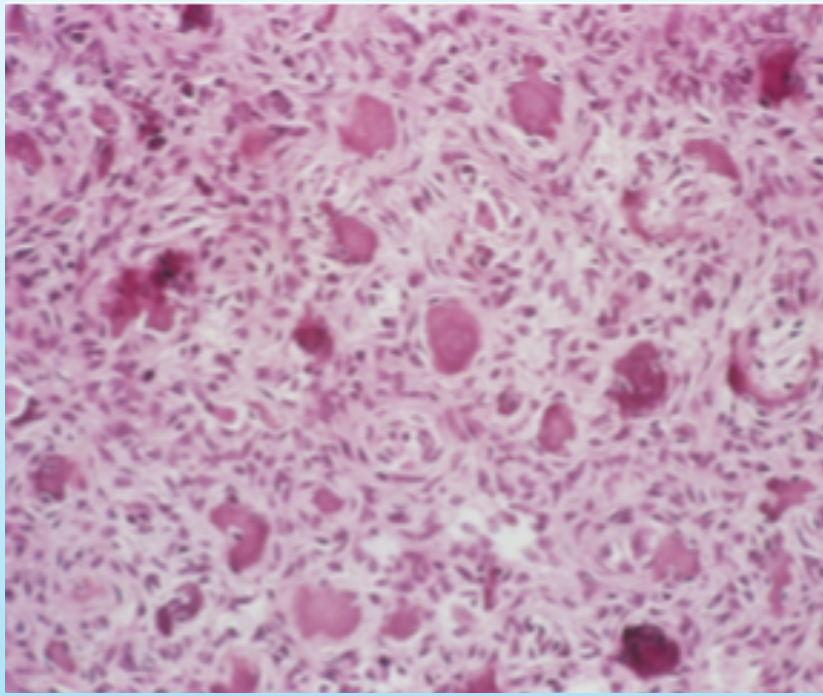
FD radiographical and histological aspect



c lesion of FD has signal
ty of intermediate to low on
l T1-weighted MR image



Frontal radiograph of knee shows well-defined lesion with smooth sclerotic margins and hazy matrix in distal femur



Histological aspect of a fibrous dysplasia lesion sho
the accumulation of fibrous tissue within the bone m

Fitzpatrick et al, 20

EF-1a Gsa (R201C)
Fibrous Dysplasia mouse model

- Lentiviral knock in for human Gsa (R201C) cDNA under EF-1a promoter
- Hemizygous
- Not lethal in germline ⟹ Mendelian inheritance pattern
- First visible lesions at 2/3 months
- Radiographically detectable skeletal phenotype at 6 months

Saggio et al. 2014

Differences with human condition: no mosaicism
hemizygousity
not lethal in germl

Experimental plan

WHAT ?
GSa (R201C) Gene Editing

HOW ?
CRISPR/Cas9 system

WHERE ?
e marrow stem cells from EF-1a
Gsa (R201C) mouse

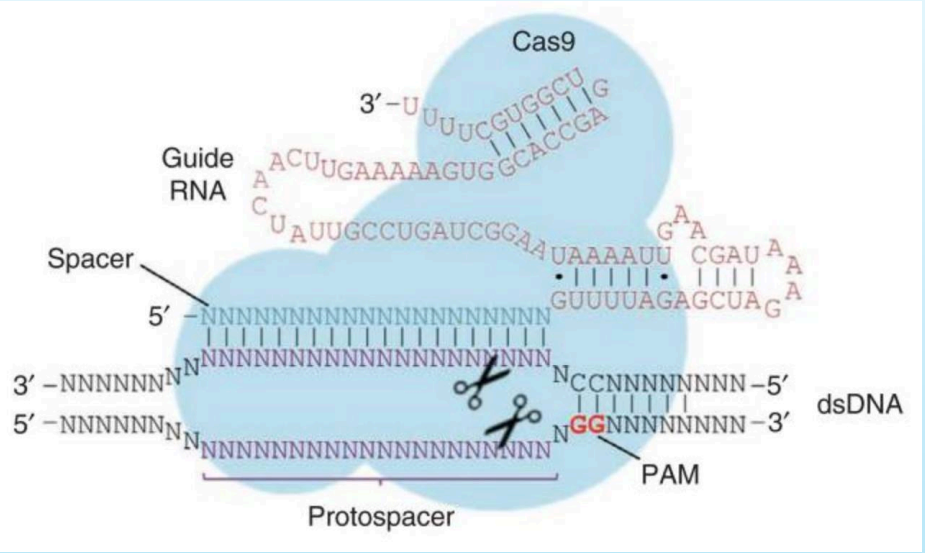
WITH ?
AAV Cotransfection

WHAT DO WE EXPECT ?
Limit degeneration by normal bone tissue
formation and by replacement of chirurgically
removed fibrous tissue

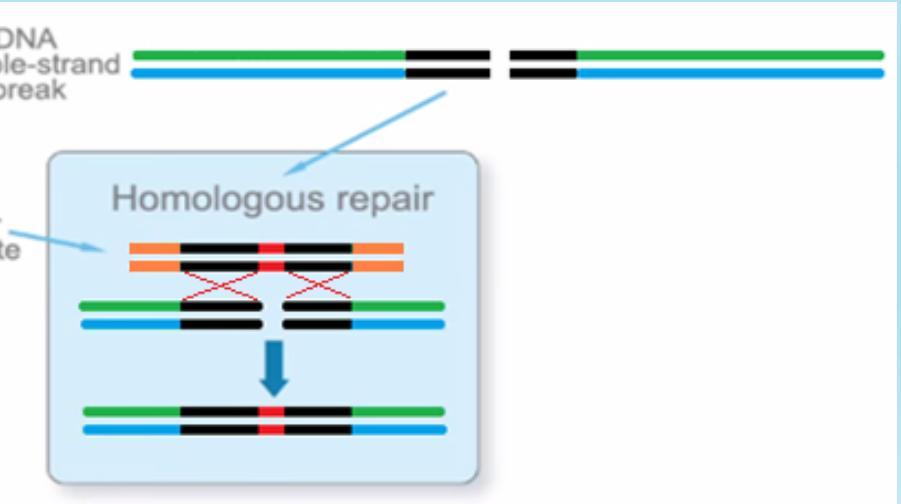
WHEN ?
2 months old mice

CRISPR/Cas9 system

(clustered regularly interspaced short palindromic repeats)



- Cas9/sgRNA complex binds to protospacer adjacent motif (PAM) site and unwinds DNA;
- sgRNA binds to target sequence in genomic DNA adjacent to the PAM site;



- Cas9 produce a double-stranded break (DSB) in the target DNA;
- Homologous repair from a donor template

Hao Yin et al, 20

Why CRISPR/Cas9

Advantages:

- Very high efficiency
- Rapid construction and easy delivery
- Multiplexing possible in vitro and in vivo
- Successful in different cell types and species

Disadvantages:

- Target selection may be limited by requirement for PAM sequence
- Possibility of off-target cleavage

Walsh et Hochedlinger, 2013

AAV VECTORS

Advantages:

No pathogenicity

Ability to infect both dividing and non-dividing cells

Low immune response from the host

Ability to deliver genes into different tissues (many serotype)

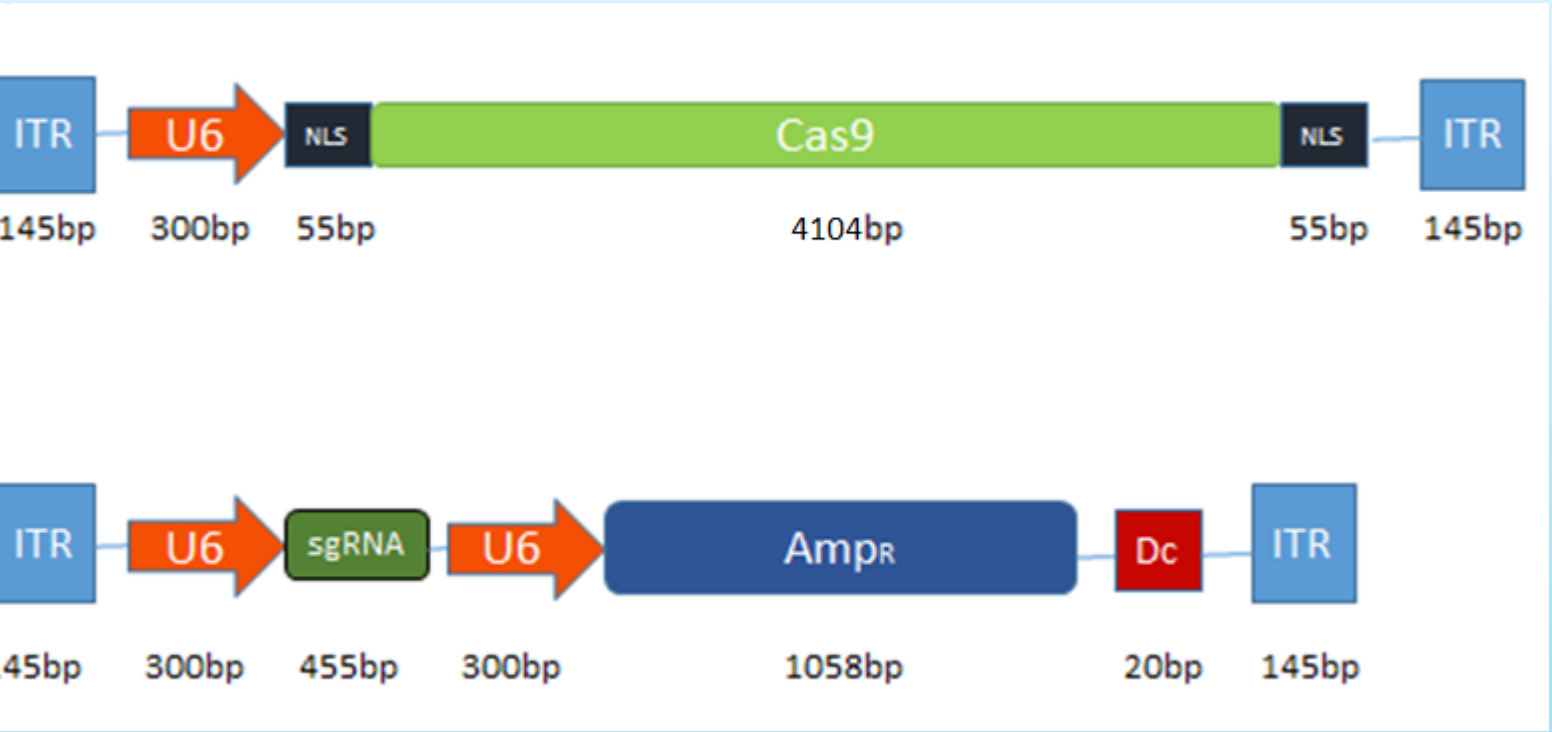
Long term gene transfer in lung, CNS, eye, muscle

Disadvantages:

- Small size: 4,8 Kb
- Possible presence of the neutralizi antibody
- ssDNA
- Variable transduction efficiency (1-80%)

Skubis-Zegadlo et al, 2013

AAV2-Vectors



- **U6**: ubiquitous and constituti promoter
- **ITR**: inverted untranslated r
- **NLS**: nuclear signal
- **Cas9**: CRISPR associated pro
- **sgRNA**: guide RNA for Cas9 targeting
- **AMP$_R$**: ampicillin resistance g (negative control)
- **Dc**: donor cassette (wild type

Adapted from Swiech L. et al, 2014

Therapy protocol

IN VITRO

- EF-1a Gsa (R201C) mouse
- BMSCs selection
- AAV construction
- Cotransfection
- Transfected cells selection
- Gene Editing efficiency analysis
- Functional rescue

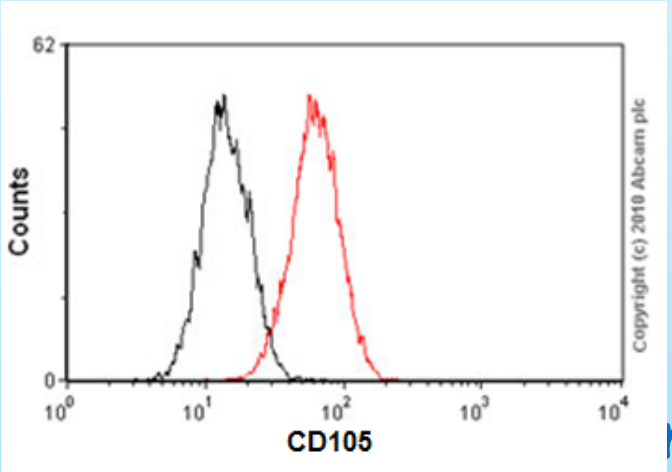
EX-VIVO

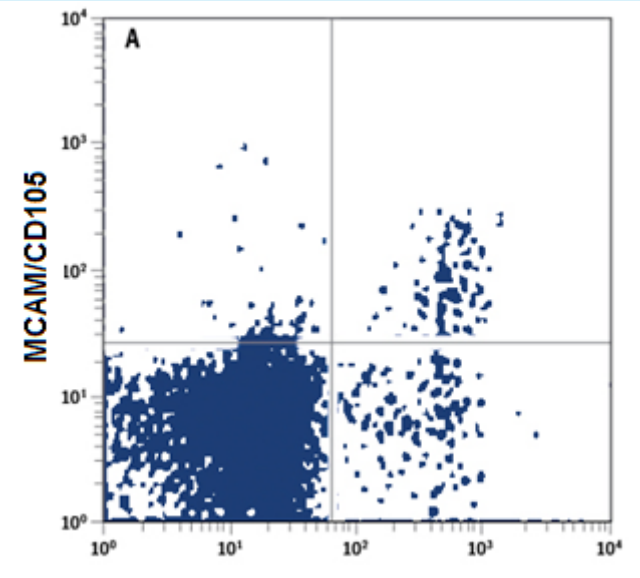
- Intravenous injection
- Chirurgical removal and local injection
- Phenotype rescue

BMSCs isolation and culture

ne Marrow Stem Cell (MSC) isolation by FACS Sorting

arker +: CD105





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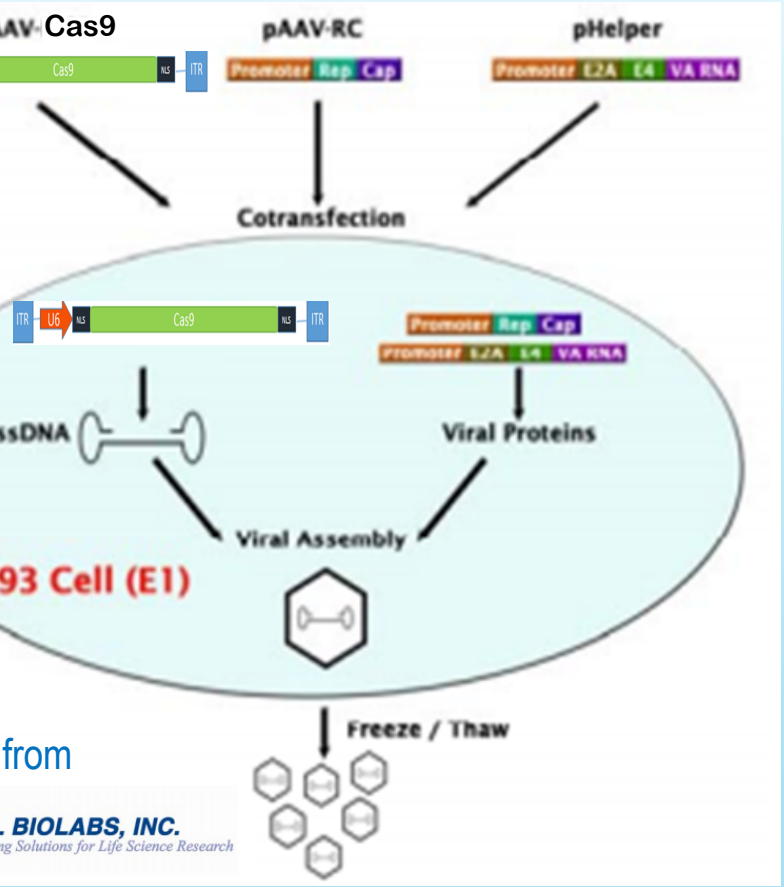
Sidhu and Tuch, 2006

GF2 and Wnts3 in culture

Zhu et al, 2010

'Helper free' AAV construction:

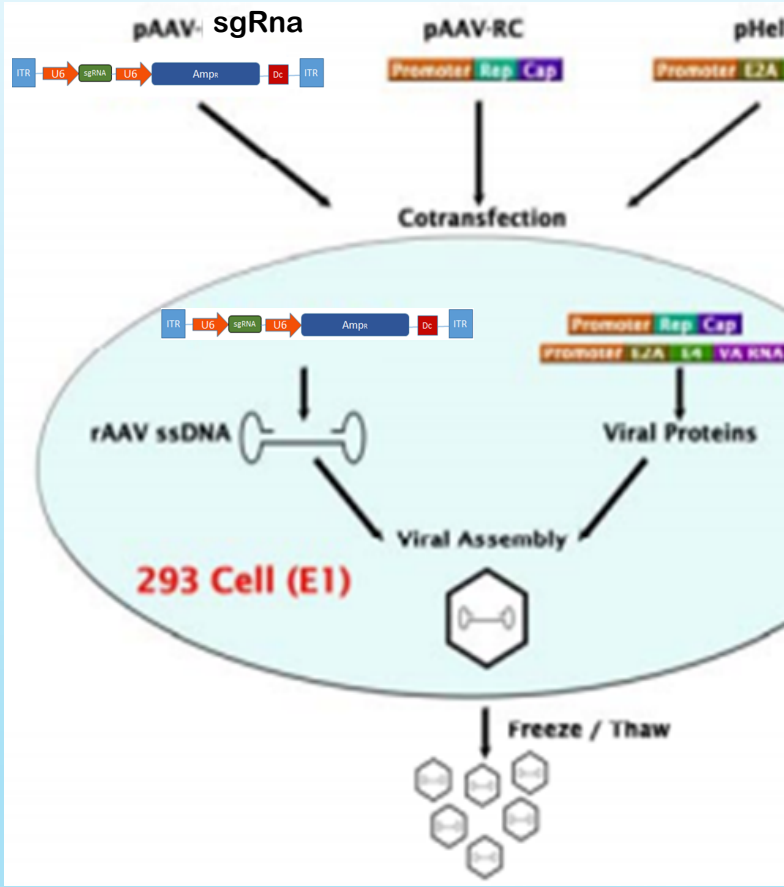
helper virus is replaced with two plasmids providing adenoviral necessary genes



AAVs spCas9

COMPONENTS:

- 293 E1 cells
- pAAV Cas9/pAAVsgRNA
- pHelper (E2A, E4, and VA RNA genes)
- pAAV-RC (Rep and Cap genes)



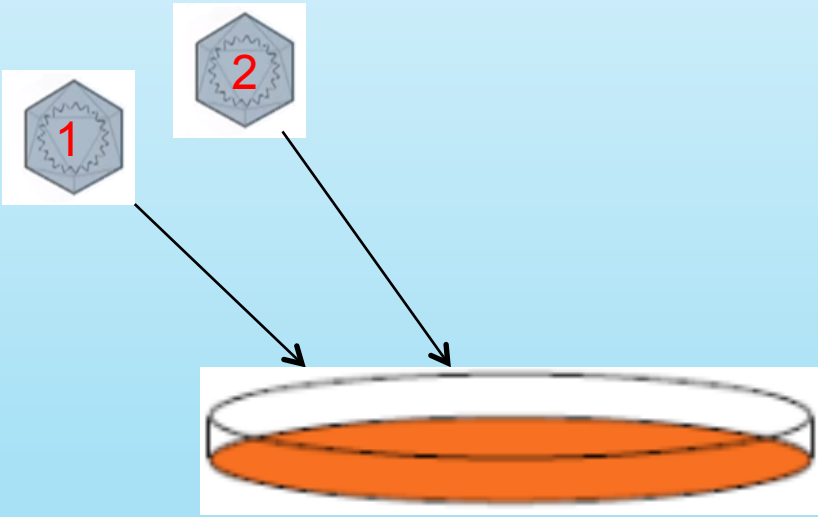
AAVs sgRna

Ellis et al, 2013

AAV Cotransfection in BMSCs

AV carrying Cas9 / AAV carrying sgRNA = 1:1

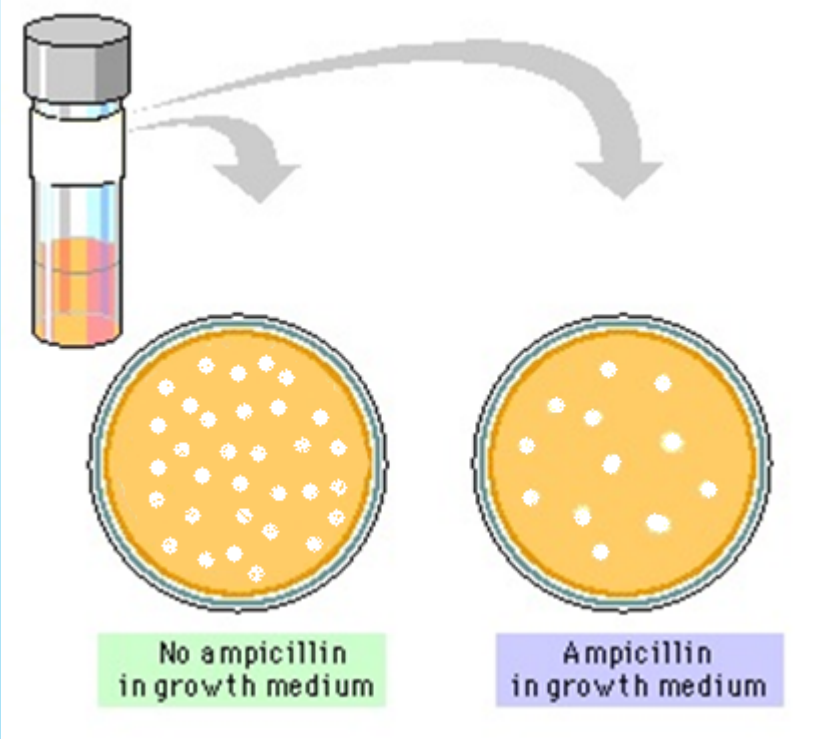
OI= 10^5 vector genome/cell
hen cells are 85% confluent



Mi et Al, 2009

Transfected BMSCs antibiotic selection and clonal growth

ransfected cells



Trasfected cells

Clonal growth

Gene Editing efficiency analysis

F-1a Gsa DNA PCR and sequencing

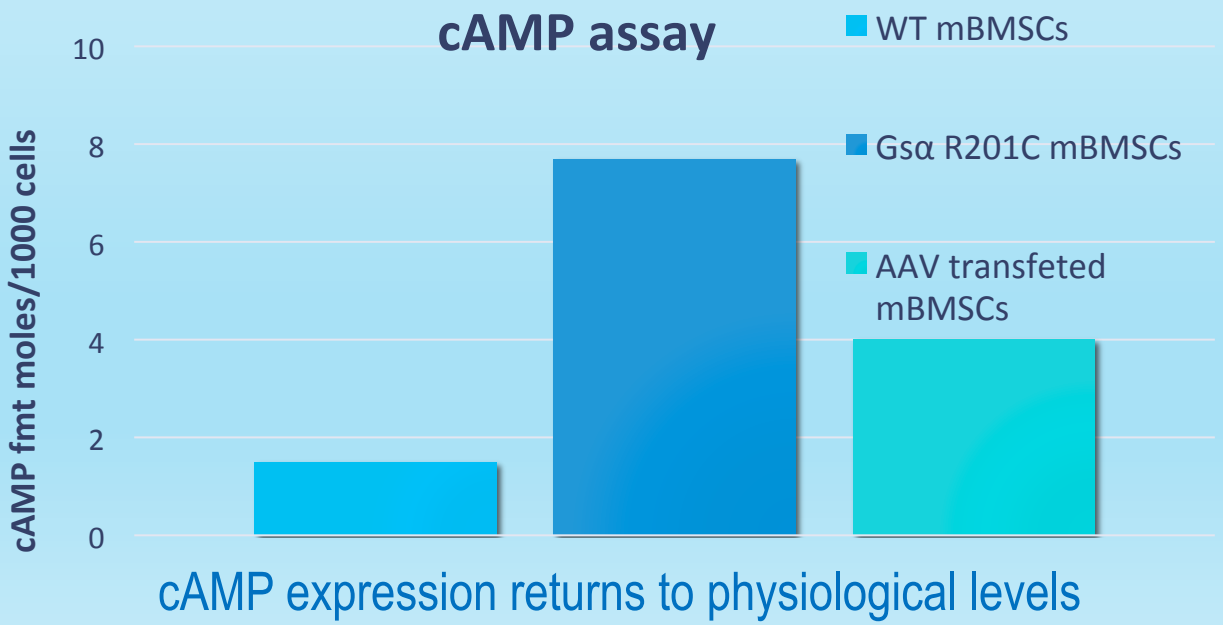
F-1a Gsa mRNA expression through SNP specific probes

Hansen et Oudenaarden, 2

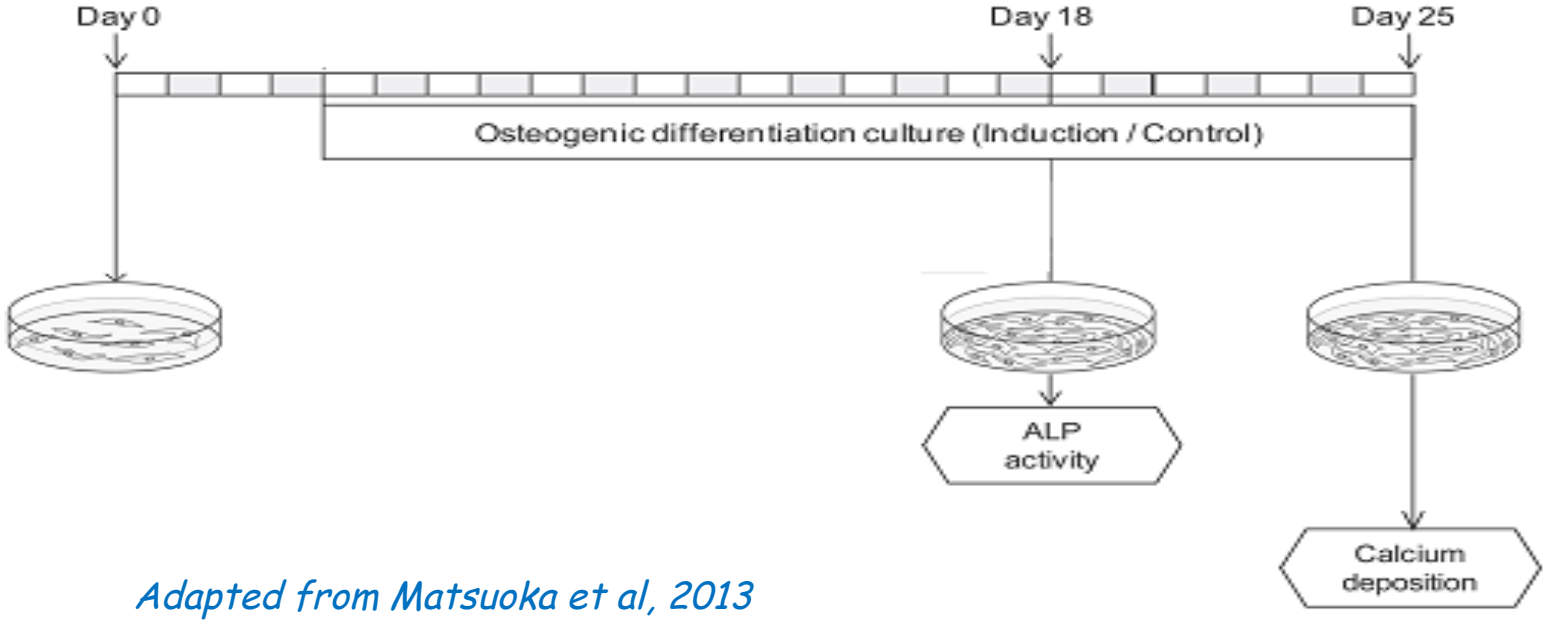
Functional rescue analysis

cAMP assay

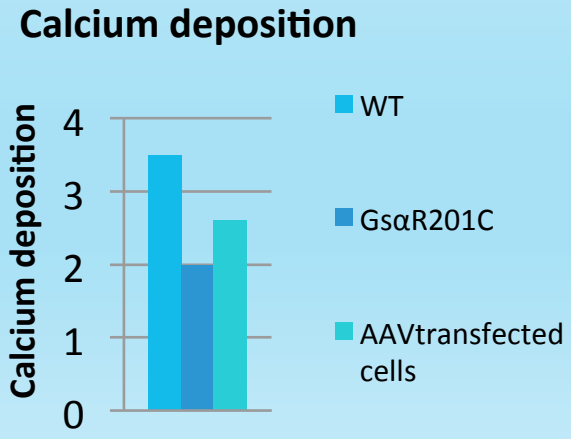
Osteogenic differentiation ability

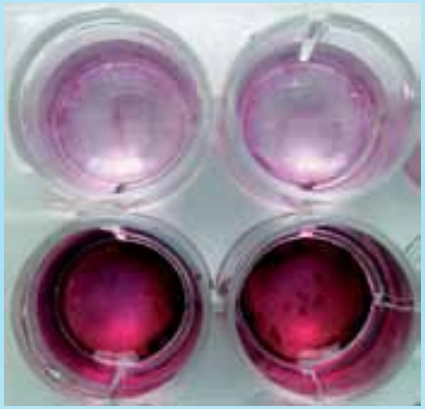


Osteogenic differentiation ability



Adapted from Matsuoka et al, 2013





Calcium deposition analyzed
Alizarin Red S staining

EX-VIVO

EF-1a Gsa (R201C) mouse BMSCs selection

AAV construction

Cotransfection

Transfected cells selection

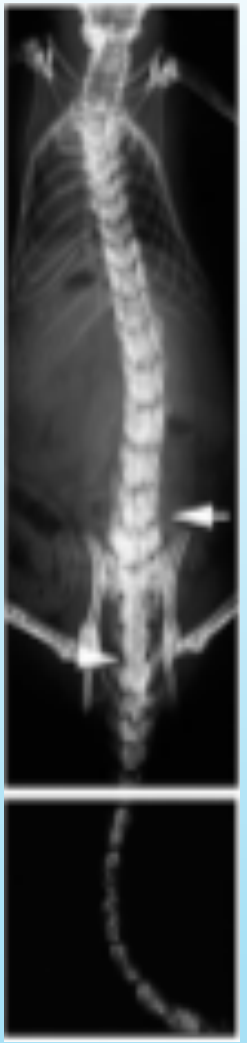
Gene Editing efficiency analysis

Functional rescue

Intravenous tail injection

Chirurgical removal and local injection

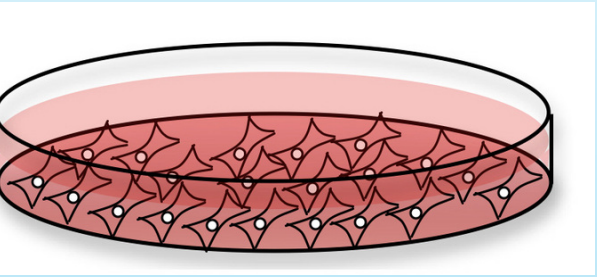
Phenotype Rescue



EF-1α Gsα (R201C) mouse

njection at 2/3 months, when FD sympthoms are visible → Human FD late detenction and therap

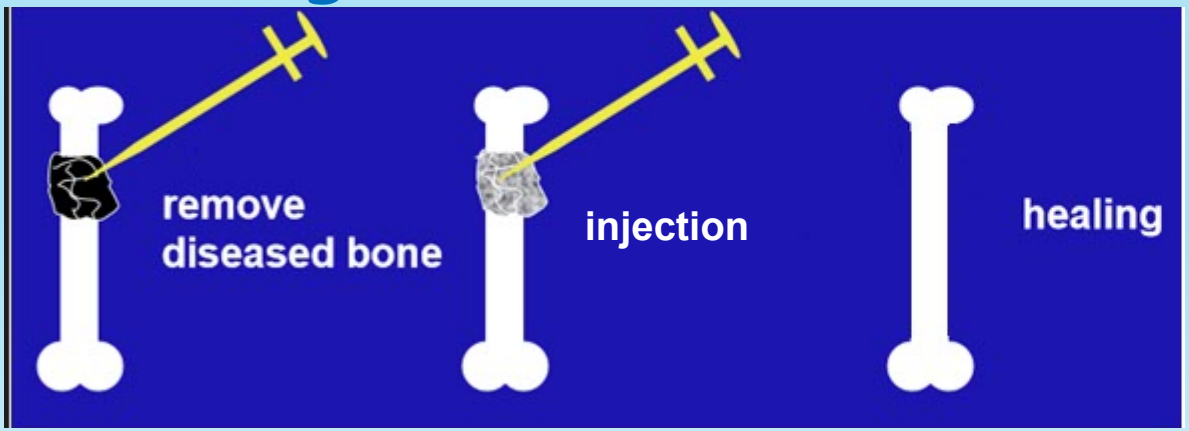
Tail vein injection



Hao Yin et al, 2014

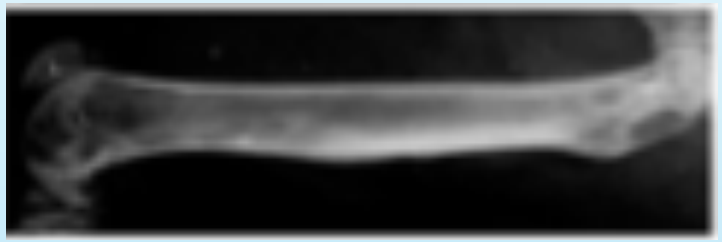
erentiated proliferative AAV transfected BMSCs

Fibrotic tissue chirurgical removal and local injectio



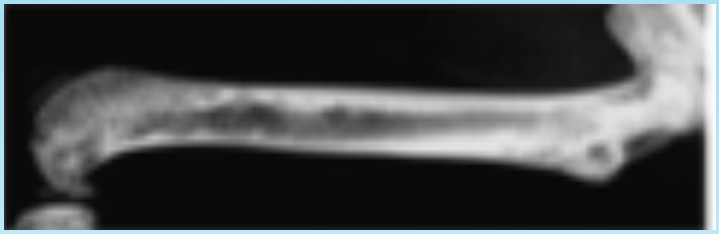
Phenotype rescue

- Femur radiography



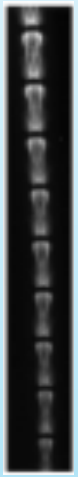
WT

Gsa (R201C)



Treated mice

- Tail and spinal column radiography



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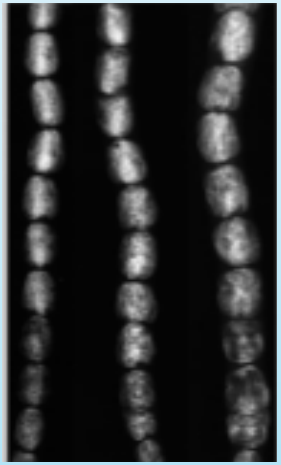
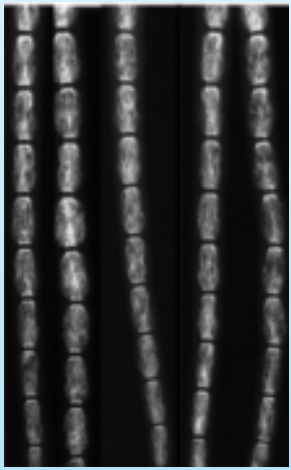


Gsa (R201C)



Treated mice

- We expect an amelioration of the phenotype
- We want to observe the eventual regeneration patterns during growth

Images from Saggio et al, 2014

Expected results and future perspectives

- We hypothesize that editing the EF-1a Gsa R201C sequence into the wild type one in BMSC, we could restore their capacity to differentiate into osteogenic cells, this would represent a new step along the way to ameliorate our mouse model for the human fibrous dysplasia. Probably the next step could be to create a chimeric mouse with both the wild type and the R201C Gsa subunit, in order to get a condition more similar to the human one.
- Our expected ex-vivo investigation has the aim to prove that these treated cells form a normal bone tissue when injected after fibrotic tissue removal. This could represent a future approach for rigenerative medicine in humans.

Pitfall and Solutions:

- Because of the slow turn over of the fibrotic tissue, we don't expect a great phenotype rescue with the intravenous tail vein injections of wild type BMSC, we hope for a better result with local injection after chirurgical removal of the fibrotic tissue.

COSTS

Materials	Costs
Crispr/cas9 kit	300€
AAV Helper- Free System x 2	1548,00 €
PCR mix	301,00€
DNA sequencing	Contact
cAMP Direct immunoassay kit	280,00€
EF-1 Gsa R201C mice	Gently donated by Saggiolab
MesenCult™ Osteogenic Stimulatory Kit (Mouse)	Contact
SNP specific probes	Contact

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Thank you for your kind attention!