Gene Therapy

SAPIENZA
UNIVERSITÀ DI ROMA

Gsalpha and G-coupled receptor diseases: Fibrous Dysplasia

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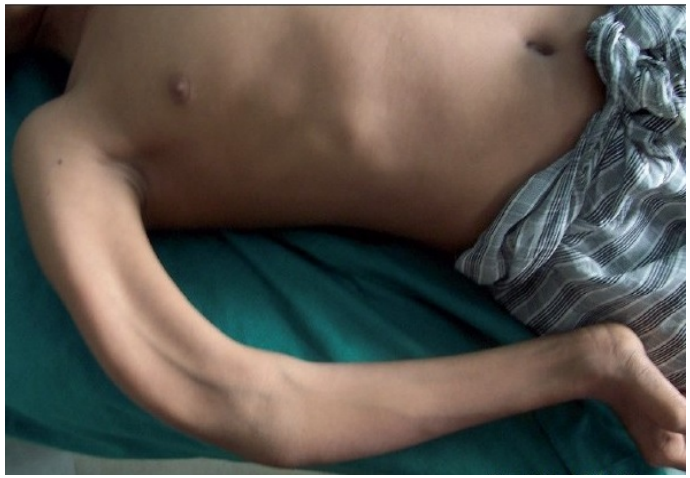
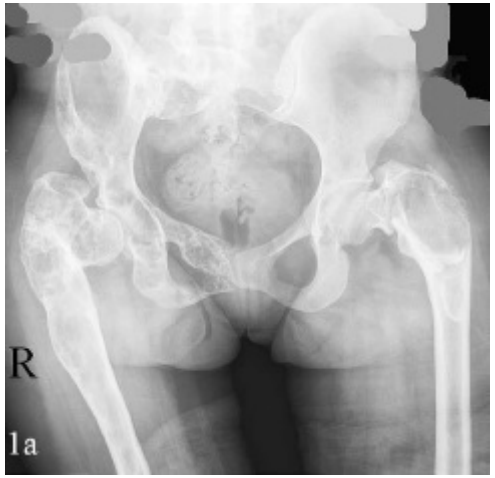
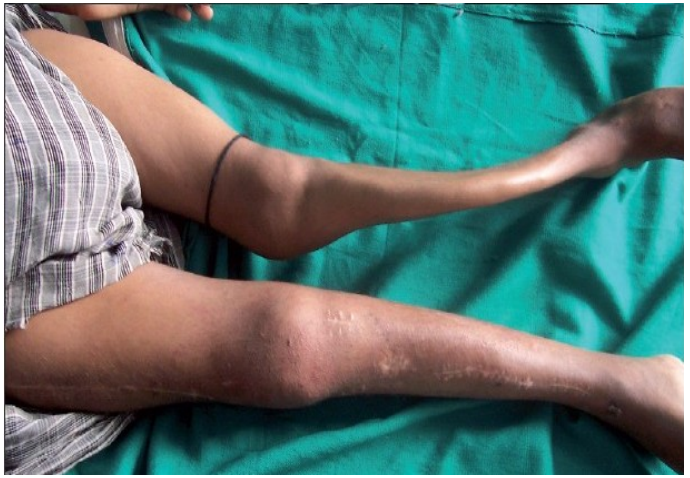
FIBROUS DYSPLASIA (FD)

OMIM: 174800







Adapted from Krina B. Patel, 2010

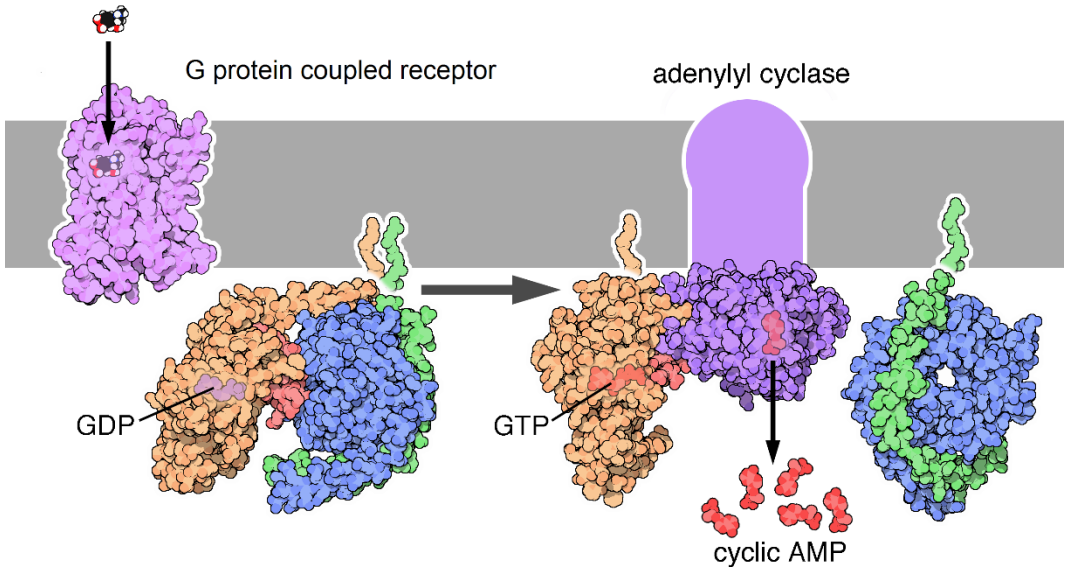
Clinical features:

- 60% of patients are symptomatic before age 10 years
- Pain and spontaneous fracture
- Leg-length discrepancies
- The most involved sites are femur, tibia, skull, facial bones, pelvis and arm bones.

Prevalence range:

- 1/100,000 and 1/ 1,000,000
- One bone involved (monostotic FD): 75% of all cases
- More than one bone involved (Polyostotic FD): 25% of all cases

FD IS CAUSED BY AN ACTIVATING MUTATION IN THE STIMULATORY G-PROTEIN α SUBUNIT



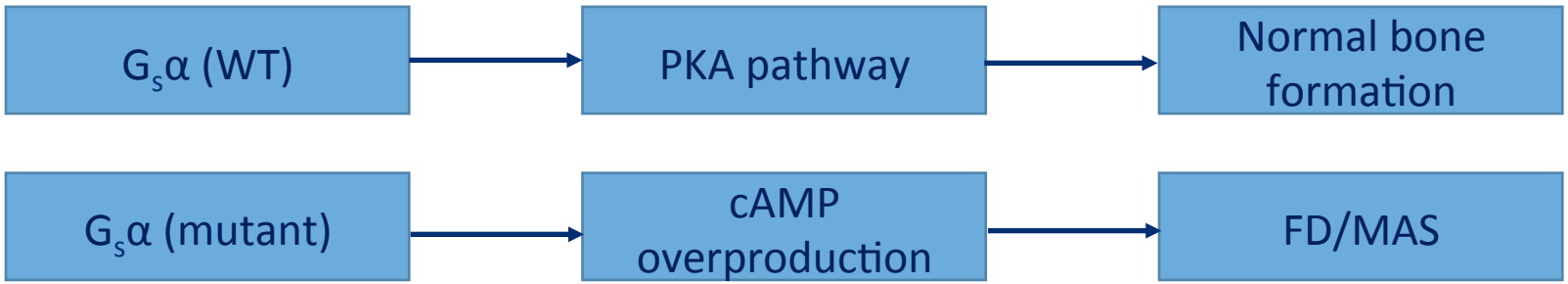
Adapted from http://www.rcsb.org/pdb/101/motm.do?momID=58

Gsα function:

- Heterotrimeric G protein (αβγ subunits)
- Gsα subunity has GTPase activity
- Gsα binds adenylyl cyclase increasing [cAMP]
- [cAMP] can activate Protein kinase A (PKA)

Gsα in Fibrous Dysplasia

- **Missense mutation** causes the replacement of arginine 201 by either cysteine (**R201C**) or histidine (**R201H**). The mutations occours **postzygotically**.
- The mutant form of Gsα cannot hydrolize GTP and remains **constitutively actived**.



OSTEOGENESIS

BONE MARROW STROMAL CELLS (BMSC)

•Bone marrow adipocytes
• Osteoblasts
• Myocytes
•Chondrocytes
• Fibroblasts

[cAMP] is involved in osteoblast differentiation:

- high cAMP concentration at early stage inhibits osteoblast differentiation
- low cAMP concentration at early stage stimulates osteoblast differentiation

BONE MARROW STROMAL CELLS R201C (BMSC R201C) FD-LIKE

Higher cAMP levels if compared with WT cells

- Lack of mineral deposition
- Upregulation of osteocalcin
- Enhanced upregulation of osteogenic markers (ALP and BSP)

Activating mutation of the Gs alpha gene

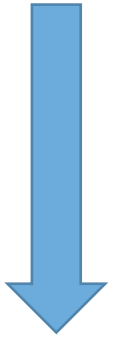
Increased levels of cAmp

Abnormal bone formation

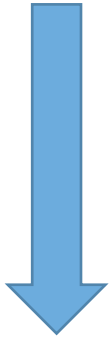
OBJECTIVES

RESTORE cAMP PHYSIOLOGICAL LEVEL IN BMSCs R201C BY CONTROLLED OVEREXPRESSION OF A SPECIFIC PHOSPHODIESTERASE (PDE) USING A LENTIVIRAL VECTOR

Insert the PDE gene under the control of a weak constitutive eukaryotic promoter (PGK) engineered with cAMP responsive elements (CRE) to correlate PDE transcription to endogenous cAMP levels.



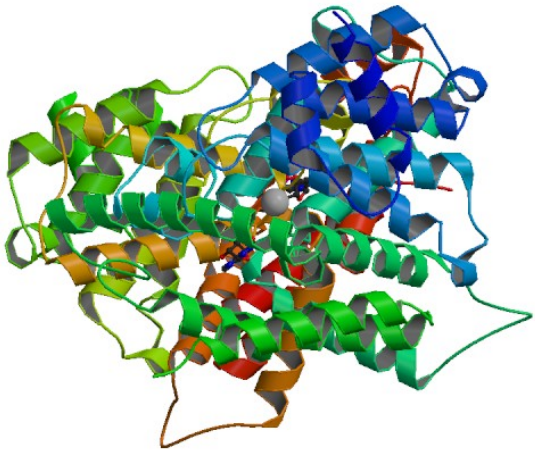
The chosen regulation mechanism is necessary to reduce the side effects on wild type BMSCs transduced with the same lentiviral vector.



Demonstrate that this approach is sufficient to restore the correct differentiation of mutated cells

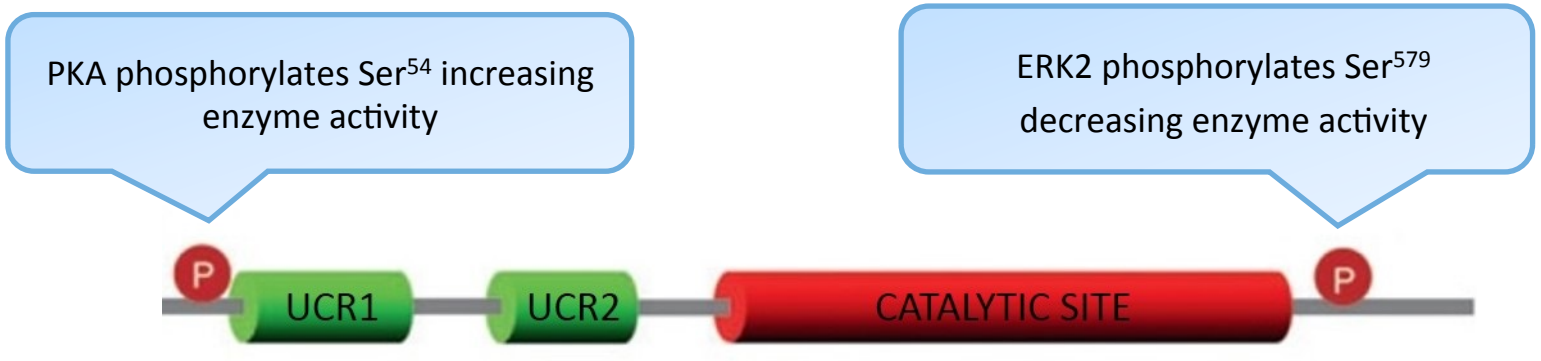
PHOSPHODIESTERASE 4

This enzyme family selectively hydrolyzes cAMP, while other PDEs are selective for cGMP or hydrolyze both cyclic nucleotides with varying efficiency.

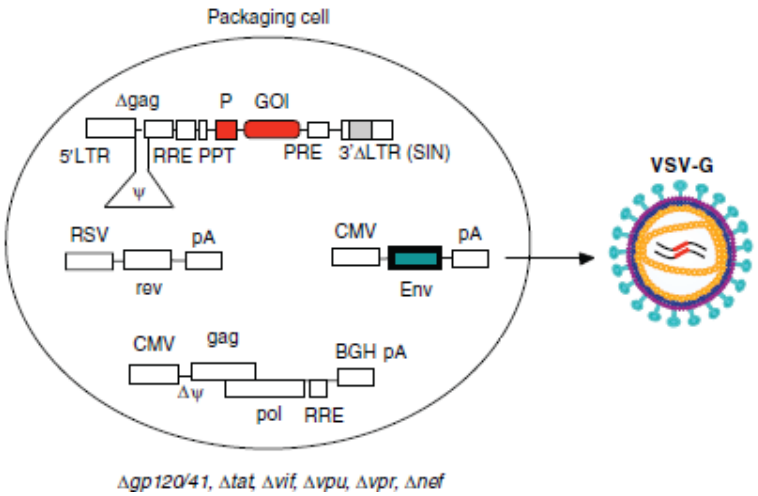


The PDE4 family can be divided in 3 different variant groups: **long, short and super-short form**.

We choose the **PDE4D3** because it's a long form and it can be regulated by cAMP levels thanks to two different phosphorylation sites



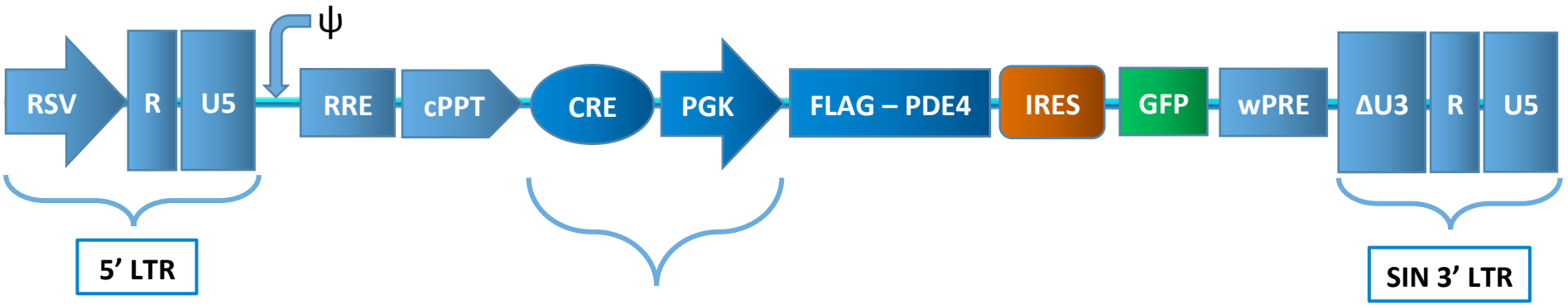
3rd GENERATION LENTIVIRAL VECTOR



Adapted from Mátrai et al., 2009

Why Lentivirus?

- Stable integration in the host genome (useful for bone marrow stromal cells).
- Possibility of use in ex vivo treatment without forcing cells replication.
- U3 deletion in third generation lentiviral vectors avoids virus mobilization



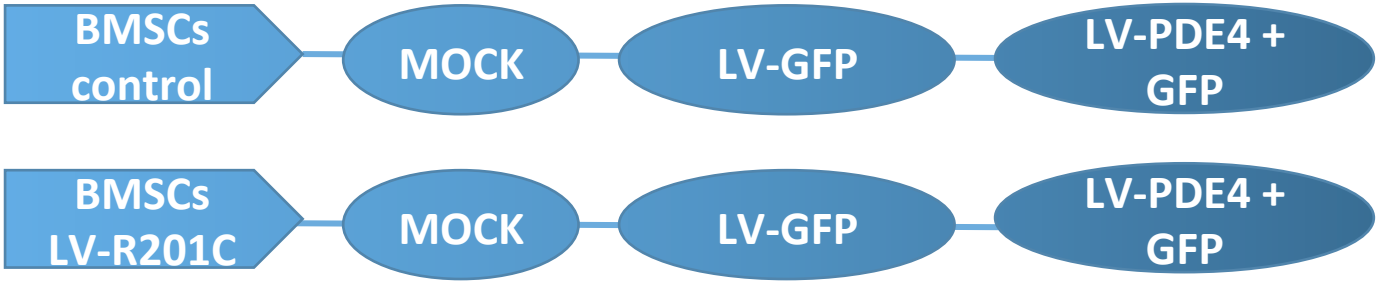
cAMP response elements (CRE) correlate transgene expression to cAMP levels
Basal transcription levels are ensured by phosphoglycerate kinase (PGK) promoter

EXPERIMENTAL PLAN

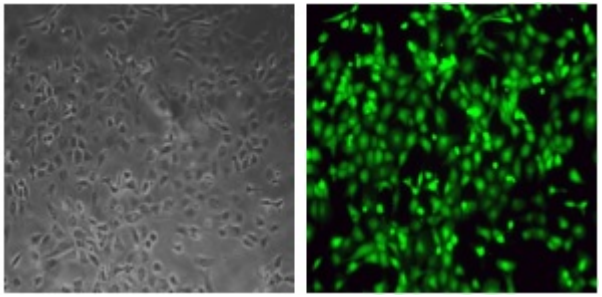
IN VITRO MODEL

- **Bone Marrow Stromal Cells**
- **Bone Marrow Stromal Cells transduced with Gsα^{R201C} Lentiviral vector**

TRANSDUCTION PLAN

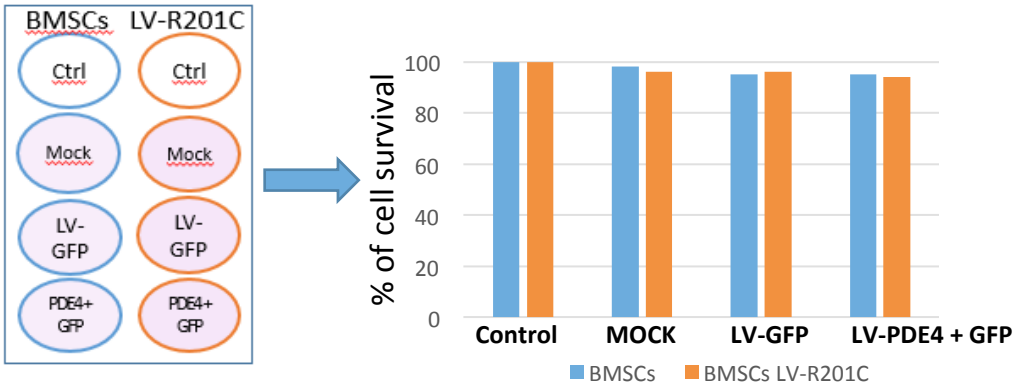


GFP is necessary to observe transduction efficiency and to sort transduced cells only by FACS.



BMSC and BMCS R201C can be stably transduced ex vivo using lentiviral vectors and they retain true organogenic potential in vivo

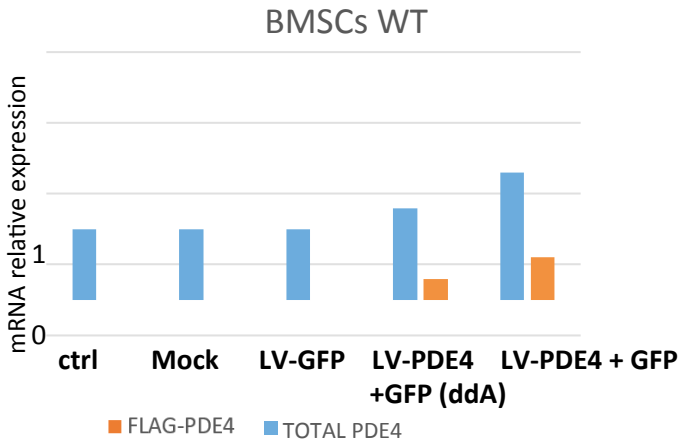
VIABILITY ASSAY (MTT)

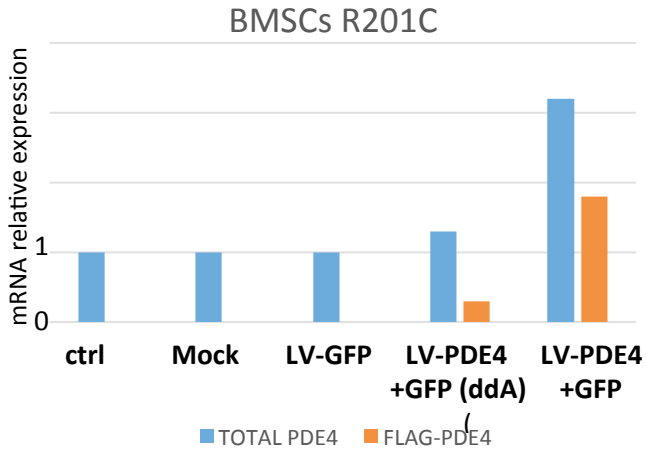


PDE OVEREXPRESSION DOES NOT AFFECT CELL VIABILITY

TRANSGENE EXPRESSION CONTROL

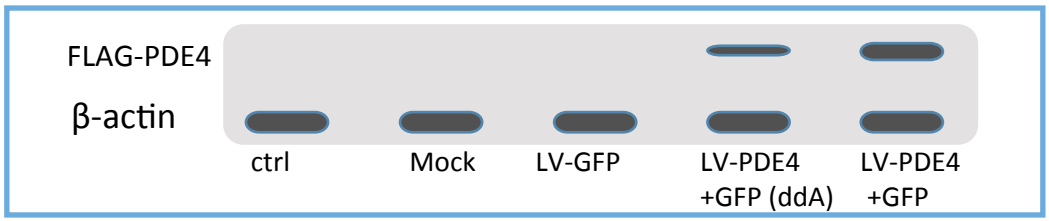
RT-qPCR for PDE4



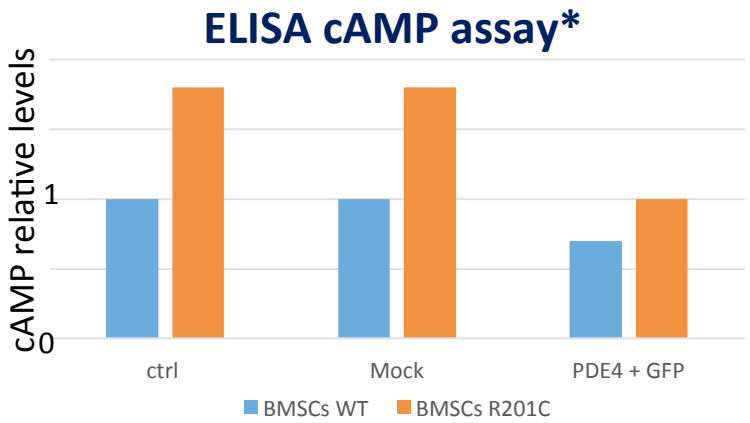


*ddA: 2',3'-dideoxyadenosine

WESTERN BLOT for PDE4 in BMSCs R201C



FUNCTIONAL ANALYSIS

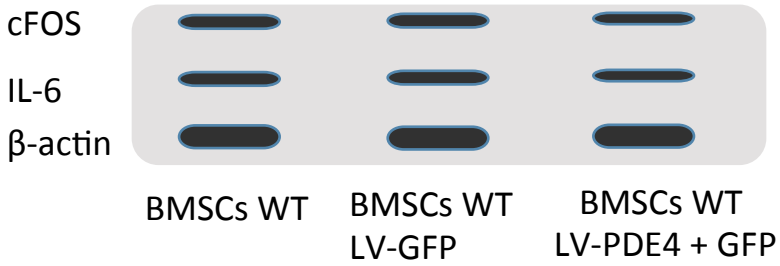


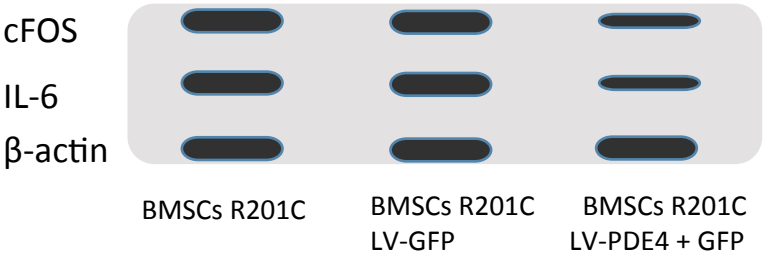
cAMP levels in BMSC R201C transduced with lentiviral vectors decrease to physiological levels

*cAMP measurement are obtained using an ELISA assay (Harlow and Lane 1988)

Fos and IL-6 (Western Blot)

cAMP increasing in BMSC R201C leads to trascriptional activation of gene cFos, which accumulates in this type of cells. IL-6 is increased in BMSC R201C cells too.



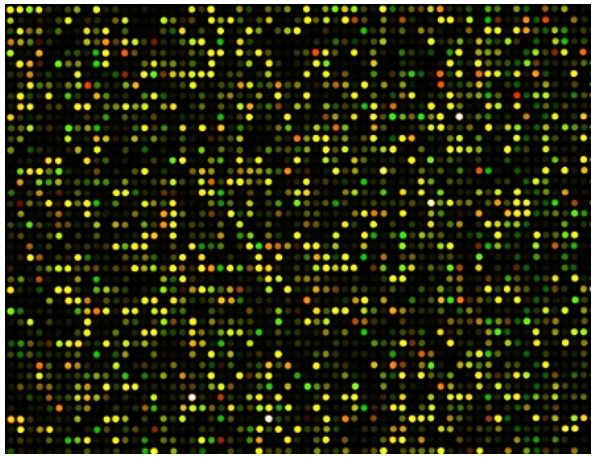


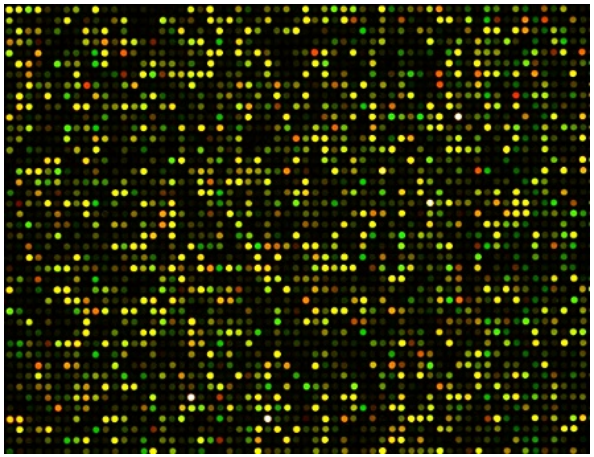
cFOS and IL-6 levels in BMSCs R201C treated with lentiviral vectors are similar to WT

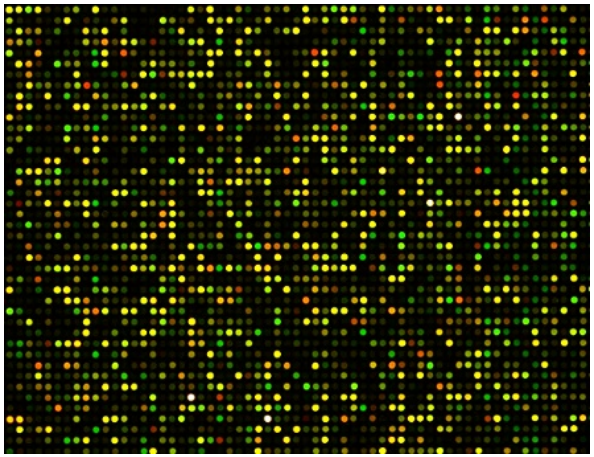
MICROARRAY ANALYSIS

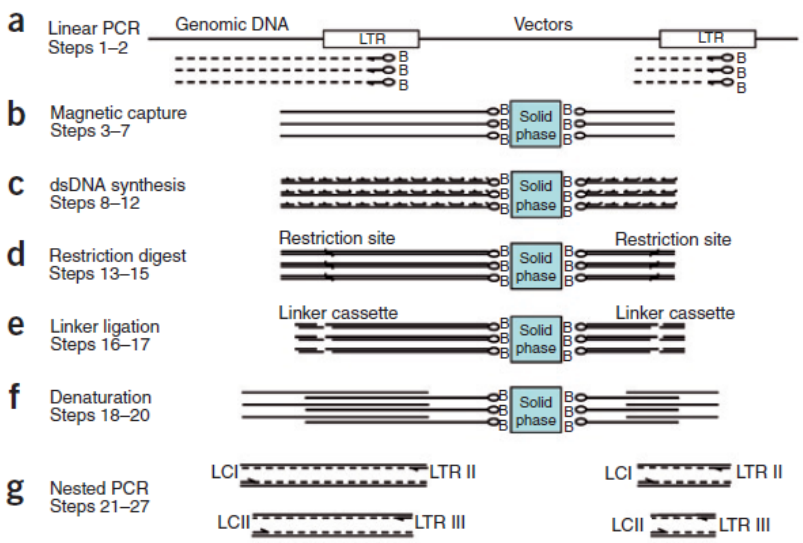
We use this method to control that the over expression of PDE4 doesn't deregulate other cellular pathways

BMSCs WT	BMSCs WT PDE4	BMCs R201C PDE4









LAM PCR

Linear amplification-mediated PCR is useful to control insertion sites. With this method we are able to identificate the **lentivector flanking sequences** and to know if integration will have an impact on important genes.

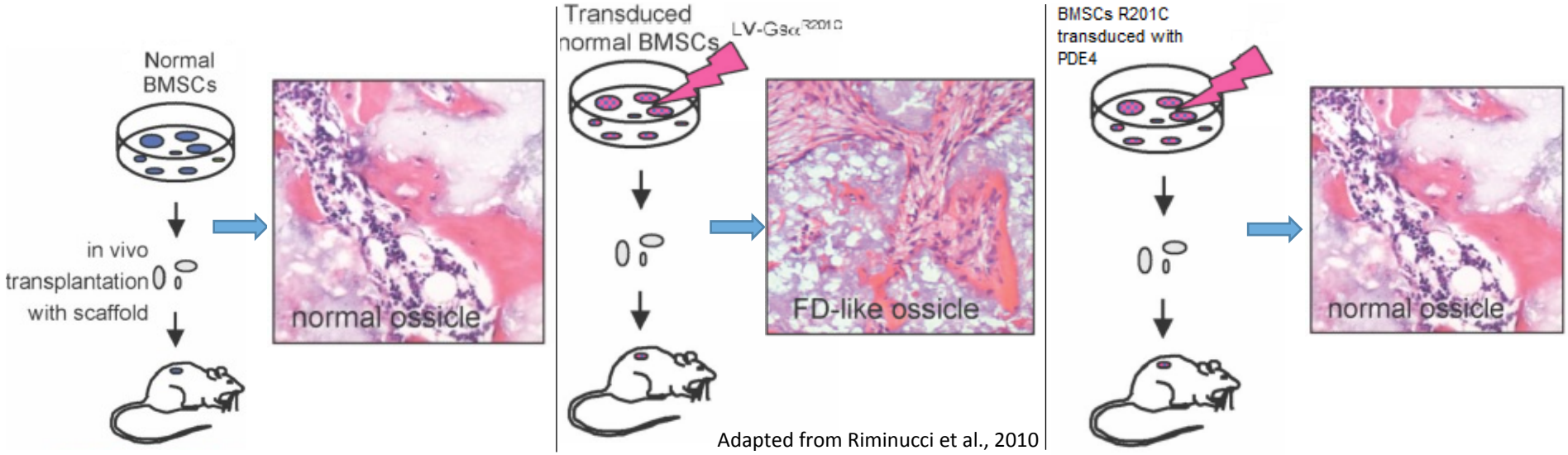
Schmidt et al., 2007

OSTEOGENIC DIFFERENTIATION
Differentiation Analysis

BMSCs WT	BMSCs R201C	BMSCs R201C LV-PDE4 + GFP

Adapted from Piersanti et al., 2009

IN VIVO MODEL
Osteogenic differentiation in SCID–mice Xenografts



Adapted from Riminucci et al., 2010

FUTURE PERSPECTIVES

ANIMAL MODEL

Creation of a transgenic $Gs\alpha^{R201C}$ mouse (Fibrous Dysplasia)

↓

Sample-taking and isolation of mouse BMSCs

↓

Insertion of the PDE4 gene under the CRE-PGK promoter, using a lentiviral vector

↓

Transgene expression controls, functional assays, insertional mutagenesis tests

↓

In vitro cell proliferation and transplantation in FD mouse

HUMAN

Sample-taking of patient BMSCs

↓

Insertion of the PDE4 gene under the CRE-PGK promoter, using a lentiviral vector

↓

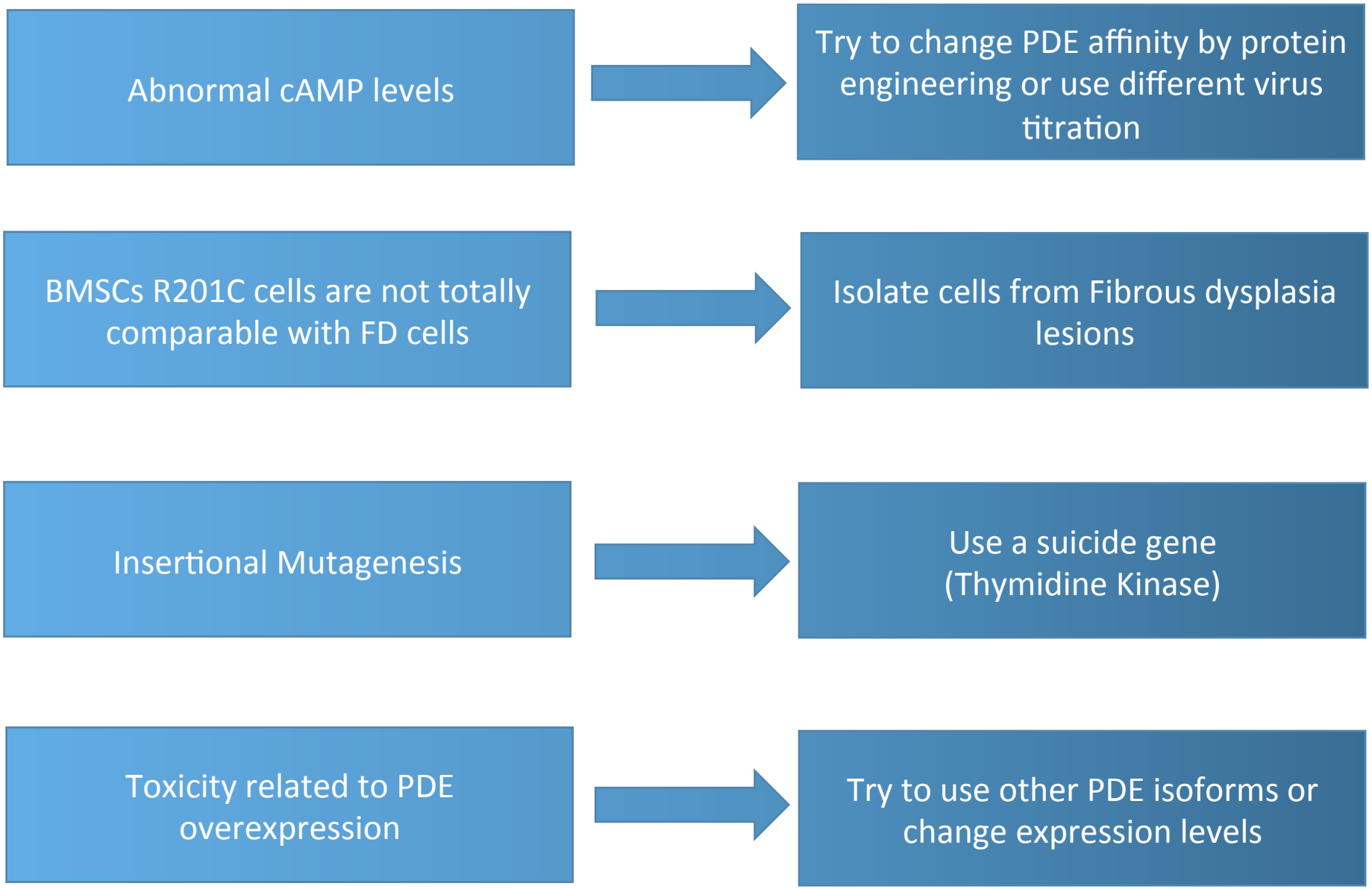
Transgene expression controls, functional assays, insertional mutagenesis tests

↓

In vitro cell proliferation and transplantation in patient

IN CASE OF INSERTIONAL MUTAGENESIS A SUICIDE GENE, SUCH AS THYMIDINE KINASE, SHOULD BE INSERTED INTO THE VECTOR; IN THIS WAY ONLY INFECTED CELLS WILL BE KILLED BEFORE TUMORAL TRANSFORMATION

PITFALLS AND SOLUTIONS



CO$TS

- Custom Lentiviral Vector (ViraSafe™ Lentiviral Expression Systems) *
- TaqMan® Array Human Osteogenesis (Applied Biosystems®) € 262
- NOD-SCID Mouse (each): € 89
- V13154Vybrant® (Life technologies) MTT Cell Proliferation Assay Kit- (1000 Assays) 1 kit € 280
- cAMP-Glo™ (Promega) Assay 300 assays (384-well plate)-V1501 € 296
- Anti FLAG™ Epitope Tag (DYKDDDDK) Antibody (FG4R) Host Mouse (Pierce) € 368
- Anti Cfos Antibody Host Rabbit (Santacruz) € 159
- Anti IL-6 Antibody Host Rabbit (Abcam) € 420
- Secondary Anti Rabbit (Pierce) *
- Secondary Anti Mouse(Pierce) *
- MicroArray Analisys (Microtech)* (from € 690)
- LAM PCR :
  - Taq DNA polymerase (Qiagen) *
  - dNTPs (Fermentas)*
  - Oligonucleotides and primers MWG Biotech*
  - Magnetic particles: Dynabeads M-280 Streptavidin (Dynal) (Lifetechnologies) € 365
  - Kilobase binder kit (Dynal) (Lifetechnologies) € 337
  - Klenow polymerase (Roche) € 88
  - Hexanucleotide mixture (50 reactions) (Roche) € 71
  - Restriction endonuclease(s) and incubation buffer(s) (New England Biolabs)*
  - Fast-Link DNA ligation kit (Epicentre) € 115
  - T4 DNA Ligase (New England Biolabs) € 50
  - Spreadex EL1200 precast gel (Elchrom Scientific)*
  - QIAquick PCR purification kit (Qiagen)*
  - DNA extraction kit (Qiagen or Roche)*

* contact vendor