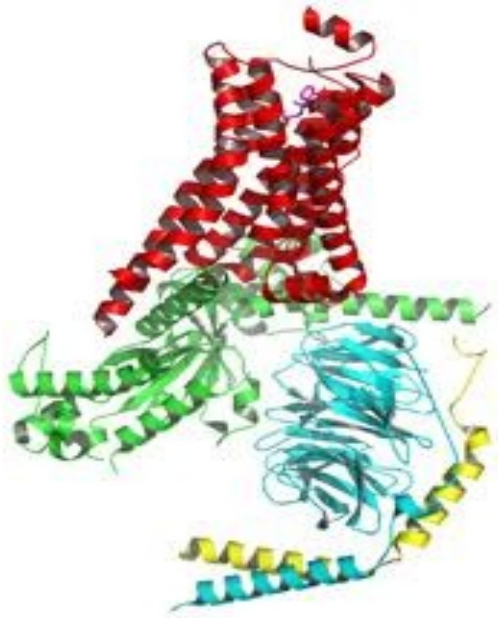
SAPIENZA
UNIVERSITÀ DI ROMA

Corso Terapia Genica
AA 2012/13
Prof.ssa Isabella Saggio

POH: new model for Gene Therapy using Lentivectors



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Progressive Osseous Heteroplasia (POH)

Genetic disorder of Mesenchymal differentiation:

- dermal ossification during infancy and progressive heterotopic ossification of cutaneous, subcutaneous, and deep connective tissues during childhood.

Autosomal dominant disorder:

- heterozygous mutations in GNAS1 (encoding the α-subunit of the stimulatory G protein of adenylyl cyclase)

- altered regulation of cyclic AMP–mediated signal transduction in MSC

Kaplan and Shore, (2000)

Progressive Osseous Heteroplasia (POH)

Clinical features of heterotopic ossificication in POH

Feature	*POH*
Sex distribution	Female = male
Genetic transmission	Autosomal dominant
Congenital malformation of great toes	−
Congenital papular rash	+
Cutaneous ossification	+
Subcutaneous ossification	+
Muscle ossification	+
Superficial to deep progression of ossification	+
Severe limitation of mobility	+
Severe flare-ups of disease	−
Ectopic ossification after intramuscular injections	−
Ectopic ossification after trauma	±
Regional patterns of progression	−
Definitive treatment available	−

Pathological and laboratory features

Feature	*POH*
Predominant mechanism of ossification	Intramembranous
Inflammatory perivascular and muscle infiltrate	−
Hematopoietic marrow in ectopic bone	±
PTH resistance	−
Hypocalcemia and hyperphosphatemia	−
Pathogenesis	Unknown

Kaplan and Shore, (2000)

Progressive Osseous Heteroplasia (POH)

MUTATIONS

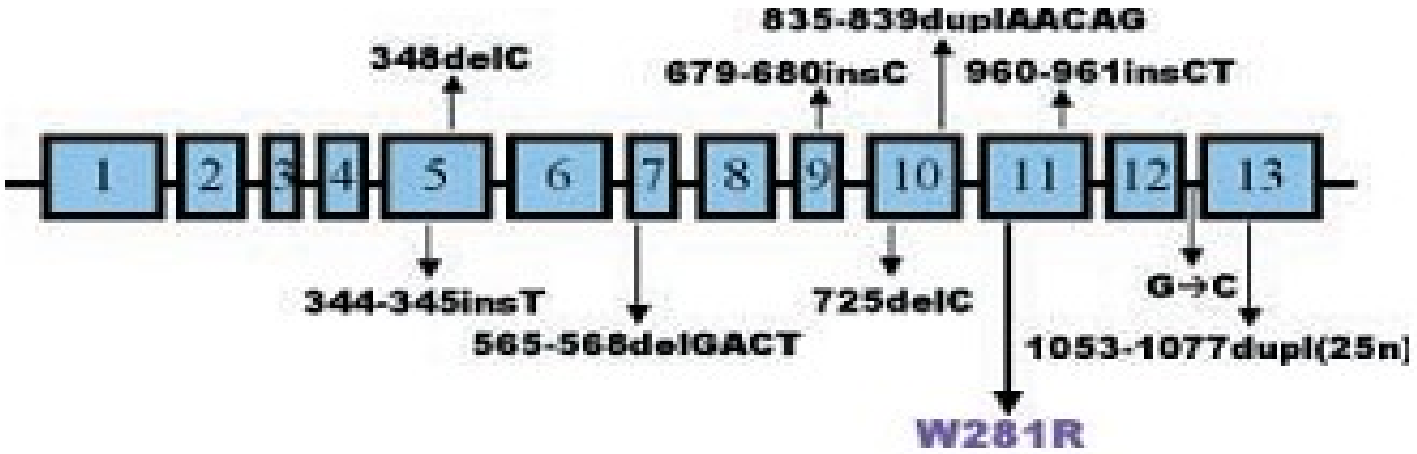
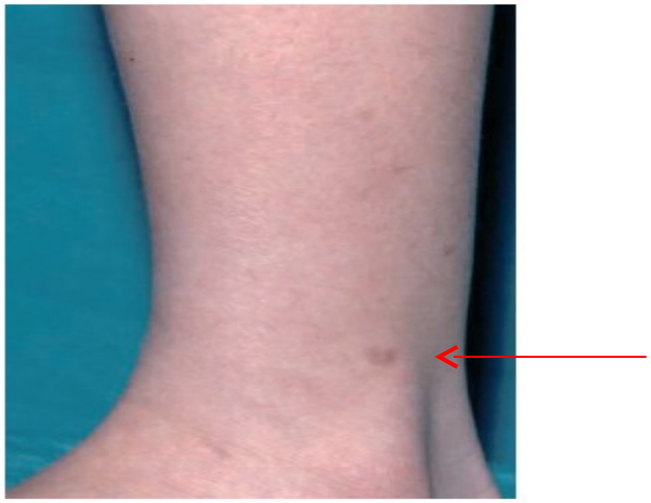


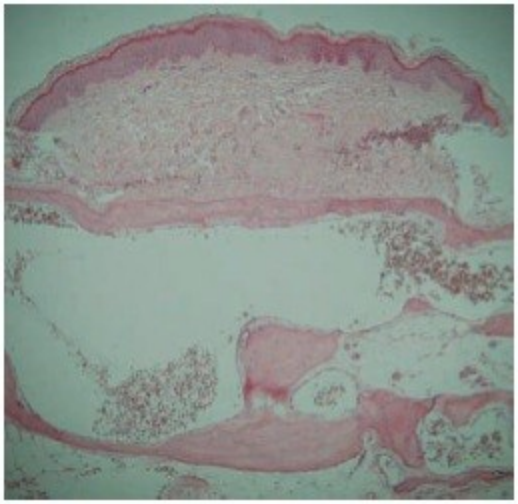
Figure 4 Illustration of the positions of the previously described heterozygous inactivating mutations identified in the *GNAS1* gene in patients with progressive osseous heteroplasia[2] and the new missense mutation described here (W281R).

Chan et al (2004)

Progressive Osseous Heteroplasia (POH)



Leg of patient aged 9 years old



Lesion on the leg shows
mature bone formation in the dermis
(haematoxylin & eosin)

Chan et al (2004)

Preliminary letteratures dates

NO CURE FOR POH

- Surgery: highly discouraged;

- In vitro studies:

BMSC (bone marrow stromal cells)
STSC (soft tissue stromal cells-adipose);

- In vivo models does not exist.

Pignolo et al (2011)

GNAS locus

Complex locus:

- contains **independent imprintig** domains that **uses multiple promoters** to generate several gene products

- 20q13.2-13.3 in the human genome

Encodes to 4 different isoforms of Gs-a Proteins:

- two longs (Gsa-1 and 2)

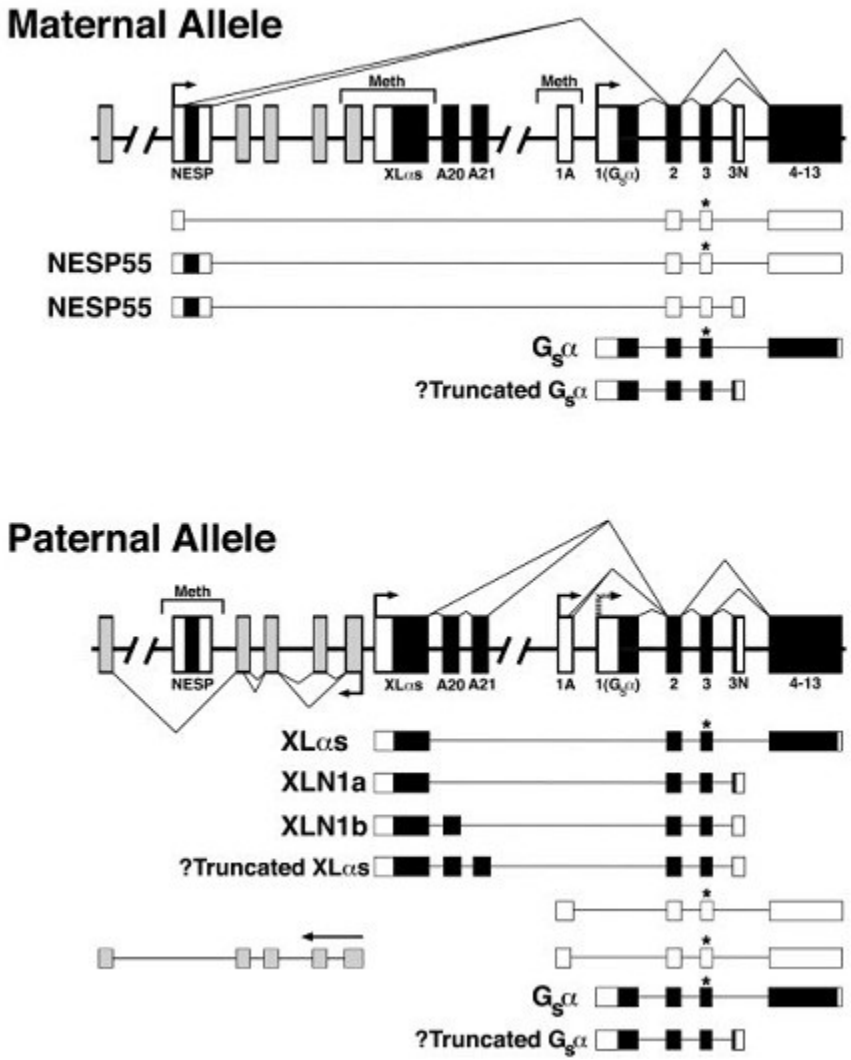
- two shorts (Gsa-3 and 4)

All are biological active!!

Weinstein et al (2001)

GNAS locus: role of genetic imprinting

The Gs trascripts are **biallelically expressed** in most tissue, but are expressed **primarily from the maternal allele in some tissue**



Weinstein et al (2004)

Gs Proteins

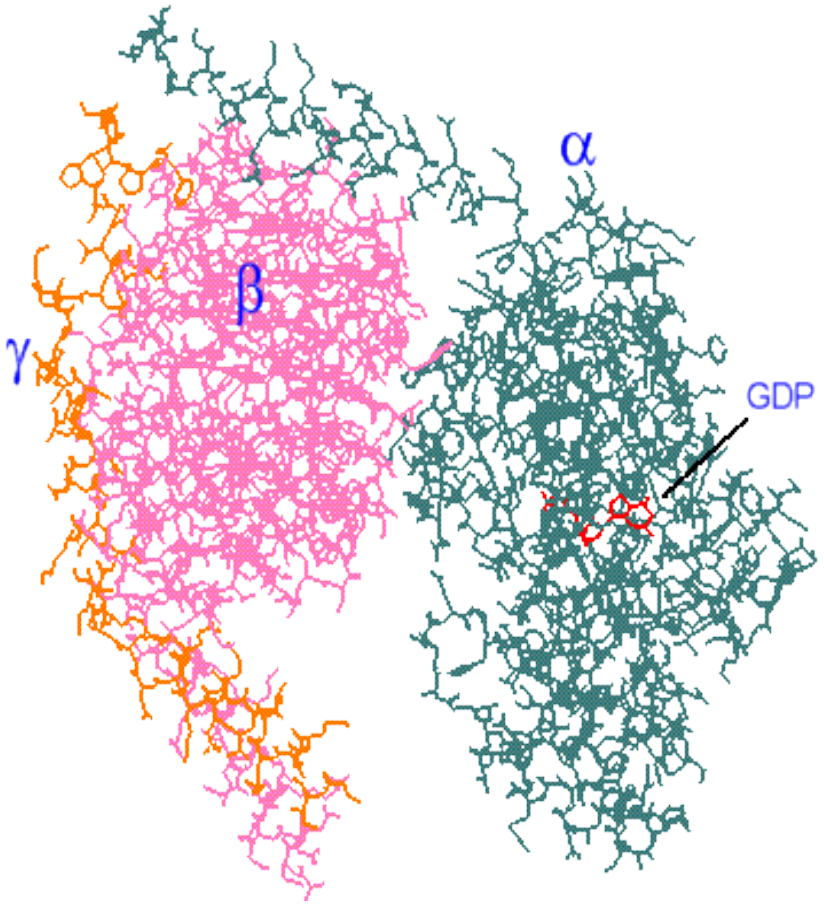
Integral components of diverse signaling pathways:

ACT AS A MOLECULAR SWITCHES

Each G protein is defined by:

- specific α-subunit (39-46 kDa)
  which binds GDP/GTP

- β-subunit(35-39 kDa)
  and γ-subunit(~ 8 kD)
  that form a complex

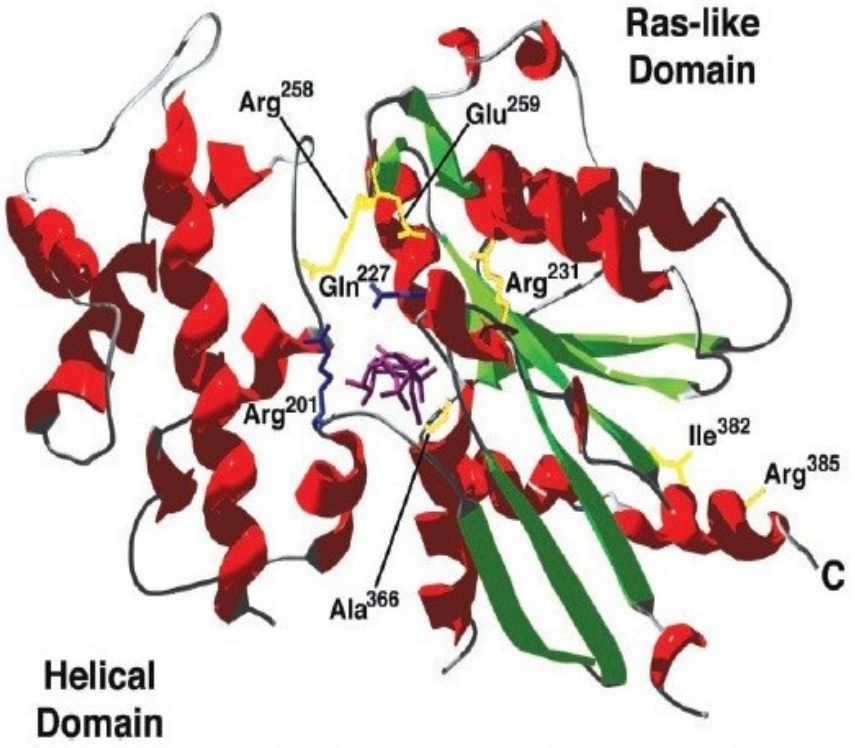


Kobilka et al (1998)

Gs-α subunit

Binds guanine nucleotide and interacts with specific receptors and effectors

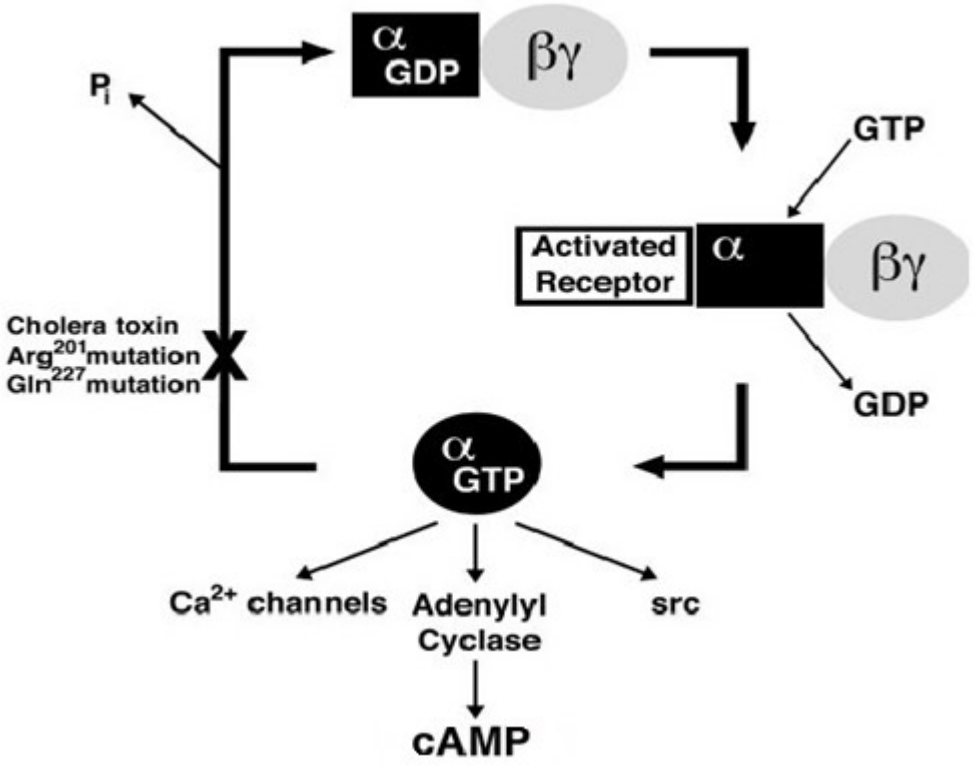
- Mutation of two residues important for the GTPase "turn-off" reaction (Arg201 and Gln227)
- Gsa is a protein found in all cell type except mature spermatozoe
- There are activation and inactivation mutations.



Weinstein et al (2001)

Gs-α Proteins

Gs is activeted and deactiveted via the GTPase cycle of its alpha subunit



Weinstein et al (2001)

Project goal

We are proposing two goals:

1. Create a disease murine models with classic POH

2. Strategy to understand the molecular basis of POH using 3rd generation lentivector

Project goal

Brief summary of the procedures:

- creation of two diffent usefull Lentivector to perform our protocols

- check point on the hability of construct

- Use of LV to:

  → generate a mouse model of POH

  → check the efficenty in vitro

- Subsequently, if everything works well, use LV protocol directly on mice

Gene Therapy strategy: 3rd generation lentivector

WHY LENTIVECTOR?!

Advantages:

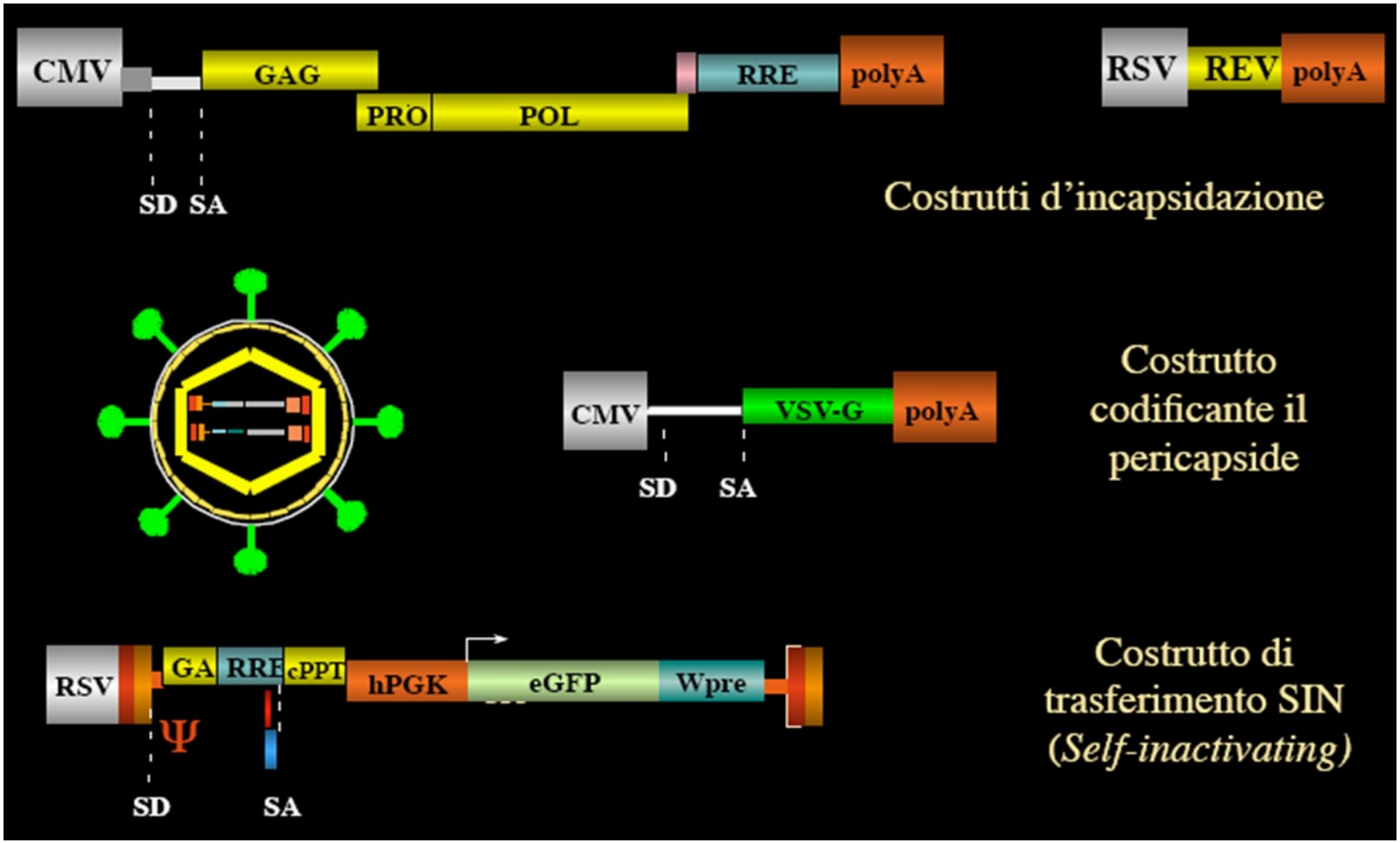
- can accomodate transgene large up to 8 kb
- stable long term transgene expression
- high titers
- easy to prepare

Drawbacks:

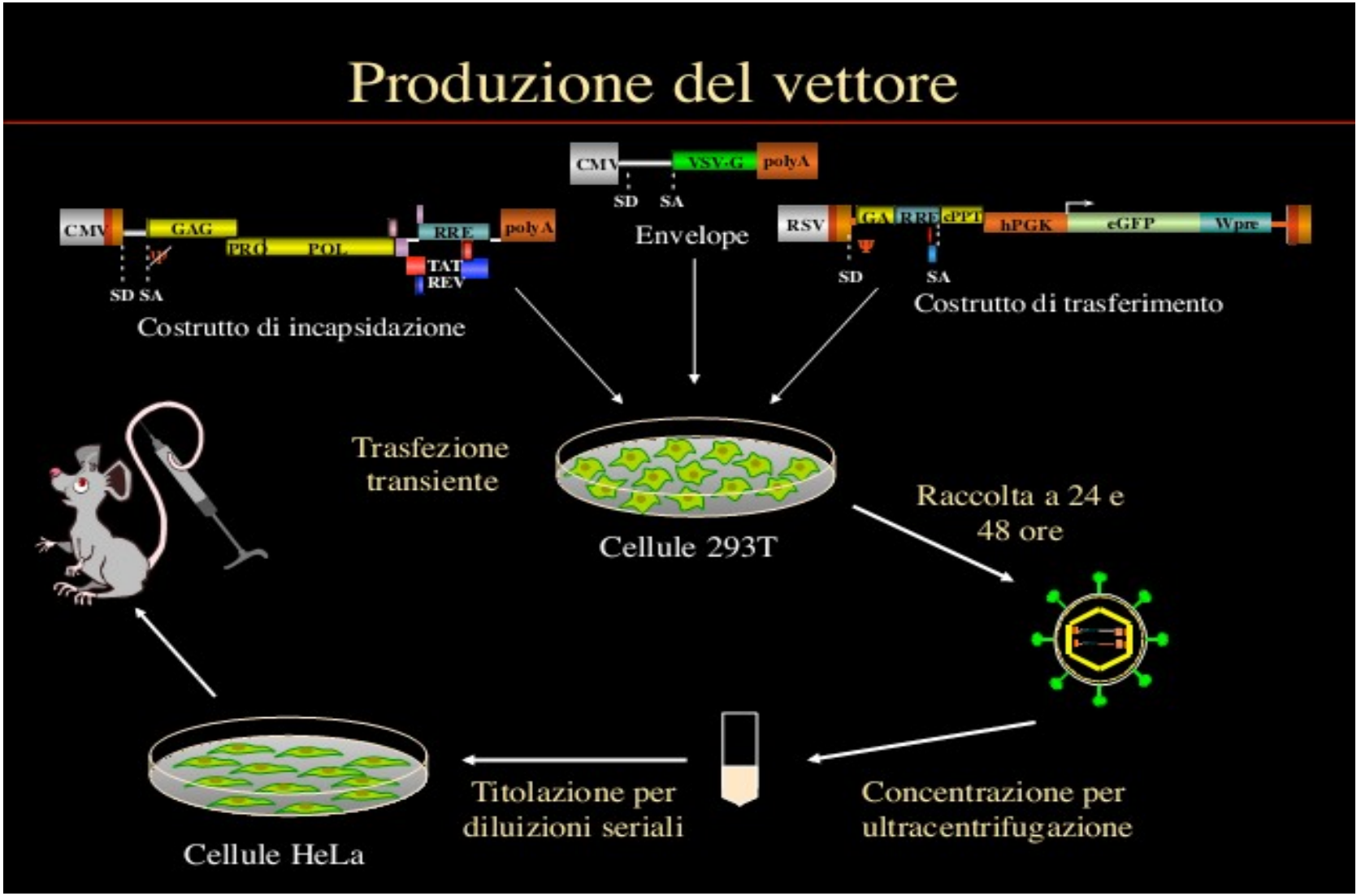
- Derives from the pathogenic HIV-1
- insertional mutagenesis

Gene Therapy strategy: 3rd generation lentivector

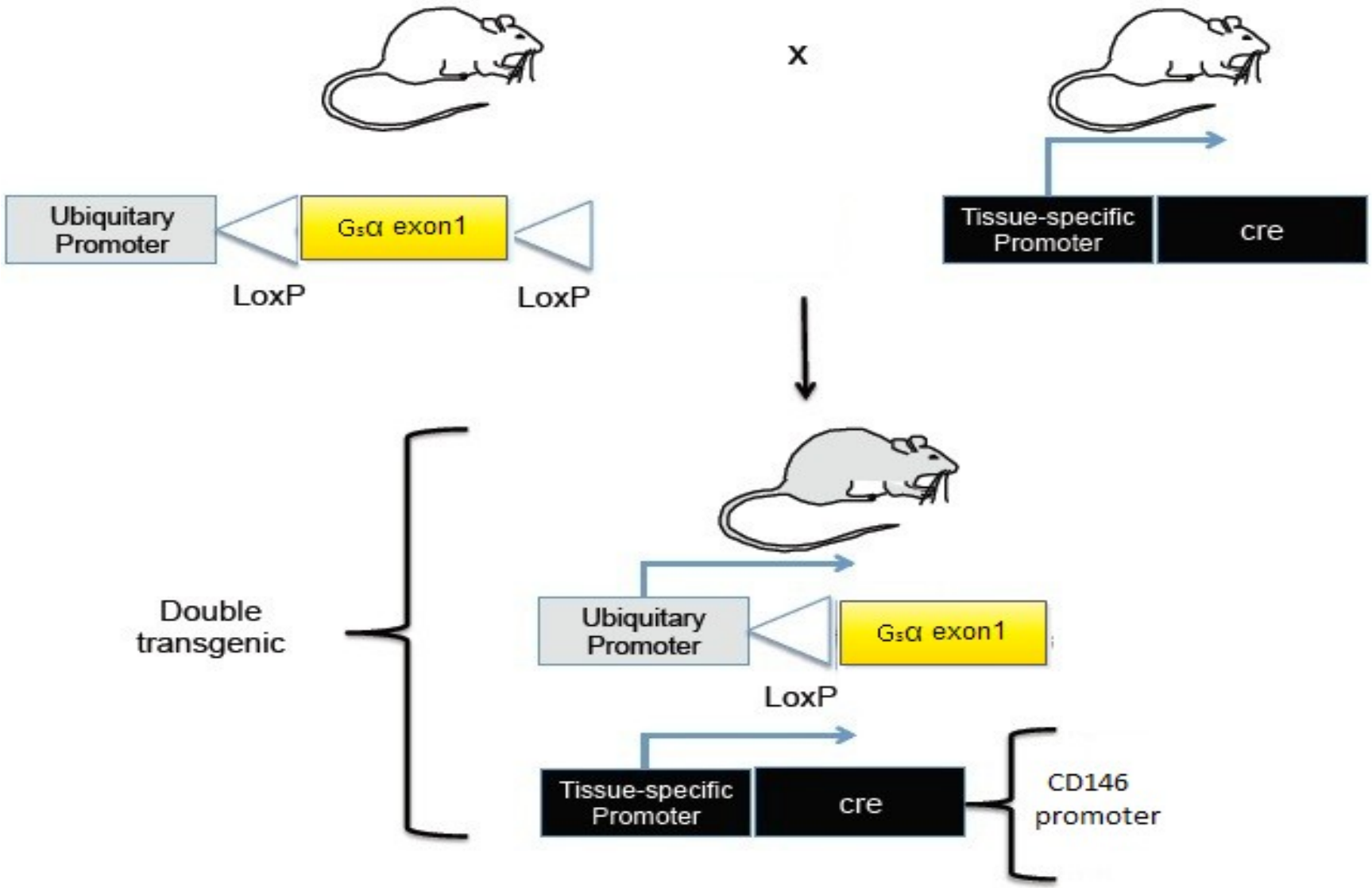
HOW PRODUCE LV IN LAB



Gene Therapy strategy: 3rd generation lentivector

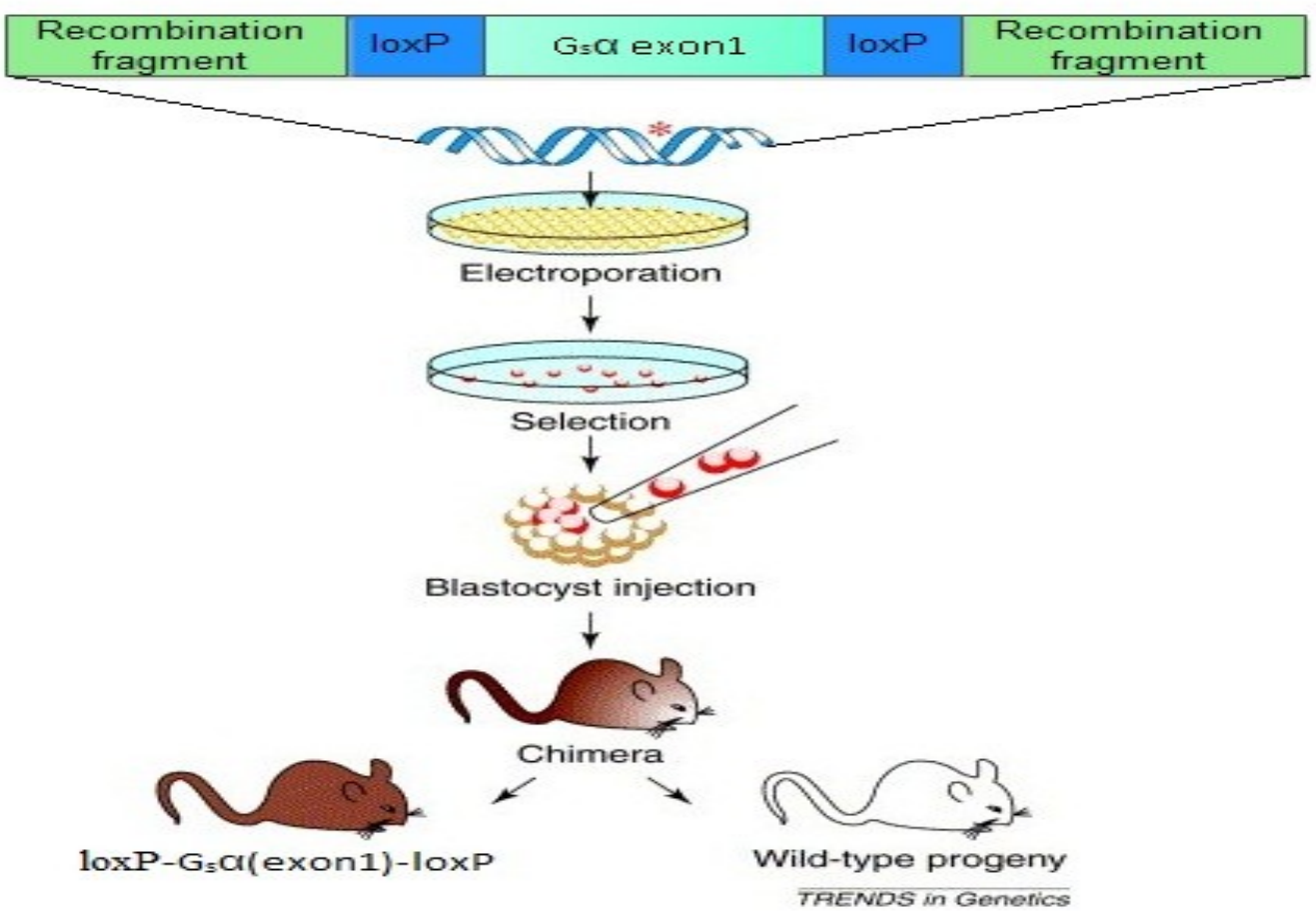


1. Murine model for POH



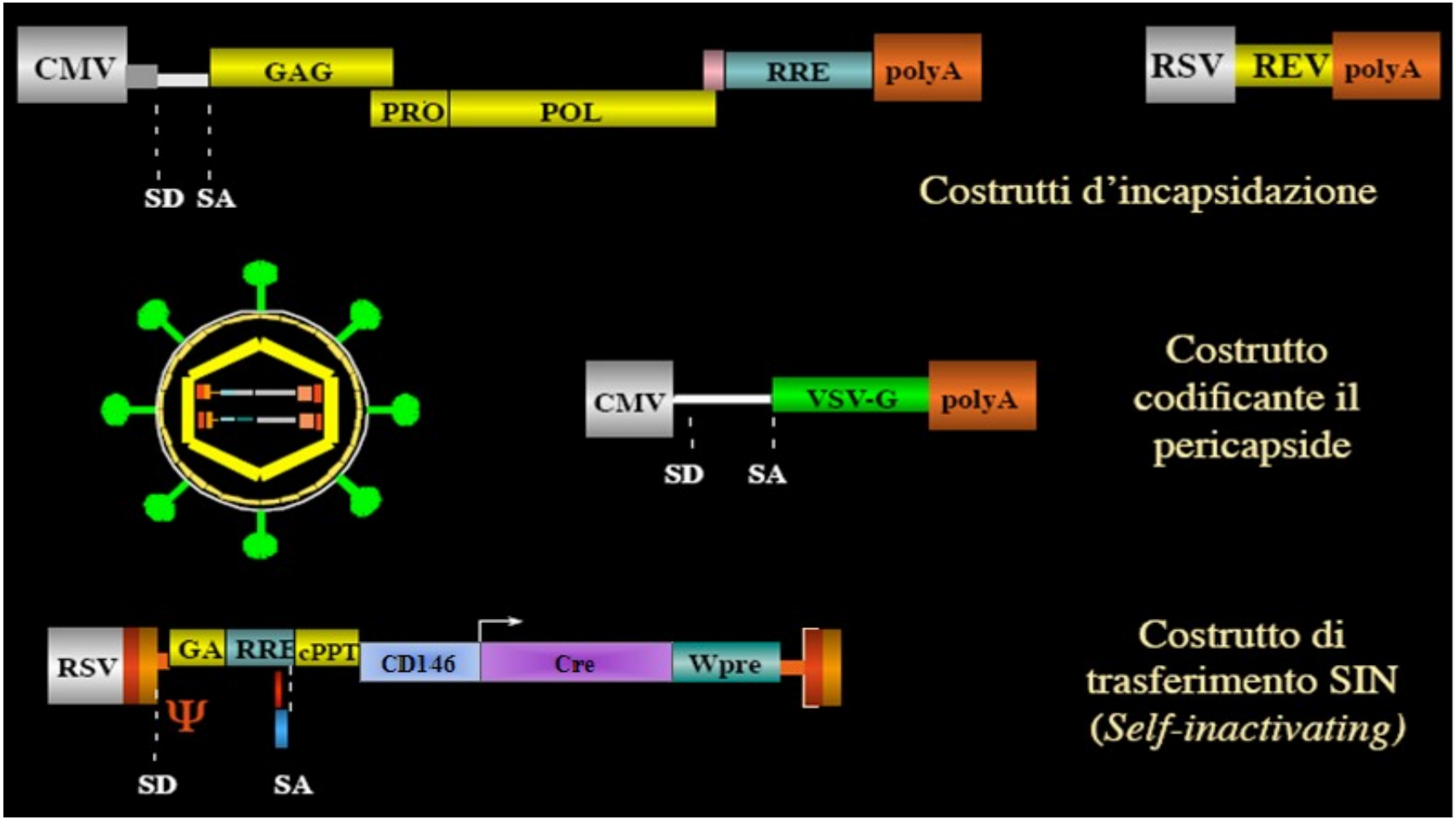
1. Murine model for POH

Generation of loxP-Gsα(exon1)-loxP mouse



1. Murine model for POH

Generation of CD146-Cre mouse



1. Murine model for POH

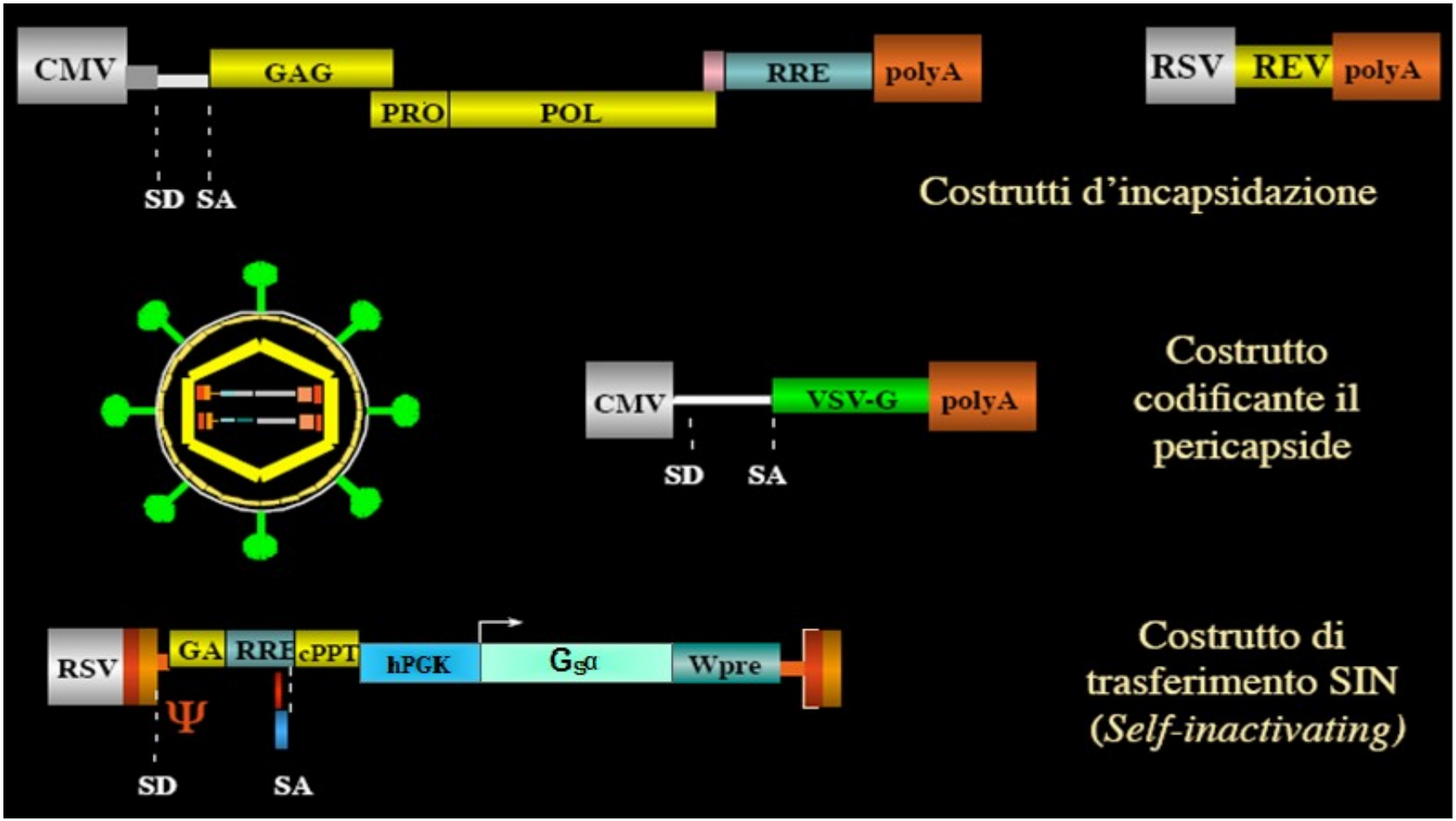
Controls on F1 looking for double transgenic mice:

- Search mutated phenotype

- Quantification of Gs-α mRNA and protein (Western blot, RT-PCR …)

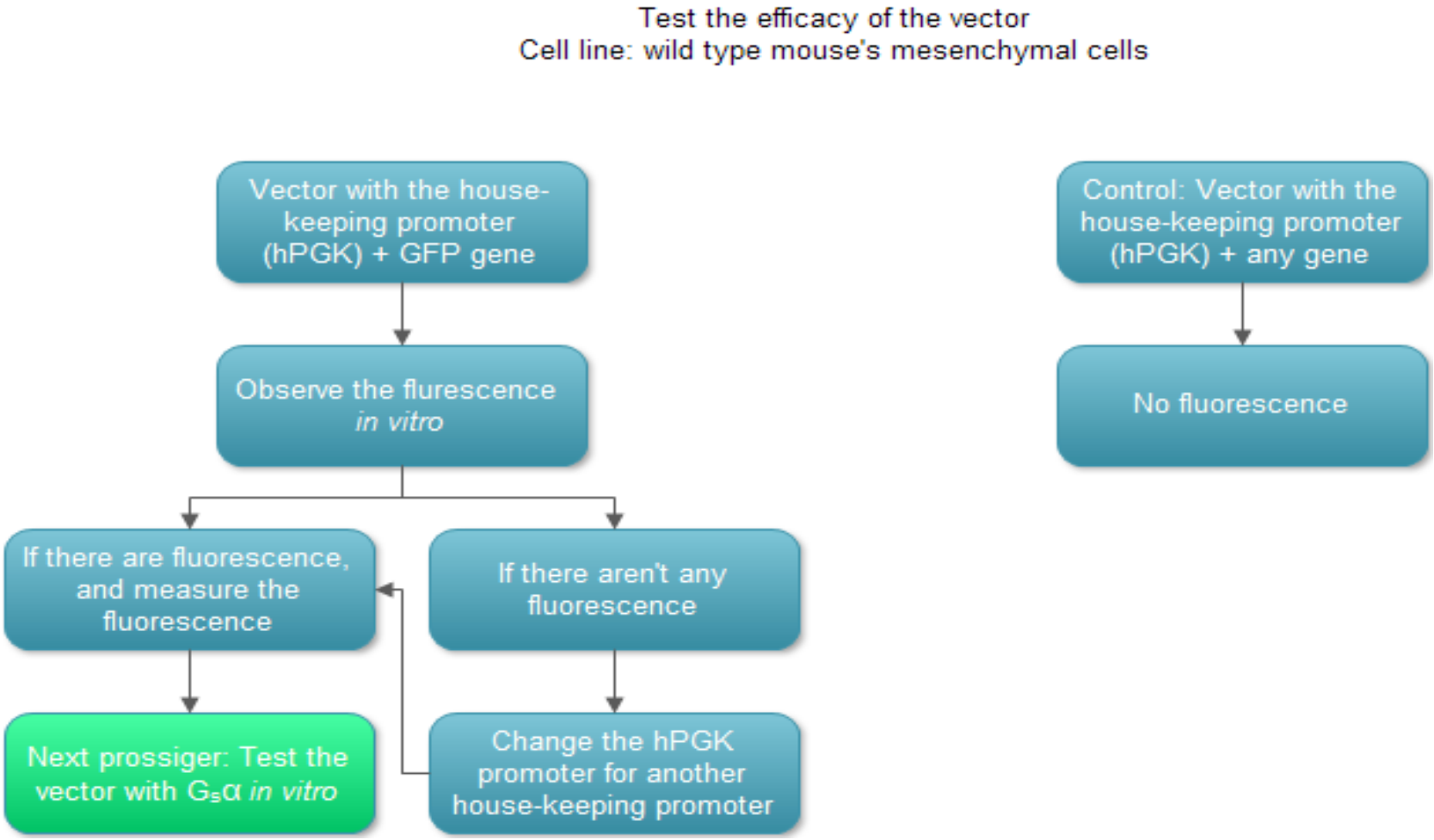
2. Gene Therapy strategy: 3rd generation lentivector

Production of LV-Gsa^{+}



2. Gene Therapy strategy: 3rd generation lentivector

Check point on the efficency of LV-GFP on MSC



POH: new model for Gene Therapy using Lentivectors

At this point, we had:

- used a transgenic approch to make animal model (Cre-Lox strategy to have a double transgenic mouse phenotypically and genotipically POH)

- test step by step if the protocols used was right performed

- construct LV-Gsa^{+}

- test if the LV-Gsa^{+} can be usefull to produce a correct form of Gsa in cells explant from our mouse POH

Now, where is the connection between Gs-α mutation and POH?!

2. Gene Therapy strategy

Gs-α loss of funtion decrease the cAMP and alters the MSC differentiation pathway

Nissenson et al (2012)

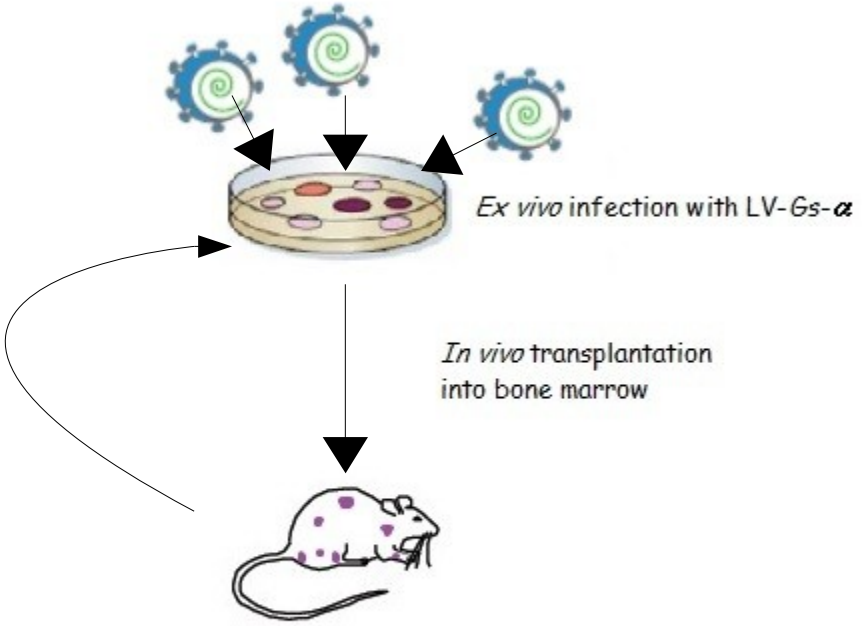
2. Gene Therapy strategy

Briefly:

- explantation of MSC

- treatment with
  LV-Gs-α

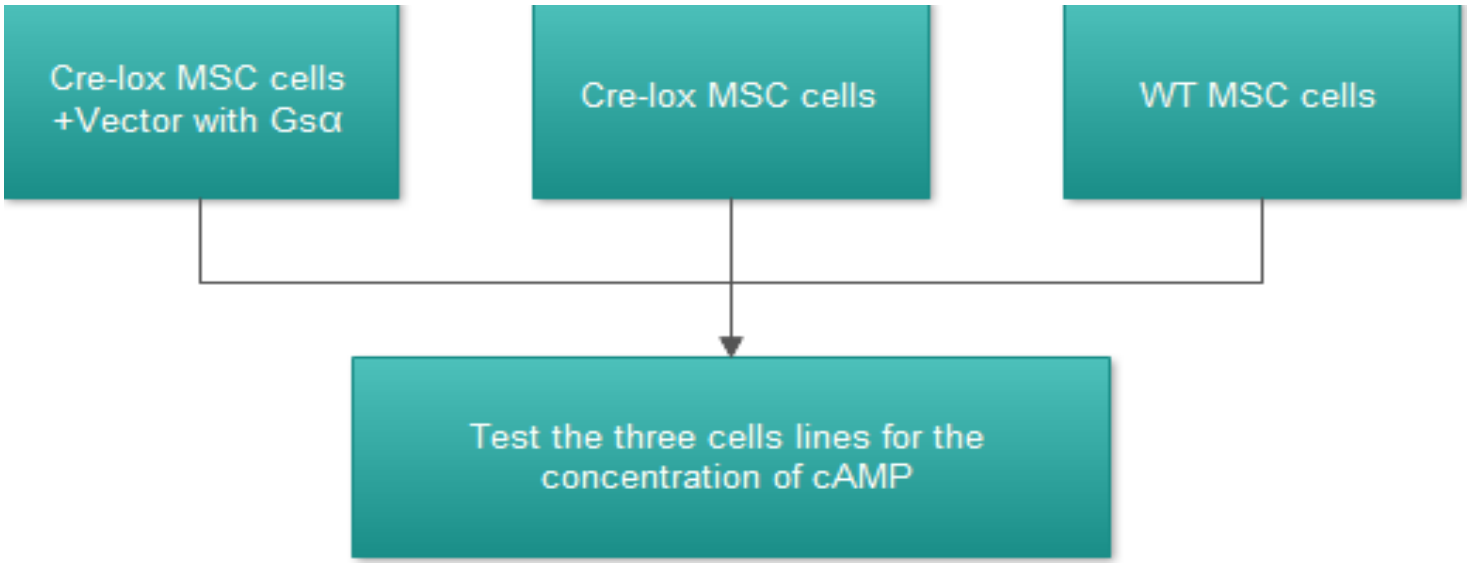
- transplantation in
  POH mouse model



2. Gene Therapy strategy

But... before go head let's check if it works!

Compared concentration of cAMP in the three cell lines.



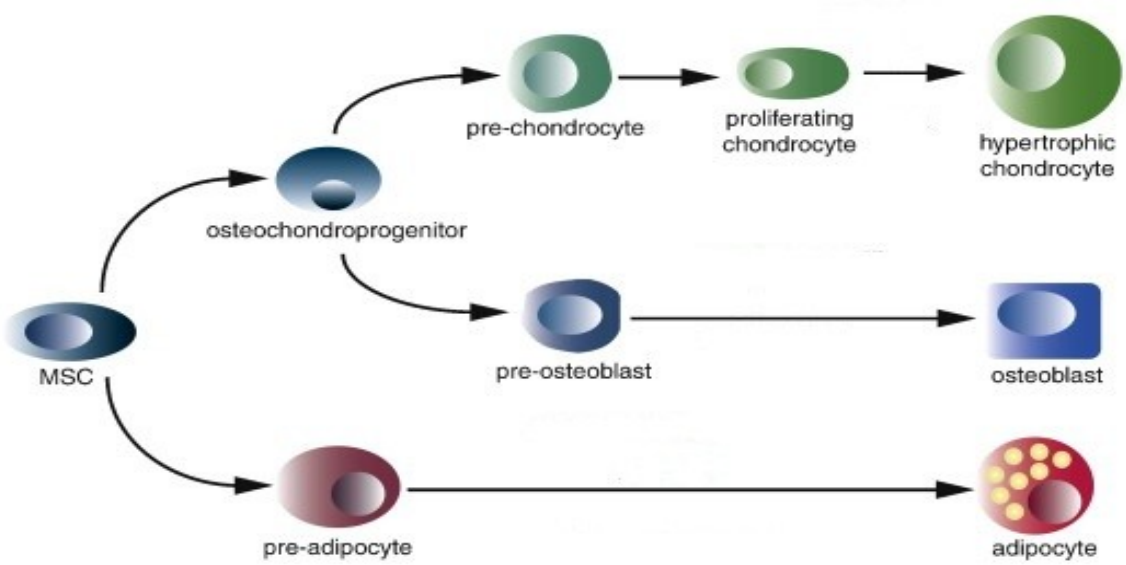
We will measure the concentration of cAMP using a commercial kit assay

2. Gene Therapy strategy

Why target MSC (Mesenchymal Stem Cells)?

- POH patients have disorder of Mesenchymal differentiation

- MSC are the multipotential progenitors of:
skeletal cells (**osteoblasts**, chondrocytes, hematopoietic-supportive stromal cells) and **adipocytes**



Karsenty (2008)

2. Gene Therapy strategy

MSC (Mesenchymal Stem Cells)

Properties:

- can be obtained from various tissues (ex: bone marrow, umbilical cord blood (UCB), placental tissue, and adipose tissue)

- isolated on the basis of their adhesive properties (ex: CD146)

- *in vitro*:
  --> differentiation is initiated by addition of growth factors and low molecular weight components

  --> characterized by their ability to adhere to plastic in culture and differentiate into various mesodermal cell lineages

MSC transplantation represents an exciting approach that could potentially treat complex diseases by providing combinatorial therapy!

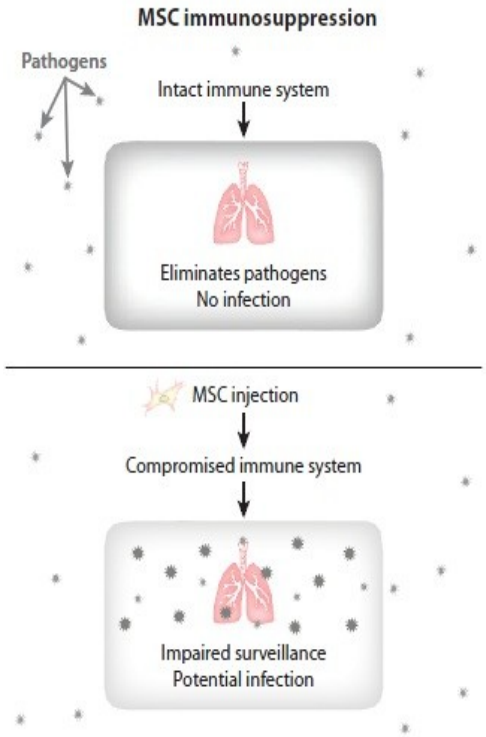
Kein et al (2010)

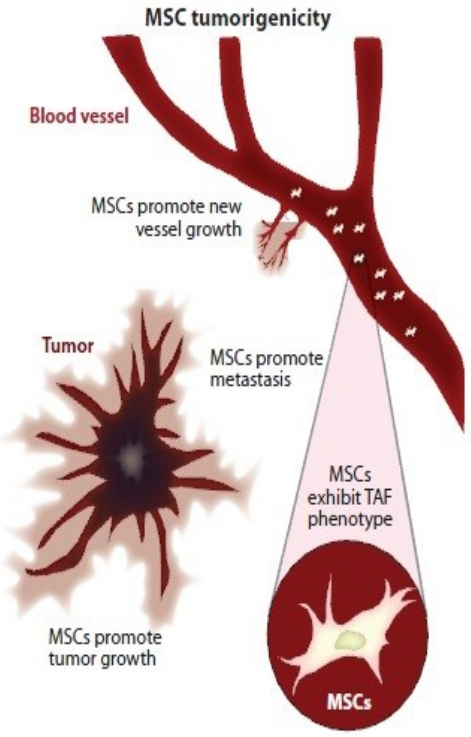
2. Gene Therapy strategy

MSC (Mesenchymal Stem Cells)

Drawbacks:

- recent preclinical studies have highlighted potential long-term risks include potential maldifferentiation, immunosuppression, and instigation of malignant tumor growth





- lack of knowledge to conclusively:
→ lineage relationships
→ differentiation properties
→ no culture conditions have been described which can maintain multipotency over time

Nature of these cells is poorly understood!

Milwid et al (2010)

Expected Results

Increase the knowledge on the molecular
mechanisms by which POH is caused,

allow to schedule a targeted therapy.

Future Prospectives

Based on new data, be able to allow in the future to implement our strategy directly on POH patients

Technics

·Tissue-specific expression (transgene expression, cAMP test, downstream effectors)

·RX analisis

·Histological analysis

·Histomorphometry

·Ex vivo differantiation assay (osteo/adipo)

·Osteoclastogenesis assay

·Molecular analysis

·.....

Material and Costs

- PCR: 1000 €
- Cloning kit: 700 €
- Lentiviral vector : 650 €
- Transgenic mouse 200 €/ month
- Mice floxed: 1000 €

  RT-qPCR: 250 €
- Western blot: 200 €
- Molecular analyse (antibodies, reagent etc) 2000 €
- Discartable material 1000 €
- cAMP kit commercial kit assay 325 €

We are supposing to spend around 16500€ to perform our project in 5 years

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Waiting for…next season!!!