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A new approach for HGPS treatment

Advanced lentiviral vector powered by small RNAs

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Gene therapy
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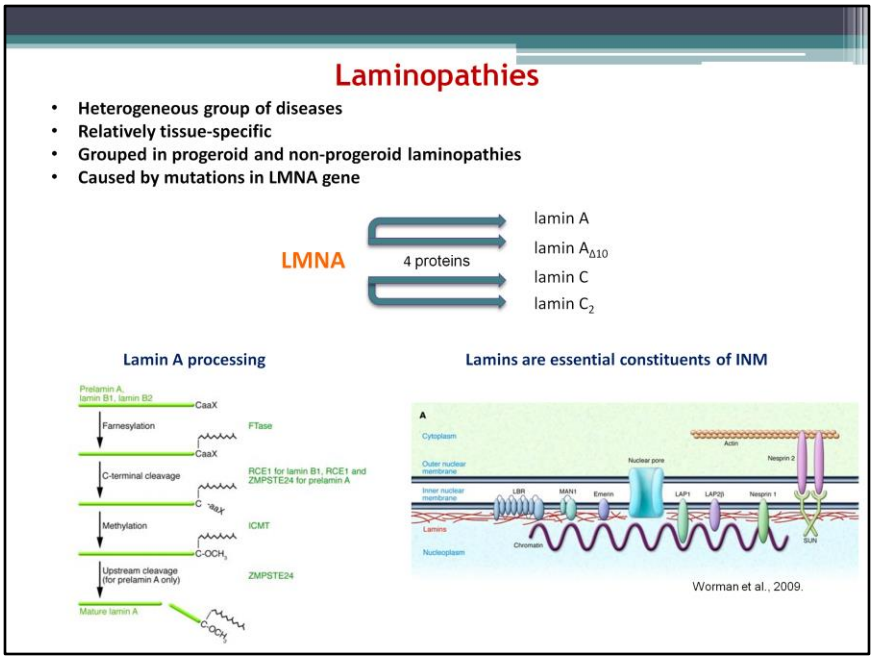
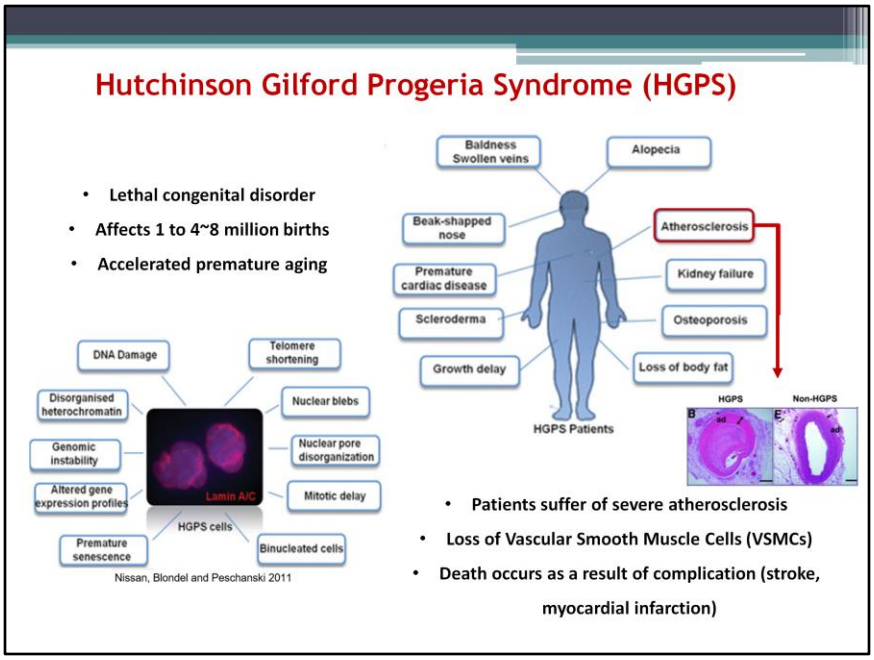


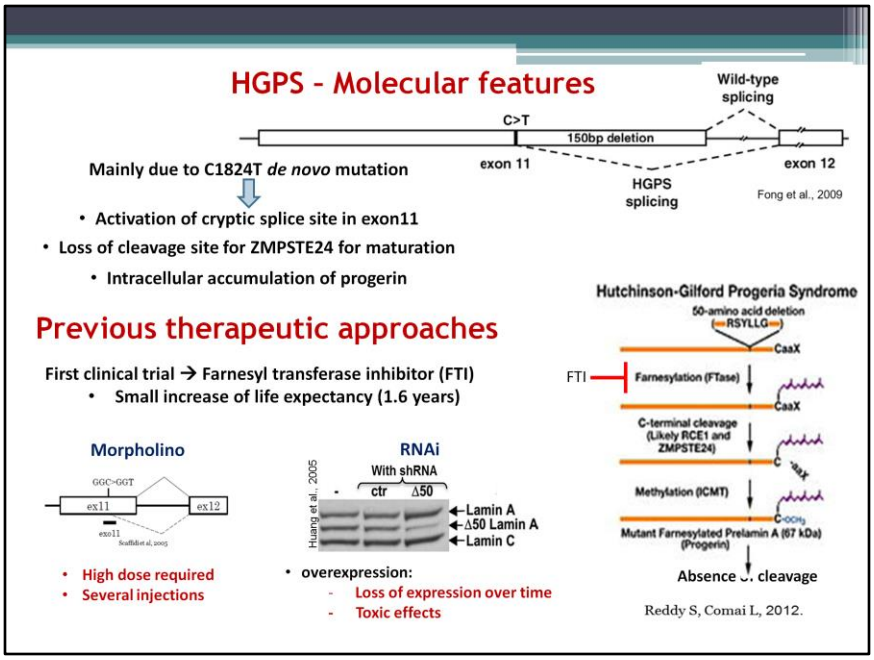
Laminopathies are a very heterogeneous group of rare diseases; each one has a specific time of onset (typically from neonatal to post-puberty age) and specific symptoms. They can be divided referring to their tissue-specificity (- striated muscles; - peripheral nerves; - adipose tissues or causing multiple tissue degeneration) or grouped into progeroid, causing early progressive aging in affected people, and non-progeroid laminopathies. All these diseases are caused by mutations in two genes, ZMPSTE24 and LMNA. LMNA is localized in chromosome 1 (1q21.2-q21.3) and it has 12 exons. LMNA, together with LMNB gene, codes for a group of proteins called lamins, that originate from alternative splicing. In particular, lamin A is the product of a multi-step processing mechanism that occurs on prelamin A, the proteic precursor derived from translation, after the splicing. Prelamin A undergoes farnesylation on a conserved CAAX sequence (C-tail) recognized by a Farnesyl Transferase (FT), then it's cutted by ZMPSTE24 (-AAX is removed), methylated and at last proteolytically cleaved for a second time, producing mature lamin A, shorter than prelamin A and not farnesylated anymore. Lamins are intermediate filaments that can polymerize to form the nuclear lamina, located between the inner nuclear membrane and the chromatin. Nuclear lamina is very important to mantain nuclear size and shape.
The specific laminopathy on which our work focalizes is the premature aging disease Hutchinson-Gilford progeria syndrome (HGPS).



Hutchinson-Gilford Progeria Syndrome is a lethal congenital disorder and it affects 1 to 4~8 million live births.

HGPS encompasses a spectrum of clinical features that typically develop in childhood and resemble some features of accelerated aging. Children with progeria usually appear normal at birth, but profound failure to thrive occurs during the first year. Characteristic facies, with receding mandible, narrow nasal bridge and pointed nasal tip develop. During the first to third year the following usually become apparent: partial alopecia progressing to total alopecia, loss of subcutaneous fat, progressive joint contractures, bone changes, nail dystrophy, and abnormal tightness and/or small soft outpouchings of the skin over the abdomen and upper thighs, and delayed primary tooth eruption. Later findings include low-frequency conductive hearing loss, dental crowding, and partial lack of secondary tooth eruption. Motor and mental development is normal. As you can see, in this histopathological picture is showed a clear fibrotic plaques formation in blood vessels, the HGPS patients suffer from severe atherosclerosis and death occurs as a result of complication of that like cardiac disease (myocardial infarction) or cerebrovascular disease (stroke), generally between ages six and 20 years. This is caused by a loss of vascular smooth muscle cells (VSMCs) and chronic fibrotic process. Average life span is approximately 13 years. (Gordon and Collins, 2015)

In cells there is an alteration of the nuclear lamin scaffold, which causes alteration of nuclear morphology. This alteration also reduces the resistance to mechanical stress. This explanation makes sense since many of the primarily tissues affected, such as skin and vasculature, which are under intense mechanical stress. Moreover altering chromatin organization can cause misregulation of sets of genes that may explain premature aging in HGPS patients, and it leads also to defective recruitment of DNA repair factors. As a result the cells accumulate damage in DNA causing genomic instability.



HGPS is present in heterozygous and in more than 80-90% of the cases it is caused by a *de novo* autosomal dominant mutation in codon 608 of the *LMNA* gene. In particular the mutation is a C-T transition at position 1824 in exon 11 that activates an efficient exonic cryptic donor splice site. This leads to the production of a truncated lamin A protein with an internal deletion of 50 amino acids (150 bp) in the C-ter domain. As result of this deletion the pre-lamin A contains the farnesylated C-ter, but it doesn't exhibit a proteolytic cleavage site of the enzyme ZMPSTE24 / FACE1 to produce the mature protein, causing intracellular accumulation of progerin (pre-lamin A with farnesylated C-ter).

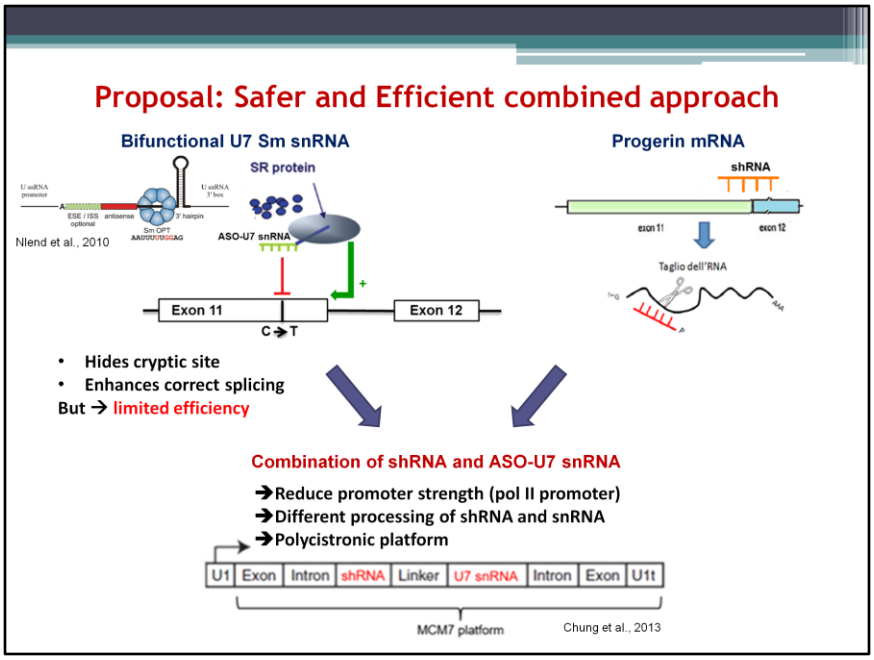
The dominant negative nature of the HGPS-causing mutant lamin A protein means that reversal of the cellular phenotype in HGPS cells requires the elimination of the mutant protein.

Farnesyl transferase inhibitors has been used in a clinical trial in in which it has shown amelioration of patient health but a small increase of their life expectancy of 1,6 years. This drug prevent the farnesylation of prelamin A.

However different strategies have been hypothesized and then tested.

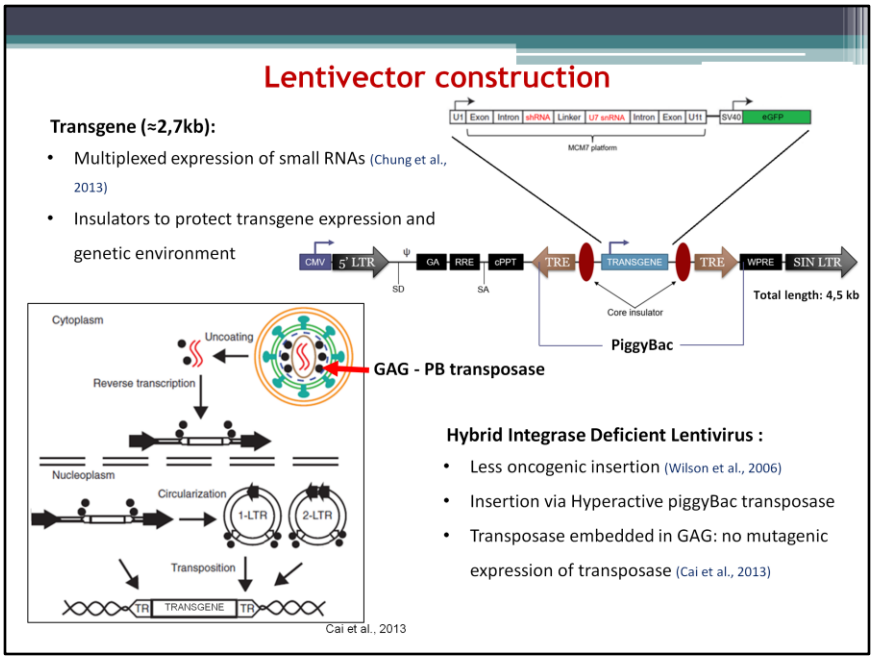
The first strategy involves the use of an oligonucleotide 25-mer morpholino (exo11) complementary to the region containing the HGPS mutation in exon 11, to block the cryptic splice site and to prevent aberrant splicing. However, the disadvantages of this

strategy are several concentration of morpholino (15-40 mM) and several times of injection.
The second strategy involves the use of shRNA to decrease the levels of progerin expression. The shRNA binds specifically transcripts that have a deletion in exon 11, but this is toxic if it expressed at high doses and the expression decreases over time.
Another strategy involves the use of ASO 365 (Anti-Sense Oligonucleotides) that contained a 2'-O-methoxy-ethyl ribose backbone. The use of ASO 365 has decreased the levels of progerin in cells, as it is located upstream of the cryptic splice site. This strategy involves the use of doses with low concentrations than morpholino (2.5 - 100 nM), but levels of progerin are reduced only about 30% and they are quickly degraded by ribonucleases.

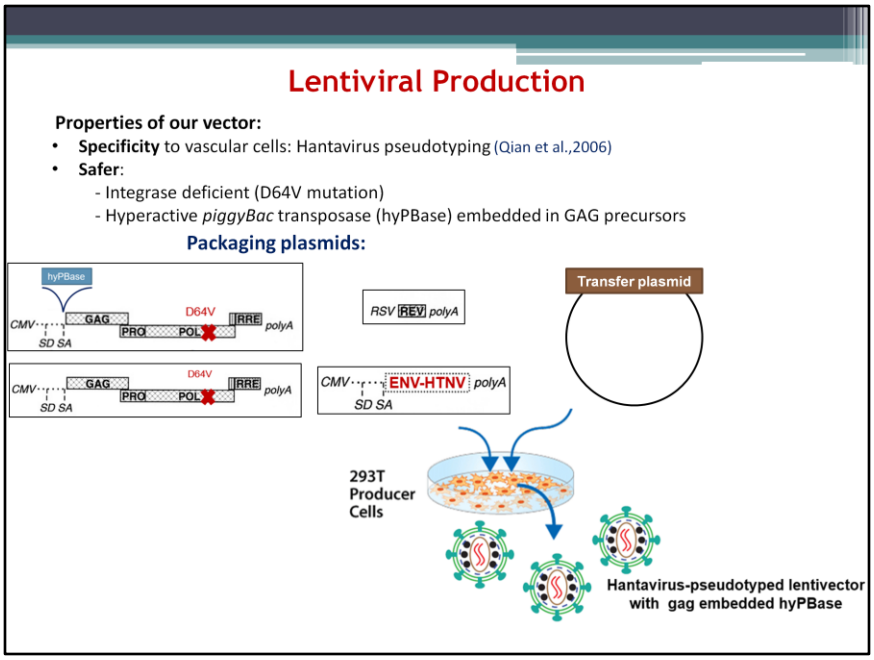


A new type of gene therapy directed toward correction of aberrant splicing through targeting the pre-mRNA has become the most promising form of therapy in many mis-splicing diseases, including those identified above. In this project, we decided to use an bifunctional antisense-U7 snRNA. It is a construct U7 Sm OPT associated to a sequence complementary to the ASO cryptic splice site. Since U7 is not normally involved in the splicing process, it has been modified with a set of three point mutations in the non-canonical Sm binding site, which make it similar to the consensus Sm binding site of spliceosomal snRNA. In this way ASO-U7 snRNA is localized inside the nucleus and it may intervene in the splicing process. The ASO-U7 snRNA thus contains the sequence complementary to the cryptic splice site of exon 11 and ESE sequences that bind proteins SR. This mechanism enables the SR to bind to sequences in the pre-mRNA and recruit at the same time component of the spliceosome improving the definition of exon. But high expression saturates the snRNA processing ability of the cell and it has a limited efficiency. Similarly it is used with similar results also an antisense-U1 snRNA.
Simultaneously with ASO-U7 snRNA we used the technology of RNAi to suppress the expression of pre-mRNA of the mutated lamin A. So we used an shRNA with a complementary sequence to exon 11 mutated mRNA to reduce the levels of expression of progerin.

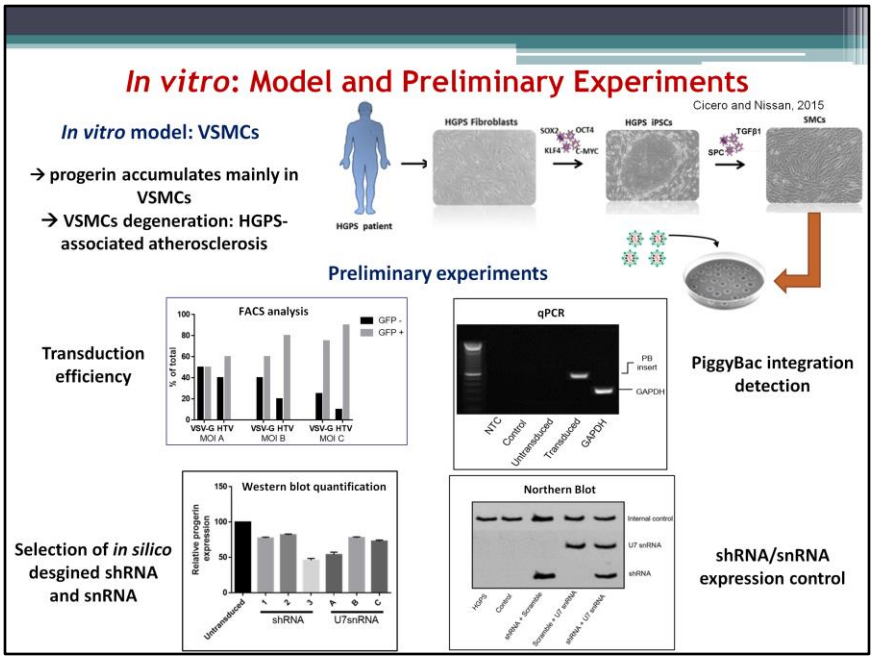
To prevent toxic effects caused by the expression to high doses of shRNA and U7 snRNA we used a less strong promoter: pol II promoter that could reduce toxicity. To enable a more permanent therapy and extend transgene expression over time, we have include ASO-U7 snRNA and shRNA expression cassette in an RNA designed for transcription in target cells: MCM7 platform. (Chung et al., 2013)



We inserted our multiplexed expression platform in a piggy bac transposon. Two insulators are at each side of the transgene to avoid epigenetic silencing of our construction and also to prevent alteration of the genetic environment due to the insert that could activate ectopic expression of downstream gene. The piggy bac is a transposon that has been modified to carry genes in a safer manner than lentiviral vector. Indeed, it's integration is not random and has been shown to have less mutagenic insertions. Using piggybac transposon, we have to use an integrase deficient lentivirus that has a mutated pol (only able to do reverse transcription). The important point is to carry the hyperactive piggybac transposase without having an overexpression that could lead to increase of mutagenesis rate. We will use an embedded GAG-piggybac hyperactive transposase that allows co delivery of the transposase and our construction by the same lentiviral vector in the cell. Then, in the cell, the transposase is released by cleavage and can mediate the transposition into the host genome.

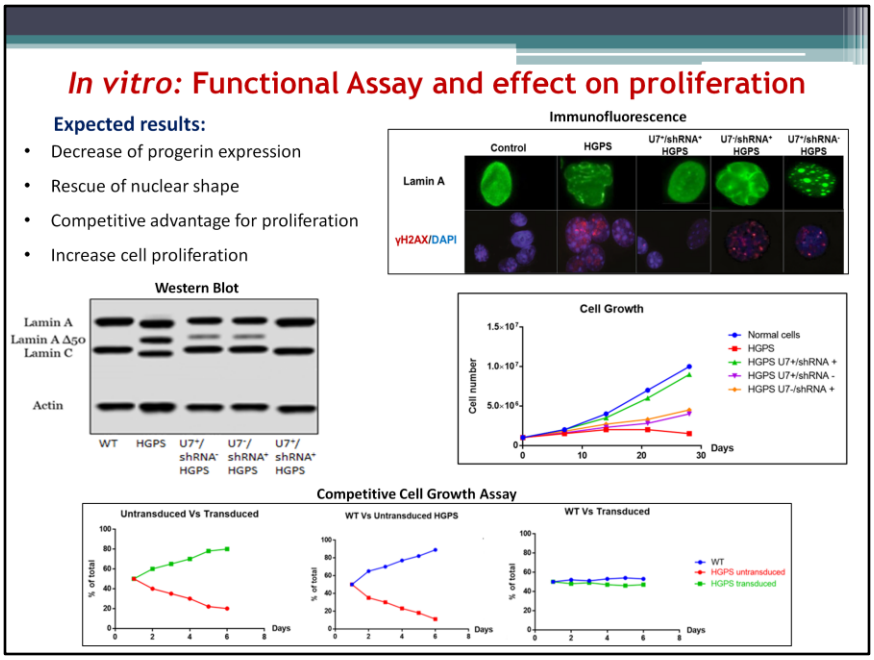


In our gene therapy approach, we want to improve the cardiovascular symptoms of HGPS patient. The VSV-G glycoprotein is not so efficient to target the vascular cells so we decided to use an Hantavirus pseudotyped lentivector that has been shown to target efficiently vascular cells *in vitro* and *in vivo*. (Qian et al.,2006)
So we use the plasmid ENV-HTV during viral production to pseudotyped our lentivector. To have a embedded GAG-Hyperactive piggy bac transposase and also a GAG that is fully functional we use one classic plasmid containing the GAG and the POL mutated sequence; we have also another plasmid in which there is an insert of the hyperactive piggybac transposase gene. Finally we transfect all the packaging plasmids described previously and a plasmid coding for REV and a plasmid containing our transfer vector into HEK 293T cells for the viral production.



For the *in vitro* experiments we decided to test our strategy on a single specific cell type. We chose Vascular Smooth Muscle Cells (VSMCs), essential to provide contractile function and structural support to blood vessels (Sinha et al., 2014). VSMCs in HGPS accumulate progerin more than the other cell types and their consequent degeneration, caused by the effects of progerin high levels, is typical of HGPS-associated atherosclerosis, one of the main causes of death in HGPS affected people (Liu et al., 2011). We delveloed differentiated VSMCs from iPSCs, whose origin comes from fibroblasts of HGPS patients (Cicero and Nissan, 2015).
Our aim is to make experiments to confirm that our lentivector reaches its purpose. After infection of VSMCs, we make a FACS (Fluorescent-activated Cell Sorting) with a flow cytometer to see how many cells have been efficiently transduced with the vector. We can visualize it thanks to the presence of GFP reporter gene in the viral vector and we compare the efficiency of transduction in VSMCs with our HTNV (Hantavirus)-pseudotyped LV and with a canonical VSV-G (Vesicular Stomatitis Virus – G protein)-pseudotyped LV: we expect to see (in different groups of analyzed cells) that HTNV-LV always give better results in terms of transduction efficiency than VSV-G-LV.
Then we can select the best shRNA and U7Sm-snRNA constructs we generated: through a western blot we detect relative progerin expression levels in differentially

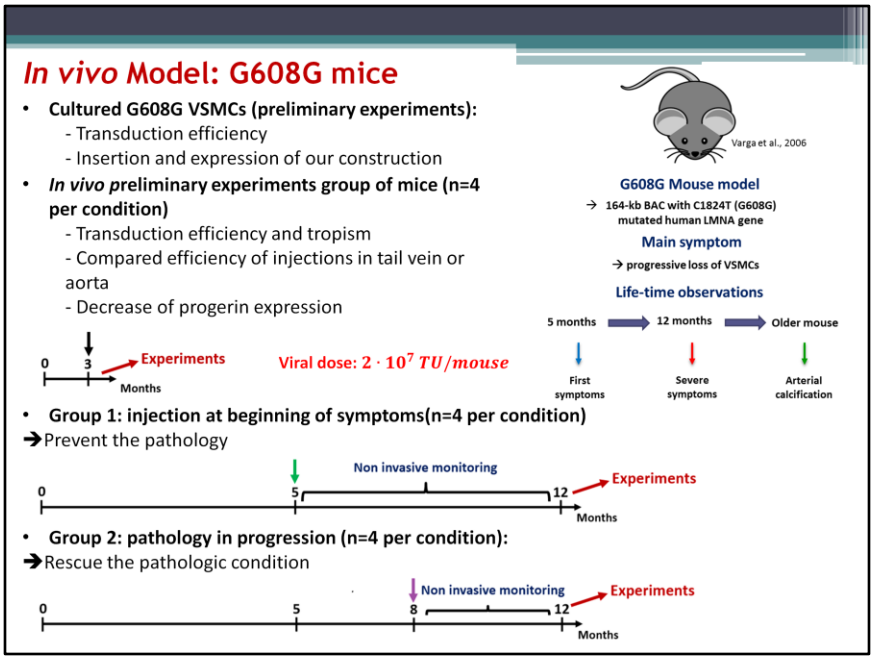
treated cell samples and we'll choose constructs that bring to lower progerin levels in cells.
Next we can do a PCR in order to see if our PiggyBac insert has been integrated in transduced cells (we use constitutively expressed GAPDH as a control). We also make a northern blot to detect the expression of our specific shRNA and U7Sm-snRNA in all transduction cases.



With the aim of examining the efficiency of our vector, we decided to test the expression of progerin, here it indicated as a Lamin A D50, through western blot. The results show clearly how in the VSMCs treated with the vector only with shRNA or only with U7, are ineffective, as compared to cells treated with either U7 that with shRNA which shows how the levels of lamin A are restored to physiological conditions, and there isn't expression of progerin.

We also evaluated nuclear shape rescue in cells treated through immunofluorescence microscopy. We expect a rescue nearly full of the nuclear form in cells transduced with U7 / shRNA.

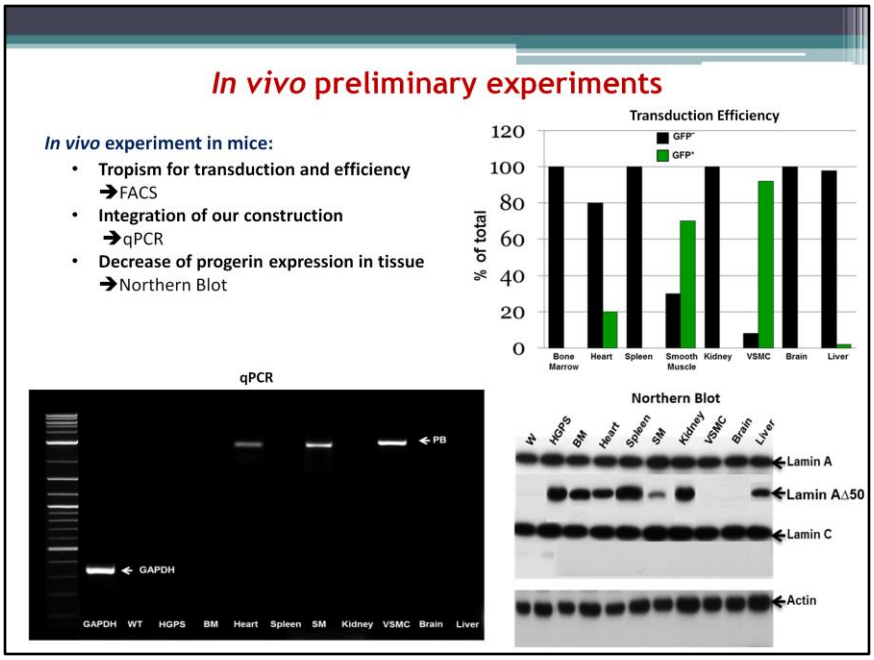
We then compared the proliferation ability between HGPS and HGPS treated cells so we could verify the correct restoring in HGPS treated. To establish an easy and sensitive assay to monitor changes in proliferation of transduced cells, we developed the CCG assay. This assay is based on the assumption that direct competition between transduced and untransduced cells will be the most sensitive and controlled way to compare growth differences. We can see how treated cells compete with WT cells more efficiently than no-treated HGPS. The results are presented here as percentage values in function of time.



For the *in vivo* experiments we decided to use a G608G mouse model developed by Varga and his collegues. It's a transgenic mice line that carries C1824T mutated human LMNA gene in a bacterial artificial chromosome generated for this aim. G608G mice don't show the external phenotype of human progeria but they acquire progressive vascular abnormalities in a similar manner of human HGPS. In particular, a G608G mouse is progressively affected by loss of Vascular Smooth Muscle Cells (VSMCs), elastic fiber breakage, thickening of the adventitia and medial layer, accumulation of proteoglycans (PGs) and collagen deposition. These symptoms are first observed when mice are 5-months-old. From 12-months-old mice show more severe symptoms, while when they are older arterial calcification can be also observed in addiction to severe VSMCs loss and extracellular matrix deposition. This loss is particularly severe in the ascending aorta. (Varga et al., 2006)

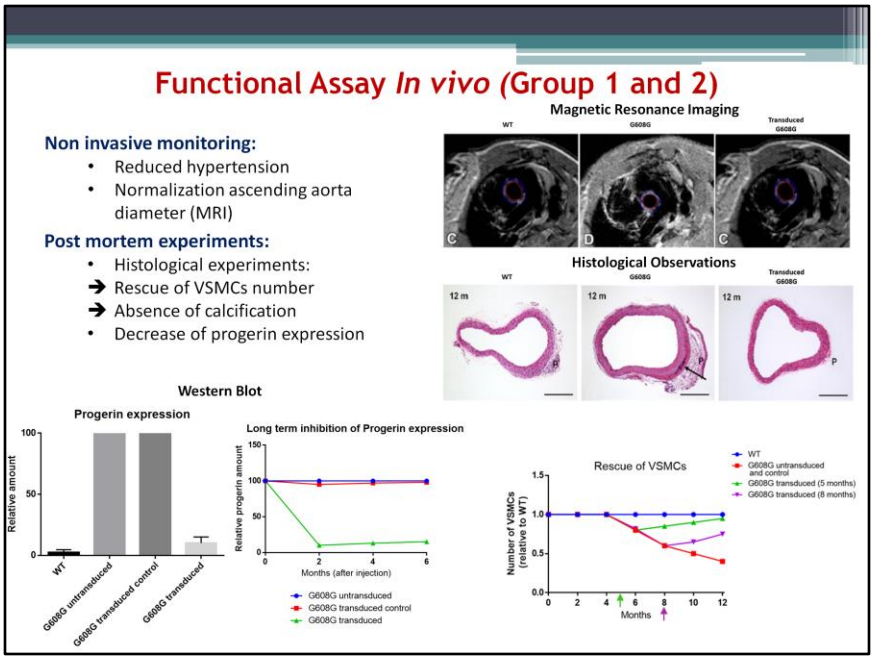
Before starting experiments in mice, we are going to use G608G vascular cells to perform preliminary experiments in vitro, like we have done in vitro. These experiments will validate our vector in the model so we can prove that we have a good transduction efficiency and that our construction is correctly inserted in the genome.
The next step is to start the *in vivo* experiments. For the groups 1 and 2 that are

described here, an injection with our lentivector (viral dose $2*10^7$ TU/mouse) and a control injection with solvent only is performed. For the *in vivo* experiments, we chose to use a lentivector without GFP in order to prevent toxicity.
We start in a small group of adult mice to control some aspects of our vector. Indeed, we want to control that our transgene target efficiently the cardiovascular cells *in vivo* and which injection could be the most efficient to also reach most of the cell of the ascending aorta. We also want to check if our construction can efficiently decrease the progerin expression in the vascular cells.
After this control, we will start our experiments in two groups: one in which we inject our construction at the beginning of the symptom (5 months) with the aim to see if we can prevent the loss of vascular smooth muscle cells. In the second group, we inject at 8 months when the loss of VSMC is already important. The experiments described later are performed at 12 months that is, for G608G mice, a timepoint when the vasculopathy is severe. Between the infection and the sacrifice of mice, non invasive monitorings (MRI, angioscopy, and blood pressure measurement) will be performed to check lumen diameter, areas of severe narrowing, stenosis and severity of atherosclerotic plaques.



After being checked for transduction efficiency, we switched to *in vivo* *e*xperimentation. *In vivo* since the GFP could be toxic, even in view of a future application in humans, we generated inside of 293T cells both vectors that do not contain both GFP vectors with GFP to verify the tropism of the virus *in vivo*.
There will be two ways to perform injections: one way is to do systemic injection in the tail vein; the other is to inject locally in the aorta to rescue the loss of VSMC that is particularly severe in this part.
Through FACS analysis, so we checked out the tropism and transduction efficiency in different organs and tissues of mice. The analysis showed that the lentivirus-HTV shows a tropism toward cardiac striated muscle, smooth muscle and VSMC. This is because the hantavirus is able to transfect so bland also striated muscle cells and smooth muscle in general. The efficiency of transduction is higher, however, it is obtained in VSMCs.
We ran a qPCR on different tissues collected to verify the integration of the construct. We used as a control gene GAPDH, and mice and wild-type transfected HGPS. Integration as expected there was a more accentuated in VSMCs, in percent lower in smooth muscle, and so bland in cardiac cells.
Finally we verified the decrease in the expression of lamin A, C and foil laminaA Δ50 (progerin) in different tissues, using Western Blot. We used as control actin protein.

Within the VSMCs in the short term there was a decrease in the levels of production of progerin by about 100%. A decrease of about 60% was obtained in the smooth muscle, and has been verified also a 30% decrease in heart tissue.



Then we will perform the experiments on the groups 1 and 2. We will inject in function of our prior findings. Then the group 1 will be injected with lentivirus or control at 5 months and the sacrifice will be done at 12 months. In the group 2, the injection will be done at 8 months and the post mortem experiments at 12 months. During the time between injection and the sacrifice, we will look at some parameters with non invasive monitoring: MRI and check of the tension. After the sacrifice, we will look at different histological aspect of our mice such as the recovery of the number of vascular smooth muscle cells after injection, the absence of calcification and the normal thickness of the adventitia. We will also check the decrease of the progerin expression at the end of the 12 months. We expect to reverse the vasculopathy as well as decrease the progerin expression in mice infected with our lentivector. We will also use a group of mice that will be sacrified at different time point to ass the long term inhibition of the progerin expression and follow its decrease after the injection with our lentivector.

Pitfalls and Solutions

- **PiggyBacinsertion may take place in proto-oncogen sites:**
  - Insert of a suicide gene as a safety in case of oncogenic process.
- **Toxic effect of GFP in vitro:**
  - Use a less toxic reporter or no reporter at all, we coud use FISH to observe transduced cells.
- **Decrease of progerin is very low with U7:**
  - To avoid this, we can change the tail position with the aim of ensure correct splicing and an efficient inhibition of aberrant splicing.

In summary

Our strategy is based on:

- High tissue-specific transduction efficiency
- High integration efficiency and safety
- Long term inhibition of Progerin expression
- Great rescue of Lamin A production
- Prevention and rescue of vasculopathy

Materials and costs

• Lentiviral production	€1900
• PiggyBac	€850
• iPSc	€2950
• RT-PCR and PCR kits	€550
• WB kit	€2100
• Cell cultures	€1350
• Imm.fluorescence kit	€280
• G608G mice (4 females and 2 males)	€1750
• Animal facilities	€30000
• Imaging techniques	€650
• Instruments used in lab (vials, eppendorf, tubes, buffers, growth medium, chemical agents)	€2500
• Salaries of 1 researcher	€30000/pp/yy

≈€220.000 / 3 years

Research Funding

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