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## Transposon-Based, Targeted Ex Vivo Gene Therapy to Treat Age-Related Macular Degeneration (TargetAMD)

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endogenous TCR genes by transient exposure to  $\alpha$  and  $\beta$  chain-specific ZFNs, followed by the introduction of tumor-specific TCR genes by lentiviral vectors. The original approach comprises four sequential steps of genetic manipulation and selection of engineered T-cells, and required approximately 40 days of culture. Within the SUPERSIST project and in view of a prompt clinical translation, we have recently simplified the editing protocol to retain the benefits of the original TCR gene-editing strategy while increasing its feasibility. This optimized gene-editing protocol can now be combined to T-cell activation and culture procedures that allow generating gene-edited T-cells with an early memory T-cell phenotype, which is associated to long-term persistence of the cells in several mouse models. These advances are instrumental to pave the way toward the development of a feasible and cost-efficient process supporting clinical testing of this new technology.

In another fundamental advance made within the project, NKI has developed highly innovative TCR capture techniques that allow identifying novel tumor-reactive TCRs from primary clinical specimens. These studies have provided major new insights in the antigenic space targeted by the immune system in human tumors. These findings were reported in a top scientific journal and granted great attention by the scientific community, as they have substantial implications for understanding the process of cancer-directed immune response as well as improving the design and application of gene-based immunotherapy. Within the SUPERSIST summary, we will now exploit these technologies to widen the reach of TCR gene editing toward a broader range of neoplastic diseases.

With the support of the SME partner MOLMED, the consortium will continue to perform the scale-up and optimization of gene-targeting protocols for both HSC and T-cells for the two gene therapy applications being developed. These processes will enable clinical testing of the new strategies. We are also designing an exploitation roadmap for SUPERSIST results, taking advantage of partnerships already established by consortium partners with leading biotech companies in the field, such as Sangamo BioSciences, for the development of ZFNs, and other big pharma.

### Expected Outcome

HSC gene therapy and cancer adoptive immunotherapy are facing an accelerated phase of development, largely because of the first therapeutic successes obtained with genetically modified cells in clinical trials of inherited diseases and lymphoid malignancies. We can envisage that adoptive T-cell therapy will soon become a major pillar in cancer treatment. The expected outcome of SUPERSIST will be the next generation of HSC- and T-cell-based gene therapy, in which conventional gene addition is replaced by the more precise and powerful approach of targeted gene editing, achieved through a combination of state-of-the-art gene transfer vectors and artificial nucleases. While two diseases have been selected for investigation within the project for their paradigmatic features, the strategies being developed are expected to be potentially applicable to several additional human diseases. Thus, the outcome of SUPERSIST should benefit the European population by providing a long-

sought perspective of cure for a number of severe diseases, leading to improvement in population health and reduction in healthcare costs, while at the same time increasing the competitiveness of European biomedical and pharmaceutical industry through the generation of new scientific knowledge, processes, and technologies.

### Major Publications

- Becattini S, Latorre D, Mele F, et al. Functional heterogeneity of human memory T cell clones primed by pathogens or vaccines. *Science* 2015;347:400–406.
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- Lombardo A, Naldini L. Genome editing: a tool for research and therapy: targeted genome editing hits the clinic. *Nat Med* 2014;20:1101–1103.

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## Transposon-Based, Targeted *Ex Vivo* Gene Therapy to Treat Age-Related Macular Degeneration (TargetAMD)

Contract No.: 305134; EC contribution: € 5,976,298;  
Total costs: € 7,769,833.46; Starting date: 01/11/2012;  
Duration: 48 months

### Background and Objectives

**A**GE-RELATED MACULAR DEGENERATION (AMD) is a chronic progressive disease that appears to result from age-associated alterations that include impaired phagocytosis by retinal pigment epithelial (RPE) cells; alteration in Bruch's membrane, RPE cell, and photoreceptor degeneration; and eventually neural retinal ganglion degradation.

AMD presents two distinct forms, a slowly progressing nonvascular atrophic form (dry or avascular AMD) and a rapidly progressing blinding form (neovascular AMD), in which choroidal vessels grow through Bruch's membrane into the subretinal space.

No treatment is available for avascular AMD. However, VEGF inhibitors have been approved and used to treat neovascular AMD, since VEGF is the major promoter of blood vessel growth. Treatment with VEGF inhibitors is effective in about 30% of patients who regain 3 or more lines of visual acuity. However, the effect of VEGF inhibitors is limited in time, thus necessitating repetitive, often monthly injections to maintain the therapeutic effect.

Since *in vivo* subretinal space avascularity depends on the anti-angiogenic activity of PEDF, the natural antagonist of VEGF, administration of PEDF to the subretinal space should inhibit choroidal neovascularization (CNV) in neovascular AMD. However, its short half-life of a few hours limits its therapeutic use. Since AMD is accompanied by RPE cell degeneration, an ideal treatment would be the replacement of the degenerated RPE cells with cells that secrete PEDF constitutively. RPE and iris pigment epithelial (IPE) cells, as a substitute for RPE cells, have been transplanted to the subretinal space.<sup>1,2</sup> However, no significant improvements were observed, suggesting that the endogenously expressed PEDF secreted by the transplanted cells is not sufficient to control CNV.

### Approach and Methodology

The overall objectives of TargetAMD are to genetically modify RPE and IPE cells to produce elevated levels of PEDF.<sup>3,4</sup> The genetically modified cells will be transplanted to the subretinal space of neovascular AMD patients to inhibit CNV by augmented PEDF secretion. Specifically, RPE and IPE cells will be transfected with the *PEDF* gene using the hyperactive *Sleeping Beauty* (*SB100X*) transposon system.<sup>5</sup> Transposons are discrete sequences of DNA that can move from one location to another in the genome via a "cut and paste" mechanism. Similar to retroviral vectors, the *SB100X* system will deliver and integrate genetic material into a target cell genome, resulting in robust and stable expression of the transfected gene. The *SB100X*-mediated gene delivery shows efficient transgene delivery, integration into the cell genome, lower immunogenicity, ability to incorporate large inserts, reduced genomic instability of the host genome, and safety of transgene integration, since *Sleeping Beauty* integration is random at the genomic level<sup>6</sup> and does not integrate into transcription start sites.

TargetAMD aims at establishing transposon-based gene therapy for the treatment of neovascular AMD. Specifically, our aims are (1) development of protocols and devices for efficient transfection by electric pulses delivery of the low number of cells freshly isolated from an iris biopsy and a peripheral retina biopsy; (2) testing the use of *SB100X* transposase as mRNA,<sup>7</sup> *PEDF* transposon vectors with insulator sequences,<sup>8</sup> and plasmids free of antibiotic resistance markers (pFAR4)<sup>9</sup>; (3) transplantation of rat RPE and IPE cells, transfected with the *PEDF* transgene, subretinally in a rat model of CNV; (4) safety analyses including distribution of the transplanted cells in rabbits and distribution of transposon integration sites in the genome; (5) production of GMP-grade

plasmids for clinical use; (6) preparation of dossier of pre-clinical studies and application to appropriate agencies to obtain approval for clinical trials; (7) development of protocols and performing a phase Ib/IIa clinical trial in which autologous, freshly isolated IPE cells will be transfected with the *PEDF* gene and transplanted subretinally in 10 neovascular AMD patients during a single surgical session lasting approximately 60 min; (8) performing a phase Ib/IIa clinical trial in which autologous, freshly isolated *PEDF*-transfected RPE cells will be transplanted subretinally in 10 neovascular AMD patients.

### Main Findings

#### Optimization of biosafety profile

1. The use of *Sleeping Beauty* transposase mRNA was repeatedly tested in ARPE-19 and primary human RPE cells from distinct donor eyes and compared with the use of *SB100X* transposase DNA. Both strategies were successful and effective with transfection efficiency of around 100% in ARPE-19 cells. However, *SB100X* transposase DNA exhibited better reproducibility; *SB100X* transposase mRNA showed higher intraindividual variations.
2. *PEDF* transposon vectors with incorporated insulator sequences showed protection from transactivation; however, in primary human RPE cells transfected with the insulator-carrying transposon plasmids, expression of the *PEDF* transgene was clearly decreased.
3. pFAR4 transposon derivatives showed increased transfection efficiencies compared with the respective pT2 transposon vectors.<sup>10-12</sup> In transfected primary rat IPE and RPE,<sup>13</sup> bovine IPE, and human RPE cells, secretion of PEDF was clearly evident and persisted at constant levels without transgene silencing. Based on these findings, the decision was taken to use both the transposase expression plasmid and the *PEDF* encoding transposon plasmid in the pFAR4 back bone for the clinical trials.

#### Transfection of low numbers of primary human RPE cells

Since the final objective of TargetAMD is the isolation of IPE or RPE cells from a patient followed by transfection and subretinal transplantation of the transfected cells during a 60 min surgical session, it is necessary that transfection is accomplished in only the few cells ( $5 \times 10^3$  to  $1 \times 10^4$ ) that can be isolated from a biopsy. Using the final *SB100X* and *PEDF* constructs at a specific ratio, validated for best transfection efficiency, protocols were developed for the transfection of  $5 \times 10^3$  and  $1 \times 10^4$  cells. For these experiments, cultured primary human RPE cells (from 20 different donors) were used. In these experiments, PEDF secretion was increased at least 20-fold, consistent with the increased *PEDF* gene expression of at least 65-fold as well as with a low relative number of copies of the *PEDF* gene integrated for  $1 \times 10^4$  and for  $5 \times 10^3$  cells. Transfection was mediated by electroporation using the Cliniporator device, which has been modified for TargetAMD purposes.<sup>14</sup>

#### Transfection of low numbers of freshly isolated bovine IPE cells

Around  $10^5$ – $10^4$  cells could be isolated from small biopsies of iris. These cells were immediately transfected with the

*SB100X* transposase and the *PEDF* transposon plasmids. Efficient transfection was verified for the small biopsy-derived cells by image-based cytometry. Using ELISA it was determined that the transfected cells secreted up to 25-fold more PEDF than nontransfected cells. Western blot analyses showed that the level of recombinant PEDF secreted remained stable for the 191 days that the cells have been followed by now.<sup>15</sup>

### Expected Outcome

Based on the results to date, the final objective of the project, namely, “the successful completion of two phase Ib/IIa clinical trials for the treatment of AMD using transposon-based gene therapy technology” will become a reality. The major challenge to the completion of the project was the ability to successfully and efficiently transfect the very few cells ( $5 \times 10^3$  to  $1 \times 10^4$ ) that can be obtained from an iris or peripheral retina biopsy. TargetAMD has achieved this objective, since low numbers of IPE cells freshly isolated from iris biopsies have been successfully transfected using the *Sleeping Beauty* transposon system in conjunction with free of antibiotic resistance marker (pFAR4) miniplasmids. We have consulted with Swissmedic as to the requirements for conducting the clinical trials and are preparing the appropriate protocols and instituting the necessary procedures for the clinical trials.

### Major Publications

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- Johnen S, Marie C, Izsvák Z, et al. Efficient *Sleeping Beauty*-mediated transposition of primary pigment epithelial cells with PEDF delivered by plasmids free of antibiotic resistance markers (pFARs). ESGCT 2013;A139:P281.
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- Johnen S, Marie C, Izsvák Z, et al. Efficient *Sleeping Beauty*-mediated transposition of primary pigment epithelial cells with PEDF delivered by plasmids free of antibiotic resistance markers (pFARs). ESGCT 2013;A139:P281.
- Pastor M, Johnen S, Quiviger M, et al. The merging of the antibiotic-free pFAR4 miniplasmids with the *Sleeping Beauty* transposon system mediates higher transgenedelivery in human cells. ESGCT 2013;A61:P008.
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## Thalassemia Modular Stratification System for Personalized Therapy of Beta-Thalassemia (THALAMOSS)

Contract No.: 306201; EC contribution: 5,020,000;  
 Total costs: 6,681,623.27; Starting date: 01/11/2012;  
 Duration: 48 months

### Background and Objectives

**T**HE  $\beta$ -THALASSEMIA SYNDROMES are a group of severe and rare anemias with monogenic inheritance, a complex systemic phenotype, and several treatment-related complications, caused by more than 300 mutations of the  $\beta$ -globin gene. Novel therapeutic protocols, most of which are based on still experimental treatments, show great promise but significant variability of success between patients. These strategies include chemical/molecular induction of the endogenous  $\beta$ -like  $\gamma$ -globin gene, restoration of clinically relevant  $\beta$ -globin levels by gene therapy, or genetic correction of the abnormal  $\beta$ -globin gene. In addition, a small number of modifiers with significant impact on disease penetrance, severity, and efficacy of treatments are known, but most remain elusive.

Improvements of existing treatment regimens and optimization and application of novel treatments will critically depend on the characterization of additional disease modifiers and the stratification of patients for customized clinical management. This requires extensive analyses based on “OMICS,” an English-language neologism that refers to different but connected fields in molecular biology and biochemistry, such as genomics, transcriptomics, exomics, proteomics, and metabolomics. The major objective of OMICS is a collective characterization of pools of biological molecules (gene sequences, transcripts, proteins, and protein domains) controlling biological structures, functions, and dynamics, including several involved in pathological conditions.

One of the most interesting observations of genomics in  $\beta$ -thalassemia is the association between genomic sequences and high fetal hemoglobin (HbF) levels, in consideration of the fact that high HbF levels are usually associated with milder forms of  $\beta$ -thalassemia. Related to this issue is the possibility to predict response to different therapeutic protocols on the basis of genomic analyses. For instance, three major loci (the Xmn1-HBG2 single-nucleotide polymorphism, the HBS1L-MYB intergenic region on chromosome 6q, and BCL11A) contribute to high HbF production. Pharmacogenomic analysis of the effects of hydroxyurea on HbF production in a collection of  $\beta$ -thalassemia and sickle cell disease patients allowed

the identification of genomic signatures associated with high HbF. Therefore, it can be hypothesized that genomic studies might predict the response of patients to treatments based on hydroxyurea, which is at present the most used HbF inducer in pharmacological therapy of  $\beta$ -thalassemia.

Transcriptomic/proteomic studies allowed the identification of the zinc finger transcription factor B-cell lymphoma/leukemia 11A (BCL11A) as the major repressor of HbF expression. The field of research on  $\gamma$ -globin gene repressors (including BCL11A) is of top interest, since several approaches can lead to pharmacologically mediated inhibition of the expression of  $\gamma$ -globin gene repressors, leading to  $\gamma$ -globin gene activation. Among these strategies, we underline direct targeting of the transcription factors by aptamers or decoy molecules, as well as inhibition of the mRNA coding  $\gamma$ -globin gene repressors with shRNAs, antisense molecules, peptide nucleic acids, and microRNAs.

### Approach and Methodology

THALAMOSS aims at developing a universal set of markers and techniques for stratification of  $\beta$ -thalassemia patients into treatment subgroups for (1) onset and frequency of blood transfusions, (2) choice of iron chelation, (3) induction of fetal hemoglobin, and (4) prospective efficacy of gene therapy. THALAMOSS is organized in the following work packages: WP1 (recruitment, patient characterization, and development of culture technologies for erythroid precursor cells); WP2 (OMICS analyses); WP3 (novel therapeutic approaches); WP4 (data management and analysis); WP5 (dissemination and exploitation); WP6 (regulatory and ethics issues); WP7 (program management).

### Main Findings

The major findings and novel achievements are related to the work packages WP1–WP4. As far as WP1, the first list of recruited  $\beta$ -thalassemia patients with characterized genotype/phenotype was delivered. This is crucial for future activities employing blood sampling, culturing of erythroid cells, and isolation of genomic DNA, RNA, and protein. The list of patient to be recruited includes 1140 entries. The analysis of the most frequent genotypes and phenotypes indicates that homozygous patients are 138  $\beta^{039}/\beta^{039}$ , 364  $\beta^{+IVSI-110}/\beta^{+IVSI-110}$ , and 36  $\beta^{+IVSI-6}/\beta^{+IVSI-6}$ . Double heterozygous patients are 55  $\beta^{039}/\beta^{+IVSI-110}$ , 146  $\beta^{+IVSI-6}/\beta^{+IVSI-110}$ , and 65  $\beta^{0IVSI-1}/\beta^{+IVSI-110}$ . The list also includes 29 homozygous sickle-cell anemia (HbS/HbS) patients.

The HbS genotype was associated in 24 cases with  $\beta^{039}$ , in 49 cases with  $\beta^{+IVSI-110}$ , in 9 cases with  $\beta^{+IVSI-6}$ , and in 12 cases with  $\beta^{0IVSI-1}$ . Within WP1 we validated three protocols for isolation and culturing of erythroid precursor cells, named protocol A, protocol B, and protocol C. These cell culture strategies will be used by the THALAMOSS network for *in vitro* production of erythroid cells. The steps common to all three methods are drawing of peripheral blood (20–40 ml), Ficoll separation of mononuclear cells, and washing. In protocol A, the mononuclear cells are cultured according to the two-stage procedure first published