

GENEGRAFT

(Contract N° HEALTH-F2-2011-261392)

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PROJECT CONTEXT

Recessive Dystrophic Epidermolysis Bullosa (RDEB) is one of the most severe inherited skin disorders affecting children and adults. RDEB is caused by loss-of-function recessive mutations in *COL7A1* encoding type VII collagen, the constituent of anchoring fibres which form essential structures for dermal-epidermal adherence (Varki et al., 2007). The patients suffer since birth from skin blistering and erosions, with severe local and systemic complications resulting in poor prognosis.

Current medical care protocols for RDEB patients are limited to palliative procedures to treat blistering and erosive lesions, wounds, and severe local and systemic complications such as fusion and contracture of the digits, esophageal stricture, severe anemia, infections, malnutrition, growth retardation and aggressive skin cancers which develop at young age and are the most frequent cause of death of RDEB patients. Current medical treatments cannot prevent the recurrence of the lesions arising from defective expression of type VII collagen. This creates a tremendous economic and psychological burden for RDEB patients whose hospital cost cares are estimated to be 900 euros per day, excluding costs of consumables for the treatment of skin lesions.

PROJECT OBJECTIVES

GENEGRAFT intends to develop a safe and efficient *ex vivo* gene therapy approach for a permanent treatment of Recessive Dystrophic Epidermolysis Bullosa (RDEB).

GENEGRAFT consists in achieving a first Phase I/II clinical trial in 3 selected RDEB patients. The approach uses autologous skin equivalents genetically corrected with a safe (SIN) retroviral vector expressing type VII collagen. The project relies on the use of a new investigational medicinal product **“Skin equivalent graft genetically corrected with a *COL7A1*-encoding SIN retroviral vector”** (Orphan drug designation (EMA/OD/099/08)).

This pilot clinical trial aims to demonstrate first the feasibility and safety of the procedure and to a lesser extent the efficacy of the treatment.

GENEGRAFT comprises 9 European teams and proposes a concerted, multidisciplinary plan of action **to develop a safe and efficient *ex vivo* gene therapy approach for a permanent treatment of RDEB**. To achieve its goal, **GENEGRAFT** will:

- ✓ Identify 3 to 6 RDEB patients with optimal clinical and biological features for a first clinical trial with satisfactory keratinocyte proliferative capacities.
- ✓ Develop a method for high titre production of an amphotropic SIN *COL7A1* retroviral vector and the establishment of a SIN *COL7A1* producer cell line. A GMP clinical grade SIN *COL7A1* vector will be produced, characterized and released.
- ✓ Transfer and adapt the protocols used to generate genetically corrected skin equivalents from pre-clinical to GMP standards. This will also include the generation of a clinical grade biobank of selected patients' cells under GMP conditions.
- ✓ Investigate the safety and lack of toxicity of the approach. Safety assessment will include the

demonstration of absence of tumorigenicity during preclinical development and the validation of quality control tests (provirus integrity, detection of RCR and other infectious agents).

- ✓ Implement and conduct the clinical trial.
- ✓ Monitor the graft and the patient during the clinical trial. The tests will include the follow-up of the immune response towards type VII collagen after grafting and the analysis of the integration site patterns prior to grafting and after 12 months of follow-up

WORK PERFORMED AND MAIN ACHIEVEMENTS

1. PATIENT ENROLMENT AND SELECTION: EBGEN STUDY ([clinical.trial.gov](https://clinicaltrials.gov))

The criteria **for patient pre-selection** have been refined to optimize the safety and feasibility of the phase I/II clinical trial. They include clinical, molecular, biochemical, immunological and general criteria and also take into account the nature of the anesthetic and surgical procedures. From a pre-selection of 70 patients from the UK and from France presenting with moderate or severe RDEB, 30 patients have now been **enrolled in the EBGen study**. The consortium has agreed that adult patients only should be proposed for the EBgraft trial, given the total surface of chronic wounds to be treated (6 grafts of 50 cm² each, *i.e.* 300 cm² per patient) and the anesthetic procedure chosen (loco-regional or neuroleptanalgesia). These 30 patients have been short-listed according to their suitability for the clinical trial taking into account their age, clinical presentation, the location, number and extend of their chronic wounds and blistered areas, as well as their immune-reactivity towards wild-type type VII collagen. Of these patients, 6 patients with optimal clinical and biological features were identified as good to very good candidates. Keratinocyte proliferative capacities of the 3 best candidates were high confirming that they were highly suitable for the gene therapy trial. In conclusion, we have identified **3 patients who are the best candidates for the gene-therapy trial** (shortlist 1) and additional 3 patients (shortlist 2) who are also very good candidates and will serve as a back-up to the “best candidate” group.

Assessment of the immune response towards type VII collagen: To predict a possible immune reaction towards type VII collagen, we had previously set up highly sensitive and specific ELISA and ELISPOT assays (Pendaries et al., 2010). These assays have now been optimized and we have modified the purification process to enhance the yield of type VII collagen recovery and to shorten the procedure. The yield has been improved by 20 fold and the duration of the process has been reduced from 2 days to 8 hours. These substantial progresses made in type VII collagen production and purification have been essential to ensure a better availability and lower costs for ELISA and ELISPOT testing. They have allowed us to perform these assays for the majority of the 30 RDEB patients enrolled in EBGen, including the 6 best candidates. These assays will also be essential during the one-year follow up period post-grafting to assess B and T-cell reactivity towards wild-type type VII collagen in the 3 patients who will be grafted.

2. CLINICAL GRADE VIRAL BATCHES AND VALIDATION

Development of an optimized γ -retroviral SIN-vector expressing type VII collagen (*COL7A1*) cDNA (transgene) and allowing efficient transduction and expression of type VII collagen in RDEB patient fibroblasts and keratinocytes was essential for the feasibility of the project. The therapeutic vector had to be produced in a packaging cell able to pseudotype the vector with an amphotropic envelope. A suitable SIN-vector was characterized, termed pCMS-EF1.COL7A1.SIN1 (E890) that was able to transfer *COL7A1* cDNA with very high efficiency into primary target cells. This therapeutic vector was integrated into a plasmid-based exchange construct, termed pbib-ETAR.fcvi-E890 that was used to

target the viral genome into a retroviral packaging cell. This packaging cell (HA820) was developed on HEK293vecAMPHO cells (Ghani et al., 2007) and was preselected for stable high titer vector production by a “tagging” vector that provided Flp recombinase recognition sites at the proviral integration site. The targeted exchange of pbib-ETAR.fcvi- E890 was performed at this chromosomal locus. Selected clones were characterized, which at this stage of the process means that selectable markers of the tagging vector were removed and instead those of the targeting construct were implemented. Targeting-specific PCR was used to confirm precise targeting. The PCR-products were subcloned and sequence-verified. The most promising producer clones were subjected to a final clean-up step that was designed to remove almost all tagging vector sequences and thereby eliminates the possibility of any recombination between the tagging and the therapeutic vector. This polishing step was confirmed by locus specific PCR, and again confirmed by sequencing of the amplification product. The **type VII collagen producer clone**, HA820-E890#14.2, was expanded as a primary and secondary seed bank (PSB, SSB). SSB-cells were used to generate test batches of the therapeutic vector and to optimize the production process.

In parallel, we have developed a **new functional titration method** based on FACS detection of type VII collagen expression that allows for rapid and accurate quantification of transduced cells. This protocol was used to perform functional titration of the viral supernatants (pilot runs approx 1.5 liter) and to precisely measure the level of transduction on primary keratinocytes and fibroblasts. We then tested the effect of different sequence modifications in the pCMS backbone to improve the viral titres of the supernatant.

Next, we tested whether a different internal promoter may improve expression in target cells which are null for type VII collagen expression (BeFa) or viral titer (in transient production) and a stable producer clone was developed by EUFETS. This new packaging cell line achieved the production of the SIN *COL7A1* vectors with high titres (2.10⁶ ip/ml up to 4.10⁶ ip/ml) allowing for the use of non-concentrated and raw supernatants for further clinical use.

Two batches of raw (non-purified) viral supernatants were used to transduce primary RDEB keratinocytes and fibroblasts with a high level of efficiency, which validated the producer clone. PSB-cells were expanded to a **master cell bank** (MCB) which was tested for safety (advantageous virus, RCR, etc.) and was **fully characterized and certified**.

The **certified master cell bank** was used to produce a **clinical grade retroviral vector batch**, which has subsequently been tested, **certified and released in March 2015 and is ready for use in the clinical trial**.

3. TOXICOLOGY AND SAFETY STUDIES

Assessment of provirus integrity: The study of *COL7A1* rearrangements was required to demonstrate the safety of the approach and for the quality control of transduced cells prior to grafting the skin equivalent onto patients. Preliminary observations led to the conclusion that rearrangements occurred during the reverse transcription step as a result of template switching activity of the viral reverse transcriptase. Southern-blot and Western-blot analyses were used to characterize integrated *COL7A1* provirus rearrangements. The presence of *COL7A1* rearrangements could be detected at the genomic and/or protein levels, although abnormal bands at the protein level were difficult to distinguish from physiological proteolytic degradation products. For this reason, we concluded that provirus rearrangements had to be investigated at the DNA level. In addition, to estimate the frequency of these events and to try to get insights into the mechanism involved, we have set up a large scale experiment to isolate a larger number of rearrangement events from molecular analysis of isolated transduced clones. Our results confirmed that provirus rearrangements occurred after the infection of target cells. Analyses of the breakpoint sequences failed to reveal any rearrangement hotspot. We could estimate the frequency of genomic rearrangement of the transgene after transduction and showed that truncated proteins were detectable in a minority of clones. Our results were consistent with a mechanism involving the reverse-transcription step. They have led us to

propose a detection method based on Southern-blot analysis of transduced bulked cell populations.

Bridging studies: In order to document the safety with the new vector that will be used in the clinic, we have designed protocols for bridging studies in mice including tumorigenicity, monitoring of the graft and biodistribution studies. The protocols and the strategy cover the gap between the first generation vector and the vector optimized during the first two periods of the project by EUFETS and INSERM.

Following the ANSM (Agence Nationale de Sécurité du Médicament) scientific advice in December 2015, we have designed and carried out two bridging toxicology studies, and have organized proviral integration site analysis on two validation runs. Studies were carried out following the principles of GLP with defined SOPs to ensure sound scientific data.

The subcutaneous tumorigenicity study showed that nude mice developed no tumour, were healthy with normal weight curves 80 days after subcutaneous injection of 2.10⁶ of genetically engineered RDEB keratinocytes and fibroblasts.

The monitoring of mice grafted with skin equivalents made of transduced cells showed that they did not present with a higher rate of morbidity compared to control groups, showed no sign of illness, no weight loss and no tumor formation. Biodistribution study based on genomic DNA analysis extracted from collected organs is ongoing, but preliminary data show no dissemination of transduced cells.

Proviral integration site analysis was performed on transfer batches of transduced cells as advised by the ANSM. A subcontractor has performed integration site analysis with linear amplification mediated PCR (LAM-PCR) with two enzymes and non-restrictive LAM-PCR (nrLAM-PCR) in duplicate. NGS and bioinformatics analysis of the samples were performed on cultured fibroblasts and keratinocytes from two RDEB patients and from the same cells following transduction with the SIN COL7A1 retroviral vector showed Overall, the analyses of SIN-RV transduced fibroblasts and keratinocytes obtained from two subjects showed a highly polyclonal vector integration profile with no preferred integration in/nearby genes previously involved in serious adverse event in gene therapy.

4. TRANSFER AND ADAPTATION OF THE KNOWHOW AND PROTOCOLS FROM RESEARCH TO GMP STANDARDS

Beneficiary 10 (FIBHJ) has joined the consortium during period 4. B10 is an academic GMP certified gene and cell therapy laboratory based in Madrid. His role is to produce the Advanced Therapy Medicine under GMP conditions for the clinical trial to be performed in Paris. B10 has selected the best GMP-grade reagents for keratinocyte and fibroblast culture from skin biopsies, for their genetic correction and for their use to generate skin equivalents. All these GMP grade raw material have been approved by the Spanish Agency for GMP-manufacturing processes. B10 has also elaborated Standard Operating Procedures (SOPS) from biopsy processing and establishment of primary cultures to skin equivalent (sheet) assembly, conditioning and packaging. As part of the manufacturing process validation, three transfer batches were successfully generated under GMP conditions from three different RDEB patients. A cell bank of transduced and non-transduced keratinocytes and fibroblasts from each these three RDEB patients was made under GMP conditions in GMP facilities. Genetically corrected skin equivalents (sheets) were generated. The validation process of the preparation of genetically corrected skin equivalents will require opening of the clinical trial. The dossier has been submitted to the Spanish Agency.

5. LARGE SKIN EQUIVALENTS AND THEIR GRAFTING ONTO LARGE ANIMALS

In order to validate the skin equivalent production process, we had to demonstrate our capability

to produce, transport and graft large skin equivalents under conditions similar to the ones which will be used to graft patients. For that purpose we have produced large skin equivalents made of normal human keratinocytes and fibroblasts and have grafted them onto the face of 8 weeks-old pig and followed up for 1 month. We could demonstrate by immunohistological examination of the grafted skin equivalent the formation of a well differentiated pluristratified human epidermis adherent to the underlying dermis. The surgeons and the INSERM team were thus able to validate the procedures for the production of large area of skin equivalents, their handling and transport, their grafting together with their dressing.

6. REGULATORY AND ETHICAL ISSUES

Beneficiary 5 (GENETHON) has organized and implemented the preparation of the dossier for the Clinical Trial Authorization (CTA) including the Investigational Medicinal Product Dossier (IMPD).

Chemical and pharmaceutical quality documentation of the IMPD:

The documentation regarding the viral vector part has been finalized. The documentation on pharmacological development including the manufacturing process, the production process and validation, the container closure system and the stability of the IMP have been finalized. The quality control is almost completed.

Non clinical evaluation (pre-clinical studies) of the IMPD includes *in vitro* and *in vivo* proof of concept studies which have been successfully completed, and safety studies for which two complementary bridging toxicology studies have been performed and are currently being analysed (see WP5). Integration site analysis has been achieved and showed good safety profiles (see WP5).

Major clinical documents of the IMPD (Clinical protocol, informed consent forms) have been finalized (see WP6). The Case report form and the Investigator's Brochure are almost finalized.

Overall, the preparation and submission of the regulatory dossier is well advanced. The Clinical Trial Application could be submitted to the competent authority and to the Ethic Committee in Q2 2017.

Additional requirements have been fulfilled, including updating of the annual report of the Orphan Drug Designation Dossier; a scientific advice with the ANSM was held in December 2015; the GMOs agreements have been obtained from the Haut Comité des Biotechnologies; a technical dossier for GMP certification of Beneficiary 10 by the Spanish Agency and a dossier to the French Ministry of Health to obtain authorization for skin biopsy exportation are about to be submitted.

6. PREPARATION OF THE CLINICAL TRIAL

The design and the logistics of the clinical trial have been thoroughly discussed and defined by the consortium. Specifically, the primary and secondary objectives have been defined as well as the primary and secondary endpoints. The anesthetic and surgical procedures have been delineated. The number and size of the grafts, the nature, number and size of the skin lesions to be grafted, the monitoring and the follow-up period have been outlined. With regards to the logistics, the procedures for certification of the clinical department, for the research laboratory and the shipment of the biological material have been identified, and contacts with the relevant administrations have been made.

EXPECTED OUTCOMES

The **new therapeutic strategy** proposed by **GENEGRAFT** addresses the clinical and molecular diversity of RDEB patients. Transplantation of genetically corrected skin will provide an essential and permanent treatment of chronic and large skin wounds because gene-corrected skin equivalents have the potential to definitely cure the treated area. Indeed, transduced epidermal stem cells and fibroblasts contained in the gene-corrected skin equivalents will maintain their division and

proliferation potential, allowing for long term expression and deposition of type VII collagen at the dermal-epidermal junction. This will allow the formation of functional anchoring fibrils. Therefore, treated areas will locally reverse the disease phenotype. They will prevent the formation of blisters, skin inflammation, retraction and skin cancer. For these reasons, this project has a **strong potential to bring clinical improvement to RDEB patients and to represent a major progress in the treatment of this devastating disease.**

By performing this research and development at the European level and in direct link with the European policy on rare diseases, the results of the GENEGRFT project will serve as a model for the treatment of RDEB and in other forms of severe epidermolysis bullosa. It is also expected that this model could be extended to other genetic skin diseases, as well as non- dermatological disorders. The results of the project will benefit the entire scientific community and will have positive impacts on the health of thousands of European citizens.

Effective treatment of RDEB will have also strong, positive knock-on effects on the quality of life of patients and their families. Restoring skin cohesion over the most vulnerable areas such as the hands, forearms, feet and legs to prevent the recurrence of skin lesions and skin cancers should result in a significant reduction in the functional and systemic complications of RDEB. Consequently, vital prognosis would improve, and the frequency and duration of hospitalization would be reduced. Further anticipated benefits include a reduction in the pain, discomfort and physical handicap, and the prevention of deformities, with a positive effect on the ability to carry out normal everyday activities.

OVERVIEW OF PARTICIPATING GROUPS AND THEIR FIELD OF EXPERTISE

N°	Group leader	Beneficiary name	Country	Expertise
1	Alain Hovnanian Matthias Titeux	INSERM	France	Genetics, gene and cell therapy, genetic skin diseases, clinical trials
2	Christine Bodemer	NEM-APHP	France	Pediatric dermatology
3	John McGrath Alya Abdul- Wahab	KCL	United Kingdom	Dermatology, genetic skin diseases
4	Klaus Kühlcke Rainer Low	EUFETS	Germany	GMP manufacturing of gene therapy products
5	Didier Caizergues Geraldine Honnet	Genethon	France	Health and pharmaceutical Legislation
6	Patricia Joseph-Mathieu Dahlia Tsakiropoulos	IT	France	Project management and technology transfer
7	John Dart Clare Robinson	Debra	Austria	Dermatology, molecular Dermatology
9	Marcela Del Rio and Fernando Larcher	UC3M	Spain	Cell therapy, Gene therapy, Clinical trial
10	Gustavo Melen and Manuel Ramirez Orellana	FIBHNJ	Spain	GMP manufacturing for cell and gene therapy



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