Project no.

**LSHB-CT-2005-512073**

Project acronym

# SKINTHERAPY

Project full name

## Gene therapy for Epidermolysis Bullosa: A Model System for Treatment of Inherited Skin Diseases

Instrument type: **Specific Targeted Research Project**

Priority name: **Priority 1 – Life sciences, Genomics and Biotechnology for Health**

# FINAL ACTIVITY REPORT MONTH 42

# Publishable Executive Summary

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Start date of the project: **April 15th, 2005** Duration: **42 months**

Project coordinator name: **Dr Guerrino Meneguzzi**

Project coordinator organisation name:

**Institut National de la Santé et de la Recherche Médicale (INSERM), Partner 1**

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# Publishable Executive Summary

## SKINTHERAPY
## Gene therapy for Epidermolysis Bullosa:
## A Model System for Treatment of Inherited Skin Diseases

Epidermolysis Bullosa (EB) is a rare genetic skin disease, affecting approximately 30,000 individuals in Europe (prevalence of about 60/million) and approximately 400-500,000 people worldwide. Most patients are children and there is no treatment available yet.

EB is characterized by an extreme fragility of the skin, which results in unremitting blisters and erosions with unceasing wound healing. The skin lesions result from the poor adhesion of the epidermis to the underlying mesenchyme, which makes the skin vulnerable to damage caused by mild friction and trauma. The genetic bases of the different clinical forms of EB have been elucidated, and a precise correlation has now been made between genetic defects of the basement membrane components and the types of EB, which have been classified within the Simplex (EBS), Junctional (JEB), and Dystrophic (DEB) forms.

In DEB, generalized blisters heal with scarring. Extensive and progressive mutilating scarring results in increasing disability and deformity of the fingers, and joint contractures. The disease may also affect other areas of the body (mouth, throat, eyes) causing discomfort and, in most of cases, difficulties in carrying out vital activities, like eating. The most severe cases are often complicated by the development of aggressive skin cancer and premature death. The physical suffering can be compared to that of severely burnt people. In addition to suffering, patients are faced with social difficulties: they cannot perform physical activities, need permanent assistance and regular medical treatments. Additionally, EB patients are excluded from the workforce not only because of their physical appearance but also because people are not well informed and aware of the condition.

The project's goal was to develop a gene therapy approach to DEB, which is caused by inherited genetic defects of collagen type VII (encoded by the *COL7A1* gene), and design appropriate phase I/II clinical trials to exploit the knowledge generated by the preclinical studies proposed by the Skintherapy consortium.

Specifically, the project aimed at developing a gene therapy technology model based on autologous transplantation of skin made *in vitro* using genetically modified epidermal stem cells. This includes the development of vectors, gene transfer technologies, and preclinical models of cutaneous gene therapy.

## Work performed and results achieved

- **Recruitment of patients, diagnosis and genetic analysis of DEB patients**

Between April 2005 to October 2008 skin biopsies from over 300 patients with EB were analysed by immunofluorescence and electron microscopy. Among them 241 were diagnosed as DEB patients. Genomic DNA was isolated from the patients' blood by partners 1 (INSERM), 4 (KALUDERM), and 5 (CIEMAT) and used for direct PCR amplification of the 118 exons of the gene and either direct automated sequencing (Partners 1, 4, and 5) or DHPLC technique (IDI-IRCCS, Rome (subcontractor) and Partner 1). Genotyping was completed on 218 patients and is in progress for 14 of them. So far, analysis of the collected diagnostic data has led to the identification of 2 hot spot mutation sites in the *COL7A1* gene of DEB patients of Spanish and Italian origin, which facilitates the screening for the causative mutations in patients newly referred to the European centres. By merging the data collected by the Skintherapy consortium with those previously obtained by each individual partner, a patients' database is now available, which is based on a simplified anonymous information file concerning 440 DEB patients. This database permits the establishment of cohorts of fully characterized patients which are potential candidates for inclusion in future clinical trials. The inclusion and exclusion criteria have been proposed by the Skintherapy consortium (Deliverable D01.3).

Of note, this activity has led to: -1) the establishment of a new prenatal test based on immunomapping of first trimester chorionic villi in families at risk for a lethal EB with pyloric atresia (EB-PA) (D'Alessio M et al. 2008); -2) the identification of the genotype/phenotype correlation in a subset of patients with inverse DEB (I-RDEB) which is characterized by severe mucosal involvement and minimal skin manifestations (Youssef et al. manuscript in submission); -3) the label of Partner 1, in association with the Department of Dermatology, Nice University Hospital, as "Reference Centre of rare diseases" for diagnosis and care of inherited EB (Centre Coordinator, Prof. Jean-Philippe Lacour); -4) the establishment of a new diagnostic centre for DEB in Madrid (Partner 5, CIEMAT).

- **Biochemical studies on collagen type VII, evaluation of the content in stem cells of keratinocyte cell cultures isolated from DEB patients and DEB dogs.**

To establish a genotype-phenotype correlations in DEB, and better define the biochemical properties of the mutated collagen VII molecules, 11 genetic mutations described in association with recessive or dominantly inherited DEB were generated *in vitro* by directed mutagenesis of the collagen VII cDNA. The biochemical properties of the mutated proteins were then assessed by Partner 4 in a collagen VII-free cell system (HEK293T cells). To

delineate the molecular mechanisms of Dominant Dystrophic EB (DDEB), expression vectors, which contained three mutant collagen VII naturally-occurring *COL7A1* mutations were used for characterization of the recombinant collagen VII mutants (Partners 1 and 4). Co-expression of each mutant with equal amounts of the wild-type polypeptide strongly decreased the thermal helix stability of all the hybrid molecules that also displayed a reduced ability to support cell adhesion. Controlled over-expression of the wild-type collagen VII gradually improved the triple-helix stability of the hybrid molecules. Considering our previous results showing that over expression of a recombinant collagen VII does not interfere with the tissue homeostasis and differentiation process both in vitro and in vivo, these data suggest that increasing the expression of wild-type collagen VII in the skin of patients with DDEB could be a valid therapeutic approach (Fritsch et al. Manuscript in submission).

From these studies it became evident that a mouse model of DEB could be advantageous for the Skintherapy project. As a part of WP2 Partner 4 has constructed a hypomorphic DEB mouse strain, which opens new possibility for gene therapy, cell therapy and protein therapy approaches of DEB (Fritsch et al. 2008).

As programmed, primary cultures of keratinocytes from DEB patients that display a constellation of different clinical variants of DEB have been established by Partners 1, 2 and 4. Primary cultures of fibroblasts were also established. All the cell cultures were tested for absence of infectious agents including mycoplasma. After extensive characterizations, one of these cell cultures obtained from a collagen VII null/null patient with recessive DEB (homozygote) was selected as “gold standard” platform for gene therapy assays. A parallel research by Partner 2 was aimed at identifying of new stem cell markers that could improved isolation transduced DEB epithelial stem cells (Barbaro et al. 2007).

- **Development of viral vectors (oncoretroviral, lentiviral, and hybrid adeno/AAV vectors) to integrate and stably express the collagen VII cDNA in DEB epidermal stem cells *ex vivo***

Experiments were focused on the ability of different viral vectors (retrovirus, lentivirus and adeno/AAV hybrid vectors) to transduce and correct DEB keratinocytes *in vitro*. Low gene transfer efficiencies were obtained with both new retroviral and lentiviral vectors. New adenovirus/AAV hybrid vectors were also developed, which yielded high levels of collagen VII secretion when keratinocytes cells were transduced. However, clonal analyses of the transduced cell cultures showed a low transduction efficiency which makes the adenovirus / AAV vectors unsuitable for gene therapy of DEB.

Expression of collagen VII from a retroviral vector is real challenge: -1) the cDNA has a size at the limit of retroviral vector capacity, -2) the cDNA sequence displays short-length repeats and cryptic splicing signals that make the corresponding provirus extremely unstable for a

reverse transcriptase-based vector system. Despite these drawbacks, the Skintherapy project has demonstrated the feasibility of a gene therapy approach for DEB at preclinical level. At present, the undesired instability of the transduced cDNA sequences within the cell genome prevents clinical applications of our technology. To circumvent these problems, we (Partners 1 and 3) have re-designed the entire coding sequence of the collagen VII (9 Kb) by introducing more than 2,000 silent base changes, and obtained the synthesis of the entire mutated cDNA. The "recoded" was cloned in both a SIN onco-retroviral and a SIN lentiviral vector to verify the hypothesis that elimination of the repeated cDNA sequences and the cryptic splice sites optimizes the retrotranscription of the proviral collagen VII sequences. Our results show that the SIN retroviral vectors carrying the "recoded" cDNA are functional in terms of expression level of the recombinant protein. The analyses aimed at assessing the stability of the transduced cDNA sequences are in progress (Partner 3). At the same time, we started a full safety testing of both vector backbones, and of a keratinocyte cell-specific promoter developed and tested in the first 24 months. The testing includes efficacy data *in vivo* on immunodeficient mice (Partner 3 and 5), genotoxicity assays in clonal human keratinocytes, and a full characterization of the integration profiles in primary cells. The data produced by this effort will be crucial for the choice of a new vector platform for future clinical trials.

- **Construction of skin equivalents made with genetically corrected DEB cells to assess the full morphological and functional reversion of the DEB phenotype**

Primary dog and human RDEB keratinocytes were transduced using the oncoretroviruses expressing either the dog (MSCV-CaColVII) or the human collagen VII cDNA (pLZRS-HuColVII) respectively. Skin equivalents were generated using : -1) dog or human RDEB primary fibroblasts embedded in organic chitosan sponges or fibrin gels to generate en artificial dermis, -2) RDEB dog or human RDEB keratinocytes before and after transduction that were seeded on the artificial dermis to make the skin equivalents. The expression level, distribution of the recombinant collagen VII in the cells and in the artificial skin was assessed and compared to that of collagen VII constitutively expressed un control skins made with wild-type keratinocytes and fibroblasts. The recombinant collagen VII was actively synthesized and it was localized at the basement membrane of the dermal-epidermal junction with no evident interference on the differentiation process of the epidermis, as determined by immunofluorescence analysis.

- **Grafting of the skin equivalents expressing the recombinant collagen VII on appropriate immune competent animal models**

To provide pre-clinical evidence of stable correction of DEB *in vivo*, two RDEB dogs were grafted with autologous genetically modified epithelia. The transplantation of the genetically corrected RDEB epithelia generated a functional, self-renewing epidermis *in vivo* which gradually was colonised by dendritic and endothelial cells and melanocytes. Synthesis of functional collagen VII was observed, together with the development of a firmly adherent epidermis that in one dog remained stable for the 15 months duration of the follow-up in the absence of blisters, infections, inflammation or immune response. In the other transplanted dog, expression of the transgene messenger RNA rapidly declined after grafting of the engineered epithelium, and blisters were observed 2 months after grafting. These data were correlated with the loss in the dog of the genetically-modified epithelial stem cells, which emphasizes the importance of an efficient transduction of keratinocytes stem cells to achieve a permanent reversion of the RDEB diseased phenotype (Pin et al. Manuscript in submission). The information drawn from this activity has substantially contributed to the establishment of guidelines for efficient preclinical and clinical trials for gene therapy of epidermal cells.

- **Developing a technology for production and downstream processing of clinical-grade viral vectors preparations under Good Manufacturing and Laboratory Practices (GMP/GLP) standards**

Partner 6 defined general Standard Operating Procedures (SOPs) for transient production of lentivirus vectors. These SOPs are also valid for the construction of a stable packaging cell line. However, no stable packaging cell clone expressing collagen VII could be achieved because no collagen VII-expressing vector suitable for clinical assays of DEB is yet available.
Partner 6 has validated the SOPs and has established flow-charts of productions.

- **Guidelines for efficient gene transfer to epidermal cells**

The feasibility of a phase I/II gene therapy clinical trial has been evaluated in a pilot clinical trial involving one patient with Junctional Epidermolysis Bullosa (JEB) compared heterozygote for mutation on *LAMB3* gene. Primary epidermal keratinocytes obtained from a 36-year old male affected by mild JEB were transduced with an oncoretroviral vector carrying a wild type *LAMB3* cDNA. Genetically engineered epidermal sheets were transplanted onto the patient thighs. Follow-up showed that the regenerated epidermis contained levels of laminin-5 comparable to those of normal controls. Specifically: (i) the transduced epithelial

stem cells permanently express the correct recombinant beta3 chain, (ii) distribution of stem cell markers (deltaNalphap63 and C/EBPdelta) was observed in both implants at levels mirroring those found in normal subjects. (iii) no neutralizing antibodies against the recombinant protein were detected. *In vitro* studies also showed rescue of the clonogenic potential of the reverted keratinocytes obtained from biopsies of the transplanted tissues. The clinical follow-up the transplanted patient is going on (longer than 2,5 years). No blisters, erosion or other lesions were observed in the regenerated epidermis (Mavilio et al 2006). These results have been used to establish guidelines for efficient pre-clinical and clinical trials for gene therapy of DEB epidermal stem cells.

- **Exploitation of results, establishment of Standard Operating Procedures, guidelines for pre-clinical and clinical trials**

The Industrial Property Committee (IPC) coordinated by Prof Fulvio Mavilio examined the knowledge that could be appropriately exploited. Despite the interesting results produced, the knowledge generated was not considered suitable for protection through a patent application. Also, the most recent studies aimed at assessing the ability of new self-inactivating (SIN) lentiviral vectors carrying transgenes drived by tissue-specific promoters gave results unsuitable for patenting. Depending on the forthcoming results, a possible patent application could be considered to protect the “recoded” collagen VII cDNA.

- **Societal aspects and dissemination of results**

The Skintherapy consortium committed itself to undertaking two-way dialogue with the EB patient community to take into account their views and any specific requests. Throughout the duration of the Skintherapy project, research progress, including any significant breakthroughs, was communicated to the EB patient community, and opportunities for discussion and feedback provided. The project was also used to engage with this community to develop their understanding of how research might be translated into candidate therapies, and what participation in any subsequent clinical trials might involve. The complexity of issues related to the development of gene therapy is providing opportunities for developing understanding of the need for good scientific practice including the precautionary principle to ensure the highest possible standards of safety and efficacy in the development of the technology and methodology. Appropriate means of communication with the patient community includes web-based information, seminars and meetings with both national patient groups, and volunteer patient and family members with a specific interest in more detailed dialogue, newsletters and other distributed written information. In addition, the project was used to raise awareness of the social and physical challenges of living with rare skin diseases, and EB in particular, with the wider public. As the European EB patient-

support organization, the partner DEBRA led on this work package with contribution from and involvement of other partners as appropriate.

- **Expected End Results**

The overall expected result was a model system for DEB treatment using *ex vivo* gene therapy. This model can be extended to other genetic skin diseases. The model system addressed the current weaknesses in related research and aimed particularly at the development of significantly improved, efficient and safe delivery systems, which are urgently needed.

## Partners

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| **Coordinator-Partner 1**<br><br>Dr. Guerrino Meneguzzi<br>Institut National de la Santé et de la Recherche Médicale<br>INSERM Unité 634<br>27 Avenue Valombrose, 06107 Nice, Cedex 02, France<br>Phone: (+33) 493377779; Fax: (+33) 493811404<br>Email: Guerrino.MENEGUZZI@unice.fr | **Partner 2**<br><br>Dr. Stefano Ferrari<br>Centro Regionale di Recerca Sulle Cellule Staminali Epitheliali<br>Fondazione Banca Degli Occhi del Veneto<br>Venezia, Italy |
| **Partner 3**<br><br>Pr. Fulvio Mavilio<br>Department of Biomedical Sciences<br>Universita di Modena e Reggio Emilia<br>Modena, Italy | **Partner 4**<br><br>Pr. Leena Bruckner-Tuderman<br>Universitaetsklinik Freiburg<br>University Hospital Freiburg - Department of Dermatology<br>Freiburg, Germany |
| **Partner 5**<br><br>Dr. Marcela Del Rio<br>Department of Epithelial Damage, Repair and Tissue Engineering<br>Centro de Investigaciones Energeticas Medioambiantales y Technologicas<br>Madrid, Spain | **Partner 6**<br><br>Dr. Anna Stornaiuolo<br>Discovery Division<br>Molmed S.p.A<br>Milan, Italy |
| **Partner 7**<br><br>John Dart<br>DEBRA Europe<br>Crowthorne, United Kingdom | **Partner 8**<br><br>Gerlinde Lenzen<br>Inserm Transfert SA<br>Paris, France |

**Publishable results of the final plan for using and disseminating the knowledge:**

| Publishable results | *Result description* | *Possible market application* | *Stage of development* | *Collaboration sought or offered* | *Collaborators details* | *Intellectual property rights granted* | *Contact details* |
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| | Treatment of canine dystrophic epidermolysis bullosa by transplantation of genetically modified epidermal autografts. | None | Proof of principle | None | Partner 1 | No IP involved | |
| | Ability of new self-inactivating (SIN), HIV-derived lentiviral vectors to lead to tissue-specific transgene expression (in vitro and in vivo in immunodeficient mouse models), through the use of K14-full length or -derived promoters | None at this stage | Preclinical studies in immunodeficient mouse models | | Joint collaborations between partner 2 (FBOV), 3 (UNIMORE) and 5 (CIEMAT) | No IP involved | |
| **New vectors for gene therapy of JEB :** | Correction of Laminin-5 deficiency in human epidermal stem cells by transcriptionally targeted lentiviral vectors | None at this stage | Proof of principle | None | Partner 3 | No IP involved | |
| | Successful in vivo skin regeneration from single stem cells genetically modified and assessed for safety | None at this stage | Proof of principle | None | Partner 5 | No IP involved | |
| | Comparative safety assessment of MLV-derived and HIV-derived SIN vectors in human keratinocytes | None at this stage | Pre-clinical study | None | Partner 3 | No IP involved | |