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Bachelor Thesis

Corrective Gene Transfer in Dyskeratosis Congenita Human Skin Stem Cells

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Abstract

Gene therapy is an emerging field in regenerative medicine that is finding a definitive cure to many diseases rooted on a genetic impairment. On the other hand, Dyskeratosis Congenita (DC) is a rare disease caused by a mutation in gene DKC1 coding for one of the subunits of the telomerase complex, standing out as a potential candidate for gene therapy; however, this possibility has not been studied yet and currently DC is treated with conventional treatments. In the present study, transgene GSE4, derived from DKC1 gene, was transduced in DC keratinocytes in order to achieve genetic correction. This correction was initially surveilled through the expression of reporter GFP gene; however, when the GSE4 keratinocytes proliferated significantly, *in vitro* skin equivalents were grown from the corrected cells in order to evaluate their behavior in an organotypic culture. The GSE4 correcting transgene achieved a 97% successful transduction in the DC keratinocytes, in contrast with preliminary studies that used GSE4 precursor but did not achieve a positive transduction. The obtained results proved that DC can be targeted at its core through gene therapy, opening the path to further research in this direction and giving a new hope to the patients of this rare disease.

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Thank you to my family: I really appreciate the support of my parents, and how they have patiently listened to all the details of my thesis even though for them it all just sounded as perfect science-fiction.

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In general, thank you to the persons that have shared with me this hectic semester. Soon we will smile at the past moments of desperation, and I am sure the reward that future has prepared for us is immense.

"[…] For somewhere inside ourselves, something tells us that life is a huge mystery. This is something we once experienced, long before we learned to think the thought."

Sophie's World – Josten Gaarder

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Introduction

Background, Motivation and Goals

Dyskeratosis Congenita (DC) is a rare disease with an extremely heterogeneous genotype as well as phenotype, including initial skin disorders which, as the disease progresses, degenerate into bone marrow failure and increased cancer predisposition. Even though, as a rare disease, not many cases have been reported, most of them end up being fatal during the earliest years of life. As a condition with more unknowns than equations, its understanding has been delayed by the scientific community for a long time.

It has only been during the last ten years that the reasons behind this rare disease have started to be disentangled, and real treatments other than just mere everyday guides for good practice have been proposed. However, none of them has targeted Dyskeratosis Congenita at its core yet. The disease is rooted on a genetic mutation at the DNA-level in one of the subunits of the telomerase complex, dyskerin.

The present study aims to find a potential cure to DC by directly correcting this genetic impairment through gene therapy. Even though such an approach promises to be the most definitive cure for this genetic disease, so far no precedents have been reported in the literature of Dyskeratosis Congenita.

The goal of this project is to infect DC patient's epidermal cells with a lentiviral vector that contains the therapeutic gene GSE4 as its genetic material. In this gene therapy, GSE4 codes for the healthy version of dyskerin gene that will replace the defective mutated one in Dyskeratosis Congenita. GSE4 behaves as the equivalent of a drug in contemporary medicine, and through this simple genetic switch, DC is expected to disappear.

In order to assess the efficiency of the proposed gene therapy, an *in vitro* skin model is developed using the corrected epidermal cells as the building blocks. The behavior, proliferation, and GSE4 expression of the corrected cells, as well as the structure of the grown *in vitro* skin model, are parameters of whether the previously DC cells have been indeed genetically converted into healthy ones.

Skin

Function

The skin is the main organ from the integumentary system, which essentially is a cutaneous membrane covering the outer surface of the body. Hair, skin glands and nails are accessory structures of the skin in the integumentary system [1].

The main function of skin is protection. Skin provides the first line of defense against external agents trying to invade an organism. However, it additionally carries out several other functions: Skin regulates body temperature by sweating and by adjusting blood flow to the dermis; it is responsible of sensory perceptions; it synthesizes vitamin D; skin carries out excretion of sweat and absorption of gases, and even more functions.

Regarding the layered structure of skin, the epidermis is responsible of protection and resistance against damage, whereas the dermis prevents dehydration, regulates temperature, and is involved in healing.

Structure

Skin is composed of two basic tissues: Epithelial tissue forming the epidermis, and connective tissue composing the dermis. In the skin, the epithelial tissue is the parenchyma which is supported by a stroma made of connective tissue.

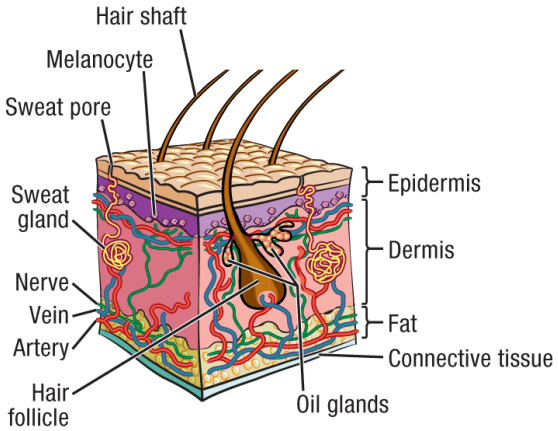

Fig 1: Sectional view of skin and subcutaneous layer [2].

Epidermis is the uppermost thin layer of skin, consisting of closely packed cells forming continuous sheets. Epithelial tissue is always avascular, therefore obtaining all the nutrients required for cell survival from the underlying connective tissue found in the

dermis. Epithelial tissue always sits on a *basement membrane* which tightly connects it with the dermis. The basement membrane is a thin, highly organized layer of Extracellular Matrix mostly composed of collagen IV (lamina densa) and laminin (lamina lucida). The dense network in the basement membrane connects to the cells in the stratum basale of the epidermis through the interaction between fibronectin and cellular integrin. At the same time, the lamina lucida connects to the fibers in the connective tissue of the dermis [1].

In particular, the type of epithelial tissue found in the skin is keratinized stratified squamous epithelium, which has an apical surface made up of several layers of squamous (flat) cells. This structure with many layers provides protection against strong friction forces. The epidermis has four layers in the case of the thin (hairy) skin, and five layers in the case of thick (hairless) skin, pictured in Fig 2.

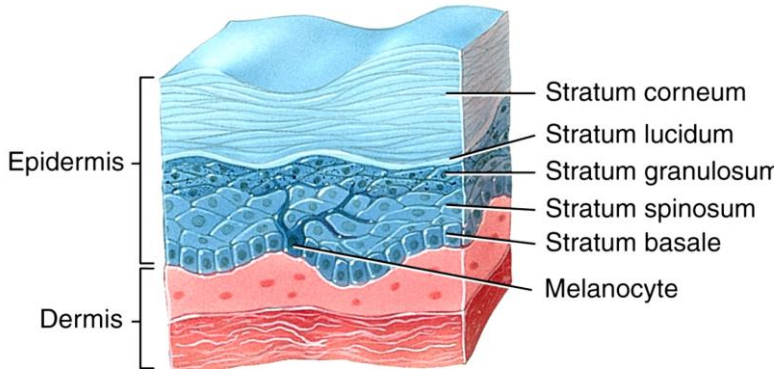

Fig 2: Layers of the epidermis [1].

i. The stratum basale, adjacent to the dermis, is where new keratinocytes are produced by skin stem cells through continuous cell division. It is the only mitotically active layer in the epidermis.

ii. The stratum spinosum is a layer of 8-10 keratinocytes.

iii. The stratum granulosum contains non-dividing cells filled with keratin granules.

iv. The stratum lucidum only exists in thick skin.

v. The stratum corneum is the outermost layer of the epidermis. It is composed of around 20 layers of dead keratinocytes filled with keratin that are continuously replaced from deeper strata. Keratinization is the process in which keratinocytes accumulate increasing amounts of keratin, the skin-specific intermediate filaments, as they move upwards to the skin surface.

Dermis is the inner, thick layer of skin. The connective tissue in the dermis carries blood and lymphatic vessels, as well as nerves [1]. The dermis is divided in two regions:

i. The papillary region lies just below the epidermis. It is made of areolar CT, a type of loose CT, therefore containing mostly ground substance and fibroblasts than ECM fibers. The papillary region contains capillary and free nerve endings.

ii. The reticular region consists of dense irregular CT, which contains an abundant extracellular matrix composed of thick collagen and elastic fibers with fibroblasts immersed in it. It also contains hair follicles, sebaceous glands and sudoriferous glands.

Fibroblasts are the main residents in the connective tissue. They are extremely versatile cells involved in the production of collagen and elastin fibers in the ECM.

The subcutaneous layer located underneath the dermis is called *hypodermis*. However, it is not part of the skin. The hypodermis is formed by areolar and adipose CT, and its role is to anchor the skin to the underlying tissues and organs. The lamellated (Pacinian) corpuscles that detect external pressure applied to the skin are found in the hypodermis.

Epidermal Stem Cells

The epidermis contains four major cell types, displayed in Fig 3:

- *Keratinocytes*: They count up to the 90% of the cells found in the epithelial tissue. They produce *keratin*, which is a tough fibrous protein essential for protection.
- *Melanocytes*: They produce melanin pigment, involved in UV protection.
- *Langerhans Cells*: Macrophages originated in the red bone marrow, carrying out immune responses.
- *Merkel Cells*: Involved in the sensation of touch along with adjacent tactile discs.

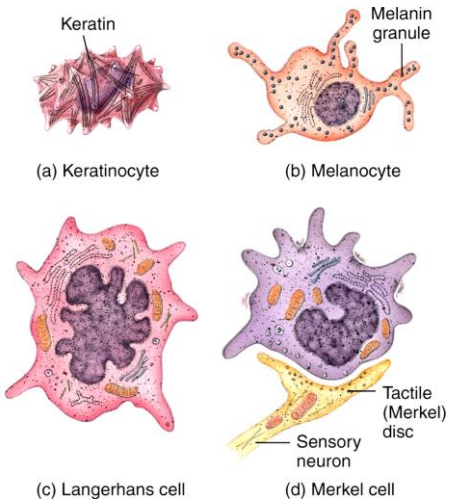

Fig 3: Main cell types in the epidermis [1].

In addition, skin contains *stem cells* which are found in the epidermis and hair follicle. These stem cells are involved in skin renewal, homeostasis and wound healing [3]. The main types of skin stem cell are Melanocyte SCs, Hair Follicle SCs, and Epidermal SCs.

Epidermal SCs are found in the stratum basale of the epidermis and are involved in the continuous regeneration of the different layers of the epidermis [4]. In the epidermis, the stratum basale contains all the alive cells whereas the stratum corneum contains only dead cells. In between, an ascending process of keratinocyte differentiation takes place. As epithelial stem cells leave the basal layer and move upwards towards the surface of the skin, they quit cellular division, switch off integrin expression and increase keratin accumulation. All these processes are part of the terminal differentiation program that converts St. Basale epithelial SCs into St. Corneum dead differentiated keratinocytes.

Among other signaling factors, the main promoter of keratinocyte differentiation is calcium gradient. In the epidermis, the lowest calcium concentration is found at the stratum basale, whereas at the stratum corneum this concentration is very high. A reason for this singularity is that dead keratinocytes are not able to dissolve calcium ions [5]. High levels of extracellular Ca induce an increase in intracellular Ca ions in the keratinocytes. On the other hand, vitamin D3 (cholecalciferol) ultimately regulates the expression of those genes involved in keratinocyte differentiation depending on the levels of intracellular calcium [6]. A general diagram of the different factors involved in epithelial stem cell differentiation is shown in Fig 4 below:

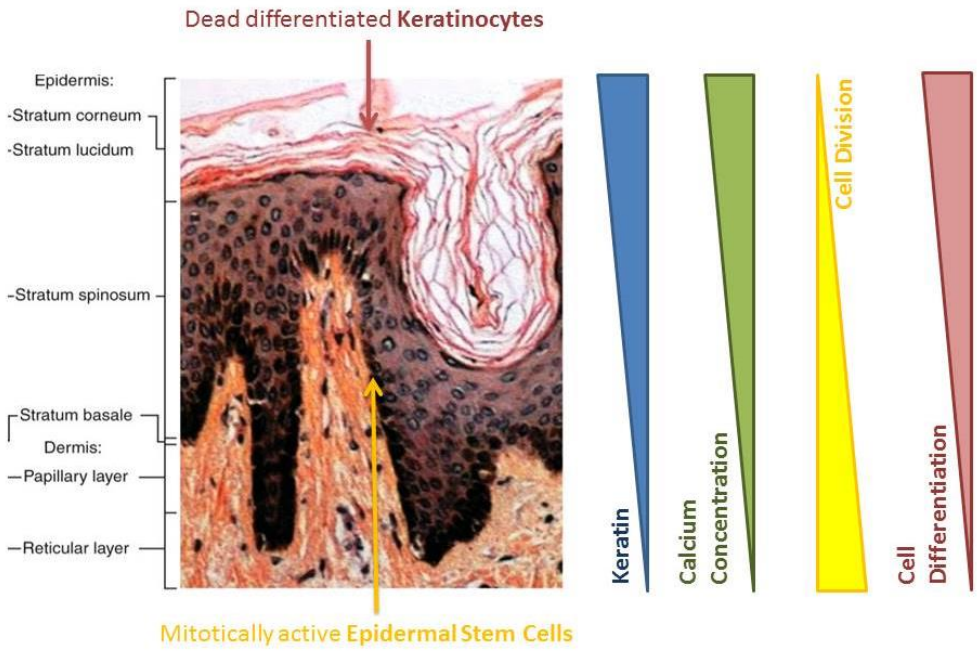

Fig 4: Epidermal Stem Cell differentiation along the section of the epidermis.

In the present study we will work with primary keratinocytes and with epidermal stem cells, which have the ability to continuously divide and the potentiality to terminally differentiate in keratinocytes. However, in general terms, they will be simply called keratinocytes along the study.

Dyskeratosis Congenita

Phenotype

Dyskeratosis Congenita (DC) is a rare congenital disease of multisystem characteristics. It was first described by Zinsser in "*Atrophia cutis reticularis cum pigmentatione, dystrophia unguium et leukoplakia oris*" in 1906, and recognized as a clinical entity by Engman (1928) and Cole (1930) [7], from whom it obtained the alternative name of Zinsser-Cole-Engman syndrome. The estimated annual incidence of DC is <1 in 1 million, and 30 cases have been diagnosed so far in Spain [8].

DC has a highly variable phenotype. The main distinguishing symptoms of DC typically are *skin pigmentation, nail dystrophy* and *oral leukoplakia*, the three of them shown in Fig 5. However, many other characteristics have been observed in DC patients, such as mental retardation, conjunctivitis or pulmonary complications. In spite of this, neither do these symptoms appear at the same time nor to all patients: the phenotype in DC is extensively wide, heterogeneous and systemic [9].

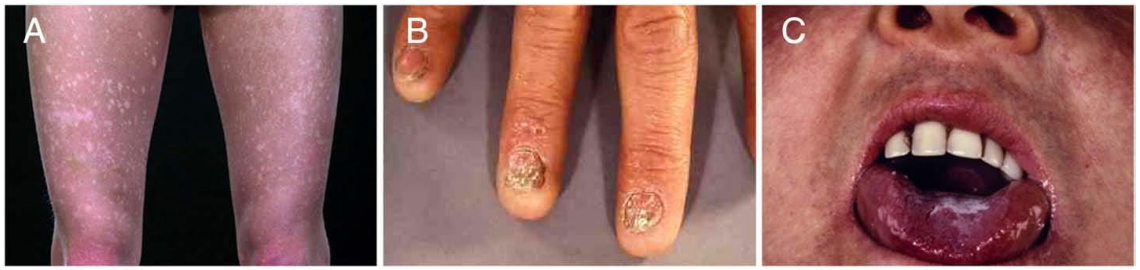

Fig 5: Main phenotypic features of DC: (A) Skin pigmentation. (B) Oral leukoplakia. (C) Nail distrophy. **[10]**

Although initially the most affected organ is skin, as the disease advances a progressive *bone marrow failure* appears in 80% of the patients. This condition is a major cause of mortality in DC patients, being most of the deaths caused by opportunistic infections enhanced by a deficiency in the hematopoietic stem cells found in the bone marrow. In addition, DC patients suffer an increased risk of *malignancy* which typically develops in the third decade of life. Squamous cell carcinoma within sites of leukoplakia as well as in the skin is the most common form of malignancy found in DC patients, although hematolymphoid neoplasms have also been observed [11].

Finally, DC includes a variety of many minor features due to its multisystem character, such as developmental delay, microcephaly or premature aging, among others. In conclusion, dyskeratosis congenita contains many disorders within the same disease: DC can be seen as a hematological disease due to BMF; it can be seen as an aging disorder, but also as a developmental disorder; DC is a cancer syndrome and an oral condition. And the list keeps increasing as the disease is further understood.

Nevertheless, from the highly heterogeneous phenotype in Dyskeratosis Congenita, in the present study we will only focus on the epithelial manifestations of the disease.

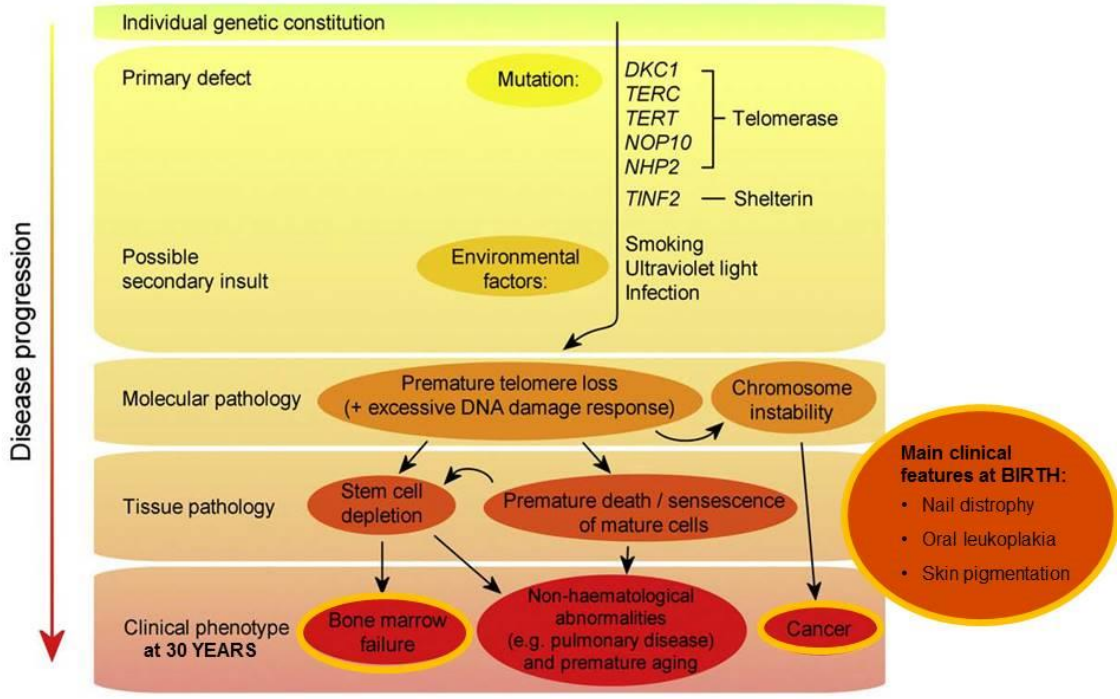

Fig 6: A model of dyskeratosis congenita pathophisiology [10]. The main phenotype features of the disease are highlighted: Initially at birth, nail distrophy, oral leukoplakia and skin pigmentation are the most common manifestations of the disease. However, bone marrow failure and enhanced cancer predisposition become the main manifestations of DC at the age of 30 years.

Causes

Dyskeratosis Congenita is a telomeropathy, meaning that a defective telomerase is the ultimate responsible of the disease [12]. The reasons behind this telomerase defect are genetic: one or more mutations in the long arm of the X chromosome at gene DKC1, codifying for one of the protein subunits of the telomerase, have been shown to cause the disorder.

Telomere is the name given to the region of repetitive sequences of nucleotides found at each end of a chromosome, whose role is genetic protection. During each DNA replication, the chromosomal ends are shortened due to an impairment of the cellular replication machinery. The function of the telomere is to behave as protective caps at the ends of the chromosomes, by avoiding the deterioration of the indeed codifying genes found at the interior of the chromosome by getting themselves shortened instead.

As a consequence, telomere shortening is a cumulative process strongly linked with aging. The role of telomerase complex is to fix this.

The *telomerase* enzyme performs telomere elongation by adding a sequence of repetitive nucleotides to a shortened telomere in order to counteract the above described effect. It carries a RNA molecule which is used as a template during reverse transcription [13].

Each telomerase is a ribonucleoprotein complex made of two main subunits (Fig 7): the telomerase RNA component (TERC) and the telomerase reverse transcriptase (TERT). TERT is the catalytic subunit, which by reverse transcription produces single-stranded DNA from the template RNA at TERC. Although a functional telomerase is composed of TERT and TERC, additional proteins are found in the complex, such as dyskerin in the case of vertebrates, which stabilizes the telomere complex [14].

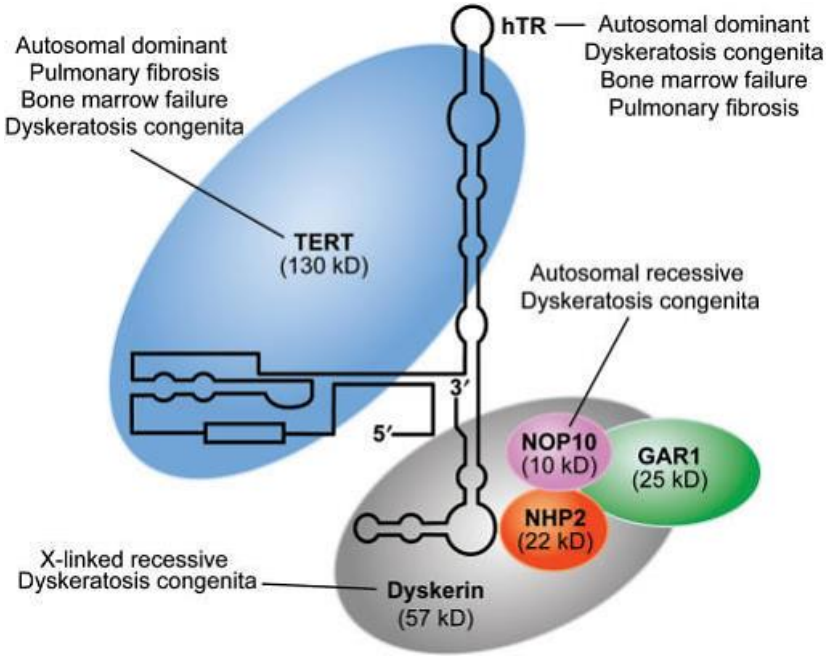

Fig 7: Structure of the telomerase complex and diseases associated with mutations in the complex subunits. Mutations in TERT, hRT (TERC) and Dyskerin lead to dyskeratosis congenita. **[15]**

The genes codifying for the different telomerase subunits are located on different chromosomes. In particular, H/ACA ribonucleoprotein complex subunit 4 or *dyskerin* is encoded by gene DKC1 in humans, located at chromosome X [16]. Dyskeratosis congenita is mainly caused in a 35% of the cases by an X-linked recessive DKC1 mutation, whereas a 5% of the cases the mutation takes place at TERT and TERC loci. The disease has been found to have a significantly higher incidence in males due to its location at the X-chromosome, whereas women are often recessive female carriers [17].

In the present study we will focus on the predominant X-linked recessive Dyskeratosis Congenita caused by a mutation in gene DKC1 (Fig 8).

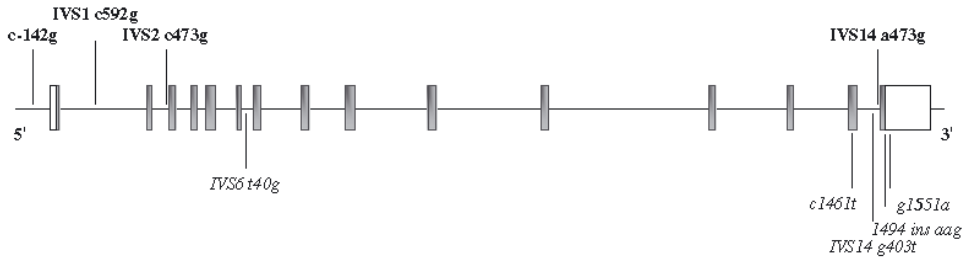

Fig 8: Mutations in the DKC1 gene, developing dyskeratosis congenita. Three splicing mutations and one promoter mutation have been found in DC patients. **[18]**

In vitro DC cells exhibit short telomeres and defective telomerase activity. Typically, telomerases are active in the early stages of life whereas becoming inactive in adult somatic cells [19], except in rapidly dividing cells such as those that line the lungs, skin cells or cells in the bone marrow [20]. As a result, since Dyskeratosis congenita patients have inactive telomerases since their birth, chromosomal instability, stem cell depletion, tissue failure and premature aging become a major risk earlier than expected in healthy individuals [21]. Furthermore, it is important to highlight that those tissues formed by rapidly dividing cells become the most damaged under DC; since replication is particularly frequent in these cells, telomere shortening is accelerated and telomerase function becomes more critical. The main manifestations in DC, such as skin disorders or BMF, illustrate the burden of these rapidly dividing tissues.

However, the origin of DC is not merely genetic. Two DC patients with a mutation in the same gene can have different manifestations of the disease. Genotype-phenoype relation is complex, and it is strongly influenced by underlying hypomorphic gene mutations, and genetic and environmental modifier effects [22].

Treatment

Up to the present moment, no cure for Dyskeratosis congenita has been officially found yet. In fact the disease has only been studied during the last fifteen years. However, since the phenotypes of the patients are so heterogeneous in terms of clinical features and seriousness, the scientific community has not reached a consensus about how to efficiently treat the patients of this rare disease [23]. So far, a variety of treatments directed to the different specific symptoms of the disease have been applied.

The general treatment indications currently given to DC patients are usually rather a guide for good practices than a real therapy: smoking and alcohol are avoided in order to preserve the liver and the lungs, moisturizing creams are commonly used to prevent skin damage and good dental hygiene is recommended to prevent early tooth loss.

Androgens and hematopoietic growth hormones have been used as *short-term therapies* for the BMF and immunodeficiency characteristics of the disease. Androgens such as oxymetholone have eventually shown to increase red blood cells production, and to reduce bleeding associated with thrombocytopenia when combined with corticosteroids [24]. This result might be explained by the observed telomerase modulation under the effects of sex hormones [21]. On the other hand, hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) have transiently increased neutrophil population in patients with BMF [25]. However, both therapies should under no circumstances be adopted together, under a risk of splenic peliosis and rupture [26].

The only currently accepted long-term therapy for BMF in DC is allogeneic *hematopoietic stem cell transplantation*, which would replace the defective DC stem cells with the healthy donor's ones [27]. This therapy would potentially cure the complications associated with bone marrow failure, but would not improve the rest of the tissues already affected by DC. In spite of the promising perspectives of hematopoietic SCT as a potential therapy, immunogenicity and graft-versus-host-disease, as in any other cell therapy, are two limiting factors to be considered [28]. Twenty-one myeloablative procedures have been so far reported by the literature; nevertheless, none of them has been considered to be successful due to the pulmonary complications observed after myeloablative hematopoietic SCT transplantation. On contrast, recent non-myeloablative conditioning protocols (i.e, reduced-intensity conditioning) have yielded successful engraftment [29]. DC patients have been reported to be extremely sensitive to myeloablation and radiation, and a recent non-myeloablative approach has shown a 22% mortality rate versus a 71% mortality rate with traditional myeloablative procedures [30].

Finally, genetic analysis might be crucial in the future for an early diagnosis of the disease. Early harvest and storage of their bone marrow could be then performed in order to be used as an immunocompatible autotransplant after anticipated marrow failure [31].

Gene Therapy

Mechanism

Gene therapy is a novel therapy belonging to future medicine, which aims to cure genetic diseases by genome manipulation. Compared with traditional therapies, gene therapy approaches medicine from a new perspective that has not been fully exploited yet: genetics. As a medicine of the future, it is expected to be personalized and preventive and will be a way to fix genetic diseases at their root.

The fundamentals of gene therapy consist on delivering DNA sequences in the cell as a drug, with the purpose of fighting a disease by allowing target cell identification and/or expression of the protein of interest. The DNA modification can include gene insertion, deletion, or replacement by another gene. Gene therapy can be classified into germline gene therapy (GGT), in which the genetic modification is heritable to subsequent generations, or *somatic cell gene therapy* (SCGT), in which it is not and the therapy results are only restricted to the actual patient. So far GGT remains controversial due to its ethical consequences, and research has only been carried out on SCGT [32]. At the same time, SCGT can be divided into *ex vivo* gene therapy, in which the cells are corrected outside the patient's body and then injected back (Fig 9), and *in vivo* gene therapy, in which genetic correction happens inside the patient's body.

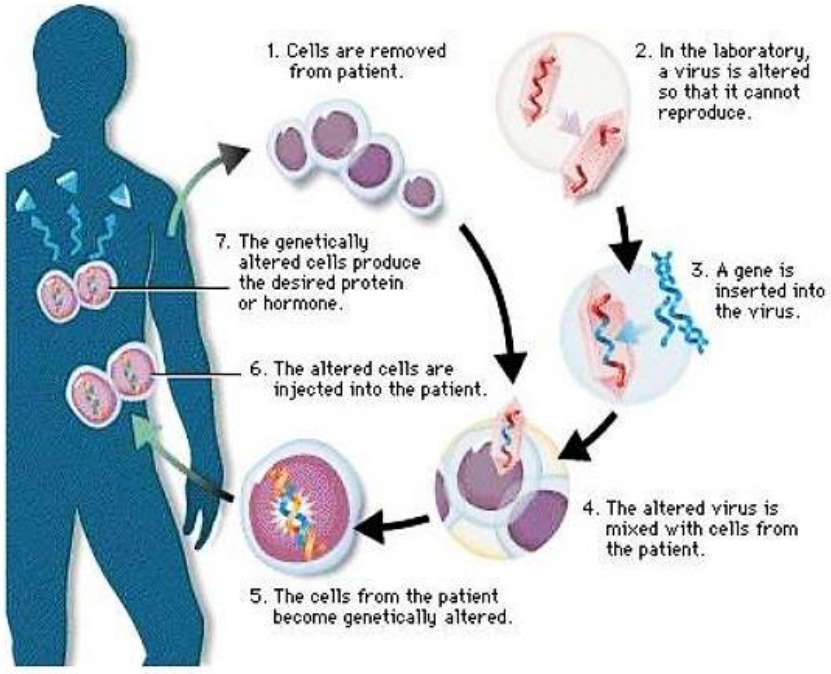

Fig 9: Steps involved in *ex vivo/in vitro* gene therapy, in which the cells are genetically modified outside the patient's body [33].

The engineered gene construct or transgene containing the DNA sequence of interest to be delivered can be designed by using different genome editing tools known as engineered nucleases. Traditionally, the restrictions enzymes or endonucleases naturally found in the cells such as EcoRI or BamHI were used. Lately, more specific and direct techniques such as Transcription-activator like effector nucleases (TALENs) or Zinc finger nucleases (ZFNs) have been developed in order to avoid multiple double strand breaks [34]. Nevertheless, the real breakthrough in genetic engineering came in the last four years with *CRISPR/Cas9*. This enzymatic complex naturally found in the prokaryotic immune system promises to be the fastest, simplest, cheapest, and most importantly, most precise and specific tool for customized genetic engineering. CRISPR/Cas9 is revolutionizing the world of genetic engineering and synthetic biology, and it was the winner of *Science Magazine*'s Breakthrough of the Year award in 2015 [35]. DNA delivery is accomplished by vectors, which will be described in the next section.

Vectors

Vectors are the carriers of the genetic material that will be used as a drug in gene therapy. The most widely used vectors in gene therapy are viruses, although recently research on non-viral vectors is being carried out since they allow simpler large scale production and low host immunogenicity. Injection of naked DNA, electroporation, and nanoparticles such as lipoplexes, polyplexes or gold nanoparticles are some of the non-viral gene therapy vectors that have been studied [36].

On the other hand, throughout evolution viruses have naturally developed a way to infect human beings and efficiently discharge their genetic material in the host cell. Gene therapy takes advantage of this simple natural vector as the most effective way to introduce DNA in a human cell. The construction of safe viral vectors is guided in packaging cells, in such a way that the disease-causing genes are replaced by the therapeutic ones.

Adenovirus, adeno-associated virus, and retrovirus have already been used in gene therapy clinical trials, being the last ones the most common scaffold in vector engineering. The genetic material in *retroviruses* appears in its RNA form; therefore it must undergo reverse transcription upon integration in the host's DNA. This procedure is carried out by reverse transcriptase and integrase respectively, two enzymes encoded by polymerase (*pol*). In general, every retrovirus requires three essential proteins that make it functional: not only the previous described *pol* gene, but also, from a structural approach, group-specific antigen (*gag*) coding for the retroviral core proteins and

envelope (*env*) coding for the viral coat proteins. However, for safety reasons, in gene therapy the retroviruses never carry these three genes required for their replication and survival and their empty space is filled with the transgene. Gene therapy retroviruses are produced in packaging cells, typically HEK 293T cell line, transfected with different packaging plasmids containing *gag/pol*, *env, rev* for regulation and the transgene *trans* of interest (Fig 10). Through this procedure, the enzymes required for building up the virus are externally produced, whereas the transgene becomes the new genetic material in the retrovirus. Upon integration in the host's genome, the engineered gene will be inserted but not the viral promoters, so there will be no viral proliferation.

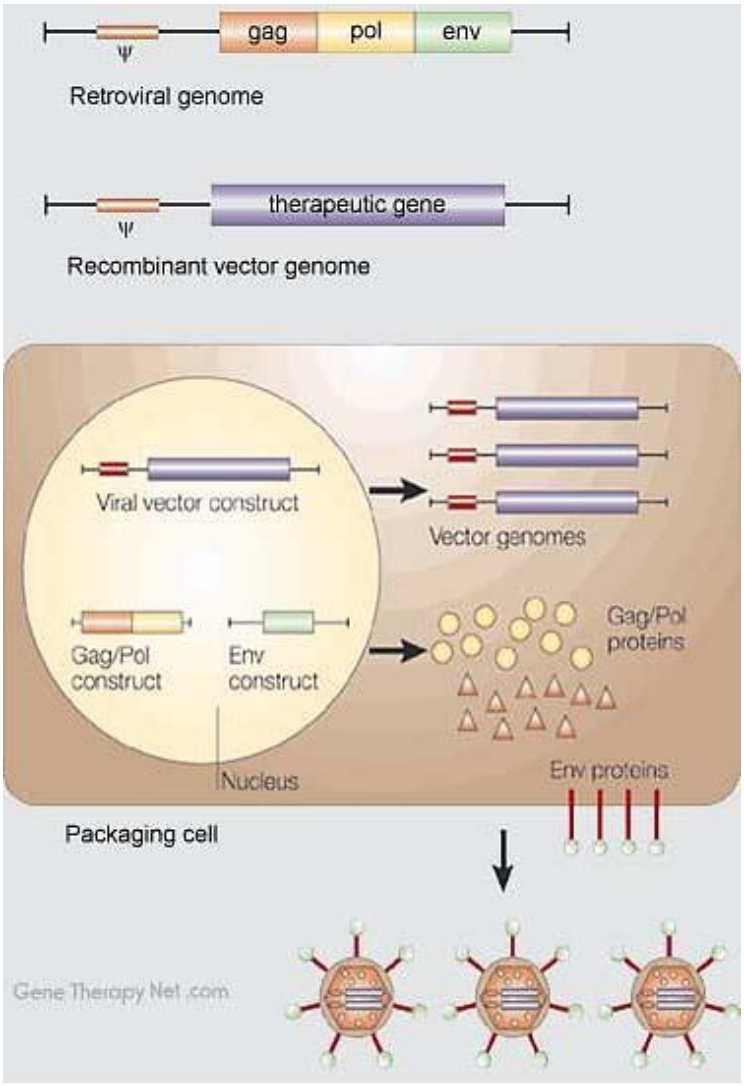

Fig 10: Production of retroviral vectors in packaging cells [34].

Lentiviruses and γ-retroviruses are the most common gene therapy vectors used from the *retrovidae* family. However, their main drawback is that integration in the patient's genome is arbitrary: gene disruption and activation of proto-oncogenes are important

factors of risks when using these vectors. In particular, lentiviruses are interesting vectors in gene therapy because they can integrate into the genome of non-dividing cells, such as in the case of keratinocytes, which cycle significantly slowly [37]; moreover, recent studies have shown that lentiviruses have lower tendency to induce cancer than other vectors [38]. HIV-1 (human immunodeficiency virus 1), represented in Fig 11, is the most common lentiviral vector.

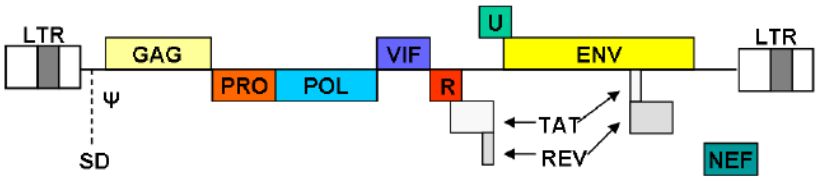

Fig 11: Scheme of lentiviral genome of HIV-1. In gene therapy, the coloured viral sequences tat, rev, gag, pro, pol, nef, vif, vpr and vpu are replaced by the therapeutic transgene.

In the present study, *ex vivo / in vitro* gene therapy will be carried out using modified HIV-1 lentivirus as the gene GSE4 (derived from DKC1 gene) delivery vectors.

History and State-of-the-art

The first evidences of gene therapy appeared in 1969 when a research group from Tenesse achieved arginase proficiency in arginase deficient animals and humans through Shope papilloma virus infection [39]. The first attempt at modifying human genome dates back to 1980, although the first successful human gene transfer was performed in 1989 [40]. One year after, the first approved clinical research took place in the National Institute of Health in the United States, under the direction of William French Anderson. The results obtained from that primitive gene therapy were temporary, but successful [41].

Even since then, 2200 clinical trials [42] have been conducted and huge technological advances have taken place in the field of gene therapy. The therapy has been applied to most of the organs in the human body: for instance, in 2003 genes were inserted into brain for the first time [43]. Currently, a gene therapy product for Fanconi Anemia treatment is being developed at phase I/II by a research group from CIEMAT [44]. The highest breakthroughs have taken place in the last five years, during which several drugs based on novel gene therapy have been approved: Gendicine in China in 2003 [45], Neovasculgen for peripheral artery disease in Russia in 2011 [46], Glybera for pancreatitis in 2012 [47] or Strimvelis for ADA-SCID disease in "bubble-babys" in

April 2016 [48]; Glybera and Strimvelis are the only two gene therapy treatments approved so far in Europe.

On the other hand, a paradigm shift in gene therapy has arisen in the last years with of CRISPR/Cas9. Its recent presentation as a new way of specifically editing the genome offers a new promising approach to gene therapy.

In conclusion, even though gene therapy is still in its infancy, it is already stablishing itself as the medicine of the future. Nevertheless, the technology still has some challenges to overcome: the current methods of gene therapy are short lived and would require of multiple rounds of treatment; also, gene therapies for multigene disorders such as Alzheimer's disease or diabetes still have to be developed. However, the greatest challenges are still the immunogenic and tumorigenic risks posed by the current viral vectors.

Skin Bioengineering

Fibrin Scaffold

Regenerative Medicine aims to stimulate the body's intrinsic regenerative capacity, mainly through the activity of stem cells. Tissue Engineering studies the strategies to artificially create tissues and organs. And, both of them, Tissue Engineering and Regenerative Medicine (TERM), are emerging fields also belonging to the previously described medicine of the future. The classical paradigm in tissue engineering consists of three main elements: a biocompatible scaffold of the tissue of interest, the cells that will seed this scaffold, and the signaling molecules allowing the similar to *in vivo* behavior of these cells in the scaffold [49].

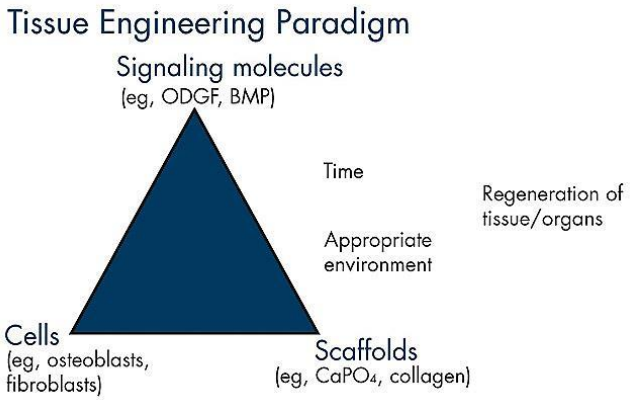

Fig 12: The three main elements in the classical paradigm of tissue engineering [49].

When tissue engineering focuses on developing artificial skin, the result is skin bioengineering. Hydrogel scaffolds have proved to be the most useful ones in skin bioengineering due to their soft tissue ECM-like stiffness and their capacity of controlled release of signaling molecules and nutrient diffusion [50], and in particular *fibrin hydrogel scaffolds* (World Patent WO/2002/072800) [51]. Fibrin hydrogels resemble the mechanisms of *wound healing* to induce *in vitro* skin growth just like it would naturally take place in our bodies [52]:

i. *Blood clotting*: Platelets aggregate into a platelet plug, then they activate and release clotting factors that will initiate the coagulation cascade (Fig 13) and ultimately end with the synthesis of fibrin. Under the presence of calcium ion found in plasma, thrombin produces fibrinogen, a precursor of fibrin.

ii. Formation of a stabilizing *matrix of fibrin* that behaves as a temporary ECM and enhances cell adhesion and platelet activation.

iii. *Remodeling*: Fibroblasts migrate to the wound site and contract the skin gap by proliferation, creating a scab. Finally, fibroblasts secrete collagen that gradually replaces the temporary fibrin matrix by permanent collagen ECM. At the same time, epithelization takes place and the stratified skin structure is formed.

In skin bioengineering, the fibrin hydrogel is formed *in vitro* by artificially mimicking the above described wound healing cascade, as represented in Fig 13. Plasma thrombin, fibrinogen and $CaCl_2$ are mixed and the fibrin matrix is spontaneously formed just as it would under the existence of a wound. On the other hand, and based on the tissue engineering strategy shown in Fig 12, the building blocks in skin bioengineering are keratinocytes and fibroblasts, which are seeded in the fibrin clot [53].

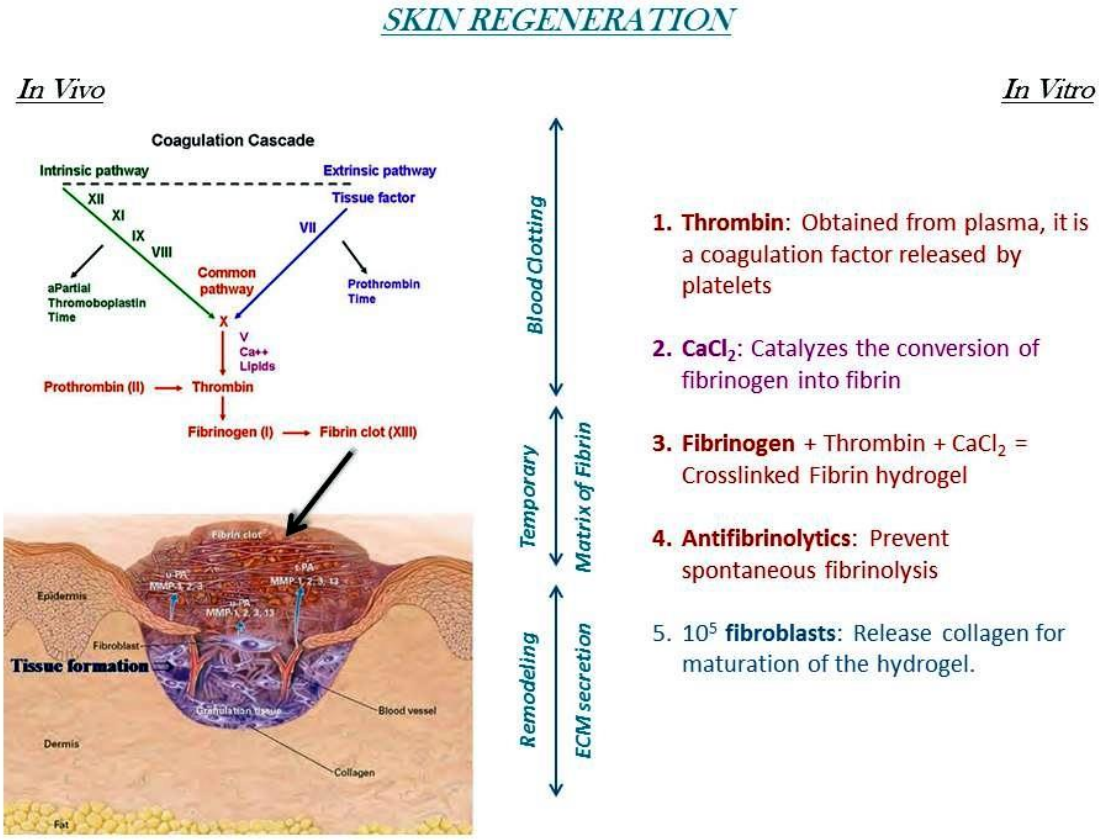

Fig 13: Comparison between the in vivo wound healing mechanism and the mimicking in vitro fibrin gelation mechanism for skin bioengineering.

Skin Equivalent Development

The third and final element in tissue engineering is the signaling molecules. Upon receiving the signaling factors released from the fibrin matrix, fibroblasts will understand that it is a wound and they will release collagen, which will ultimately form the final, mature ECM matrix. This part of the hydrogel would resemble the skin dermis.

Above the *in vitro* dermis, the epidermis is created from keratinocytes and epidermal stem cells, which are initially seeded into a cellular monolayer. However, the calcium gradient in the keratinocytes media also serves as a signaling factor during this process

of epithelization, in which the keratinocytes are exposed to an air-liquid interface resembling the apical and basal epidermal surfaces respectively, thus inducing terminal epidermal differentiation and stratification into a mature epidermis [54]. The final structure of the skin equivalent is displayed in Fig 14:

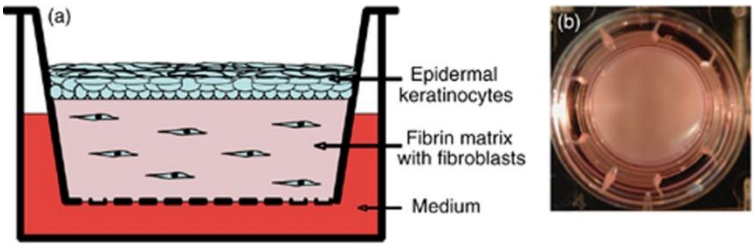

Fig 14: Fibrin-based Skin Equivalent structure [51]. (A) Schematic of a skin equivalent in a cell culture with a PET membrane (1 μm pores). The surface keratinocytes are exposed to air, whereas the matrix is few by medium from below. (B) Skin equivalent after incubation for 17 days at the air/liquid interface showing an intact and dry surface of stratum corneum, indicating successful epidermal stratification.

In the present study, a bioengineered skin equivalent will be created from Dyskeratosis congenita keratinocytes corrected by gene therapy.

State-Of-The-Art

Dyskeratosis Congenita is a disease that has not been understood until the most recent fifteen years, and that has not been targeted at its root by any therapy yet. Due to its genetic origin, gene therapy would be the most logical treatment for such a disorder; nevertheless, no projects on gene therapy for Dyskeratosis congenita have been reported in the literature [55]. In the present section, the most recent approaches of regenerative medicine in DC and the models of the disease will be analyzed. In addition, the studies from which the present gene therapy project is based on will be described.

In vitro DC models

Lately, several groups have tried to model Dyskeratosis congenita in mice with varying success [56]. One of the earliest *mice models* was carried out by Ruggero *et al* in 2003: they developed a line of DKC1 mice and reproduced many features of DC in the model, such as reduced telomere length, increased predisposition to cancer, and decreased cellularity in the bone marrow. However, their model reported additional peculiarities that had not been observed in DC patients [57]. In 2008, Hockemeyer *et al* reproduced hyper pigmentation of the skin, bone marrow failure and nail dystrophy in their mice model; however, their model was developed from the mutation in a gene that does not exist in human DC [58]. In general, the problem with the developed mice models is that none of them provides a faithful replication of human DC due to its highly variable phenotype.

In 2010, Agarwal *et al*. published an article in *Nature* that has created high expectations in DC modelling among the scientific community. The project, carried out by research groups from Harvard University, the National University of Singapore and Nankai University in China between others, studied the disease in *induced Pluripotent Stem Cells (iPSCs)* from DC patients [59]. Recalling that DC is caused by a defect in human telomerase, and considering that telomerase is always active in those continuously dividing cells, the group studied whether reprogramming somatic DC cells into iPSCs would restore telomerase limitations. The results showed that reprogramming did indeed restore telomere elongation and self-renewal; however, upon differentiation of the DC iPSCs, telomere length decreased back to the original pathological low levels. This indicated that DC cell reprogramming would be useful for studying *in vitro* the mechanisms of the disease, but not for curing it at the somatic level.

Characterization of DC keratinocytes

On the same year 2010, a study stablishing the phenotypic differences between DC fibroblasts and DC keratinocytes was published by Gourronc *et al*. This group studied how in DC patients, both fibroblasts and keratinocytes exhibited short telomeres and proliferative defects and how the exogenous expression of the TERC component of the telomerase in these cells would reverse these defects in keratinocytes but not in fibroblasts. [60] This result relies on the natural difference in telomerase regulation in healthy cells: under normal, healthy conditions, keratinocytes have some active telomerase whereas telomerase in fibroblasts is not detectable [61]. Therefore, the exogenous expression of TERC would return the DC keratinocytes to their healthy state: some telomerase activity in keratinocytes but none in fibroblasts. This study carried out by Gourronc *et al*. shows how in the epidermal manifestations of DC, the significantly affected tissue is only the epidermis, not the dermis. Based on this study, in the present project only DC keratinocytes will be corrected by gene therapy.

GSE4

Once all the fundamentals on the basis and on the therapy that will be studied in the present project have been reviewed, it is important to recapitulate on what the present research aims to achieve: In this study, X-linked recessive DC keratinocytes will be transduced with HIV-1 lentiviral vectors in order to correct the defect in dyskerin through gene therapy. The last element to describe in this strategy is the transgene that will be delivered as a drug by the lentiviruses: gene GSE4, a recently discovered short dyskerin-related peptide.

The first outlines of GSE4 date back to 2008, when a research group from CSIC research center in Spain discovered its precursor, GSE24-2 during a screening for cisplatin resistance during chemotherapy [62]. GSE24-2 was the name given to the 55 amino acids-long genetic suppressor element (GSE) belonging to the *pseudouridine synthase domain (TRUB) in DKC1* gene. GSE24-4 was found to induce impaired telomerase inhibition into those cells that had been exposed to chemotherapy or telomerase inhibition – meaning that these cells recovered a high telomerase activity. In addition, the study discovered that the promoter of the hTERT telomerase component was constitutively activated in GSE24-2 cells, meaning that telomerase was indeed activated under the expression of GSE24-2. This study was the first one in suggesting that expression of the dyskerin motif GSE24-2 induced the recovery of telomerase activity and prevented premature senescence in DC cells in a dominant manner.

Four years later, the same research group, in collaboration with scientists from The Children's Hospital of Philadelphia, published a second article in which they further studied the effects of *GSE24.2*, either in the form of cDNA or as a peptide, on an X-DC mouse line [63]. The two forms, both produced in bacteria and chemically synthesized, appeared to reduce the pathogenic effects of DKC1 deficiency. In addition, the study revealed that point mutations in two highly-conserved GSE24.2 residues, known to be involved in the pseudouridine-synthase activity of dyskerin reversed the therapeutic effects of GSE24.2.

Ever since then, GSE24.2 has been patented by the research group (EP 1947175 A1); the European Medicines Agency has approved GSE24.2 as an orphan drug for DC treatment (EU/3/12/1070-EMA/OD/136/11); and a genetically customized antiaging cosmetic cream based on GSE24.2 peptide has been commercialized by Advanced Medical Projects® in collaboration with the University of California [64].

At the end of 2015, the same research group from CSIC along with further collaborators isolated a new shorter peptide derived from GSE24.2: GSE4. *GSE4* is an eleven amino acids long peptide that exhibits the same therapeutic properties as its precursor; namely, increased telomerase activity, reduced cell senescence or activation of hTERT promoter [65]. The interesting feature of GSE4 is that its shorter size presents some additional value over previous GSE24.2: the chemical synthesis would have a lower cost, cellular delivery would be easier, and manipulation and development of variants would also be easier. The role of the present project is to perform the next logical step in the study of GSE4 in DC patients after the publication of this paper: studying the *in vitro* development of cells expressing GSE4.

GSE4 corresponds to the N-terminal region of GSE24.2, which was partially degraded into smaller peptides by mass spectrometry. GSE4 fragment was cloned from the GSE24.2 plasmid using PstI and BamHI restriction sites into the final plasmid pRRL-CMV-GSE4-IRES-eGFP.

In the present study, this pRRL-CMV-GSE4-IRES-eGFP plasmid (Fig 15 A) will be the engineered genetic material in a HIV-1 lentiviral vector (Fig 15 B). Plasmid pRRL is characterized for having adjacent U3 and R regions [66]. Since the lentiviruses are synthesized in packaging cells, the final vector does not include the gag, pol and rev regions present in Wild Type lentivirus. From a general framework, the transgene GSE4-IRES-eGFP is limited at the ends by Long Terminal Repeats (LTR) regions. LTRs allow the lentivirus to insert its genetic material into the host genome. The

expression of the gene of interest is regulated by CMV, the promoter of cytomegalovirus. The incorporation of an Internal Ribosome Entry Site (IRES) sequence allows the coexpression of two coding sequences under the same promoter; in our case, IRES allowed the simultaneous expression of DC and GFP gene under CMV promoter [67]. After the transgene there is a wPRE post-transcriptional regulatory sequence.

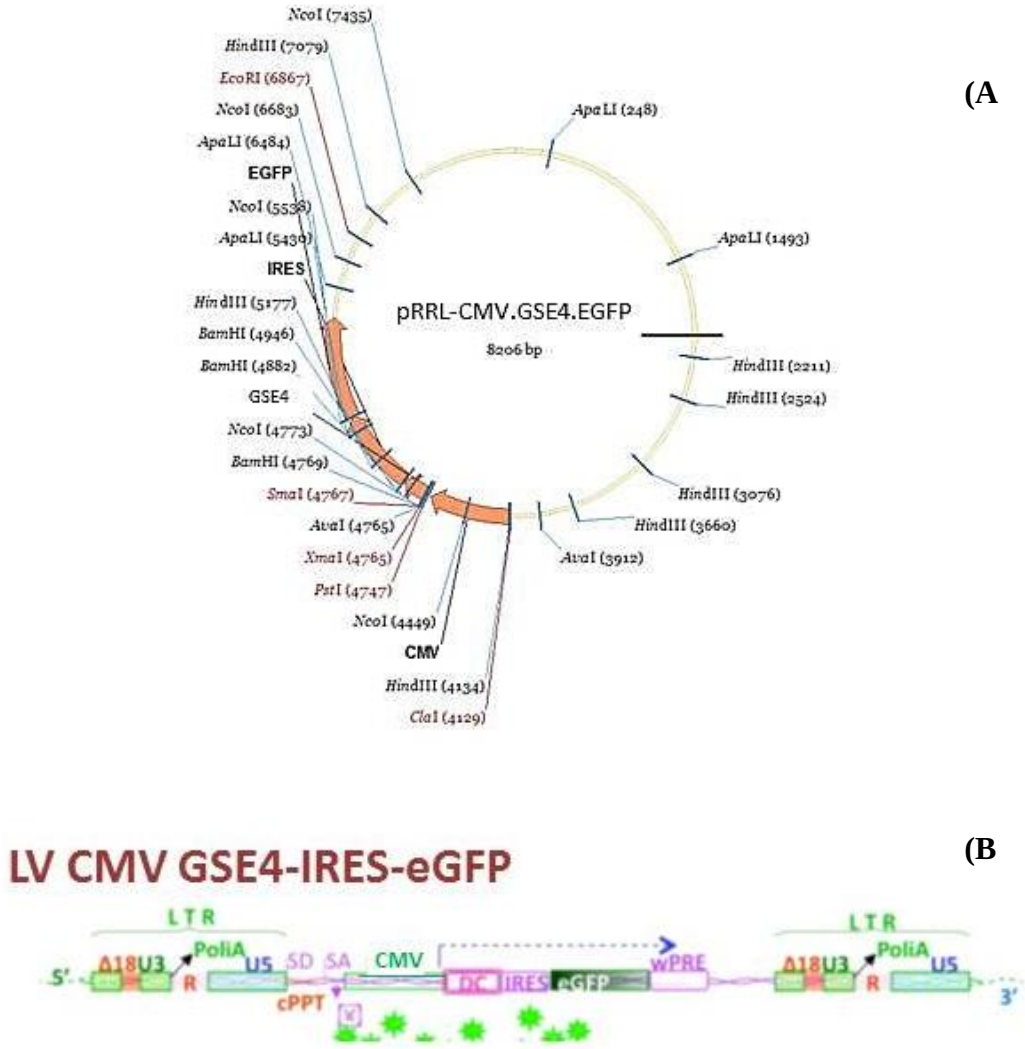

Fig 15: Structure of the GSE4 fragments that will be used in the present study [65]. (A) Plasmid **pRRL-CMV-GSE4-IRES-eGFP** that will transfect the packaging cells for creating the vector. (B) Lentiviral vector **LV CMV-GSE4-IRES-eGFP** that will transduce the DC keratinocytes during gene therapy.

Materials and Methods

The experimental procedure followed during the present project could be divided into three consecutive stages, each of them focusing on a different technique, but the three of them having a common ultimate goal: *in vitro* gene therapy. The diagram below represents a global overview of the experimental procedure:

Fig 16: Schematic overview of the steps followed in the present experimental procedure of *in vitro* gene therapy.

The main cells used in the experimental procedure were primary keratinocytes (epidermal stem cells) obtained through skin biopsy from patients suffering from DC as well as healthy patients. Skin biopsy was obtained from patients after approval from the Ethics Committee of the collaborator hospital upon informed consent. The distinguishing feature of study in the present project was Dyskeratosis Congenita; therefore, the variable groups along the experimental procedure were:

Keratinocytes	Lentiviral Infection: Control (GFP)	Lentiviral Infection: Transduced (GSE4)
DC patient	#64	#64
	#157	#157
Control (Healthy)	Healthy patient	

Table 1: Groups of study in the present experiment.

1. **Gene Therapy:**
    - *Aim*: To genetically correct Dyskeratosis Congenita keratinocytes.
    - *Description*: The lentiviruses that would work as vectors in gene therapy were prepared in packaging cells. These lentiviruses contained the GSE4 gene that would be inserted into the target keratinocytes upon their infection.
    - *Location*: Bioengineering laboratories (UC3M).
2. **In vitro Skin:**
    - *Aim*: To culture a skin equivalent using the corrected keratinocytes.
    - *Description*: The previously transduced keratinocytes were co-cultured with fibroblasts in a 3D fibrin hydrogel. The 3D co-culture self-organized into real skin structure, which is divided into layers.
    - *Location*: Bioengineering laboratories (UC3M).
3. **Histology:**
    - *Aim*: To process the *in vitro* grown skin into histological slides in order to assess them using optical microscopy.
    - *Description*: The different grown skin models underwent formaldehyde fixation, ethanol dehydration and paraffin embedding before being sectioned using rotary and cryostat microtomes. Then, H&E staining allowed distinguishing the modelled skin structure.
    - *Location*: Histology Laboratory (CIEMAT).

The above outlined experimental stages will be next in detail described.

1. Lentiviral Transduction of Keratinocytes *(Gene Therapy)*

Cell culture.

293T [human embryonic kidney, (ATCC)] cells was the cell line used as packaging cells for the lentivirus. Six p100 dishes (Fisher Scientific) were prepared, each of them containing 2 million of 293T cells. DMEM [Dulbecco's Modified Eagle Medium, (Fisher Scientific)], which had 10% of inactivated Fetal Bovine Serum [FBS, (Fisher Scientific)] and 1% of antibiotic (Penicillin-Stretomycin), was used for 293T cell culture. However, prior to transduction, 293T cells were cultured with inactivated keratinocytes medium (Kci) in order to ensure compatibility with the target cells.

Along the whole experimental procedure, all the keratinocytes from the three patients previously described were cultured using specific keratinocytes medium (Kca). Kca was made of Dulbecco´s modified Eagle medium containing Ham's F12 medium [F12 Nut Mix 1X + Glutamax (GIBCO®)], 10% fetal bovine serum, 1.3 ng/ml of triiodothyronine [T3, (Sigma Aldrich)], 5 mg/ml of insulin (BioWhittaker), 10 ng/ml of epidermal growth factor (EGF), 24 mg/ml of adenine (Sigma Aldrich), 0.4 mg/ml hydrocortisone (Sigma Aldrich), 8 ng/ml cholera enterotoxin and antibiotic/antimycotic 1% Keratinocyte Serum Free Medium (GIBCO®). However, during transduction inactivated keratinocytes medium (Kci) was used in order not to repress viral activity. The keratinocytes were cultured over a layer of feeder cells (ATCC), which are metabolically active but mitotically inactive irradiated fibroblasts. During the experimental procedure #157 keratinocytes reached confluence, and the cell culture was subcultured.

Lentivirus production.

293T packaging cells produce non-replicative lentivirus vectors. The different elements composing the virus capsule and polymerization machinery were added to 293T cells along with the transgene of interest. Chloroquine (Sigma Aldrich), which worked as a membrane permeabilizer, was also added. The exact viral components to add were:

- Gene *gag-pol* for the reverse transcriptase: 5 µg.
- Gene *rev* for the regulatory proteins: 2,5 µg.
- Gene *env* for the viral envelope: 2,25 µg.
- Gene *trans* (GFP and GSE4-IRES-eGFP): 9 µg.

The composition of gene *trans* varied depending on the group that the viral vector to construct would infect. As shown in Table 1, there were two different groups in lentiviral infection: the GSE4-IRES-eGFP transgene would be used for *transduced* groups, whereas GFP transgene would be used for *control* groups. In both cases, the pRRL plasmids containing the transgene were kindly provided by Dra. Perona. As an illustration of the composition of the engineered plasmids, the control transgene is represented in Fig 17, whereas the GSE4 + GFP transgene was already introduced in Fig 15. This control transgene included uniquely the Green Fluorescence Protein gene, which only worked as a reporter and did not have any therapeutic function.

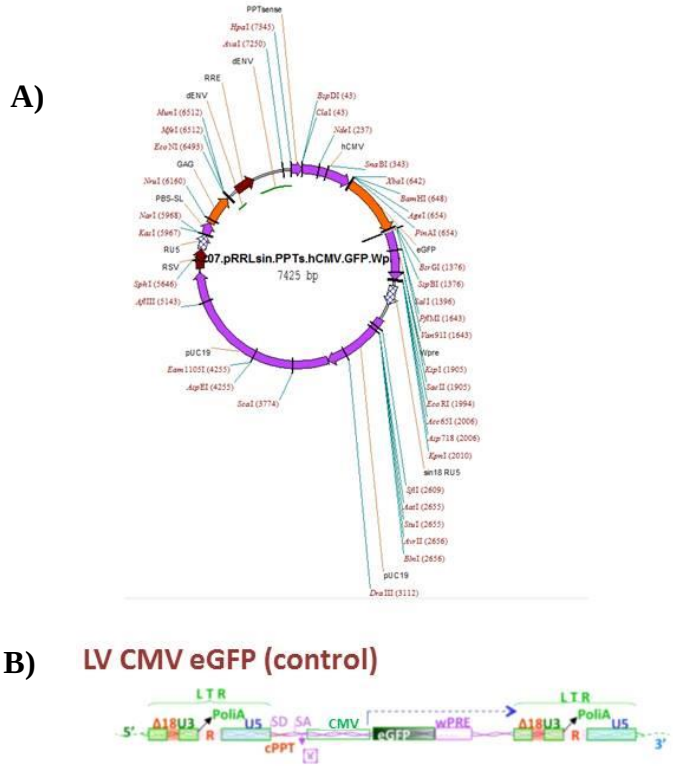

Fig 17: Schematic composition of the control transgene [65]. (A) GFP control plasmid. (B) GFP control LV vector.

Transduction.

In order to carry out viral infection, 3,5 mL of the inactivated media containing the 293T packaging cells along with the viral vectors in the supernatant were added to the corresponding target dish, either GSE4 groups or control groups. This media was filtered using 0,2 µm PuraDisc filters (Fisher Scientific), so that 293T cells were not added to the keratinocytes culture. Finally, Polybrene [hexadimethrine bromide, (Sigma Aldrich)] was used as a transduction enhancer. 1 µL of Polybrene was added per 1 mL of viral media, so in total 3,5 µL were added to each keratinocytes culture. The cell

cultures were incubated at 37 ºC during 6 hours, after which medium was changed and incubation at 37ºC continued. One day after the first viral infection, which could be assessed under blue-light microscopy (BX53 Olimpus) for detecting fluorescence in the keratinocytes culture, the procedure was repeated using a different plasmid concentration.

Transduced keratinocytes were selected, isolated and subcultured in 75 mL T-flasks (Thermo Fisher) containing 2 million feeder cells and 15 mL of activated keratinocytes medium each. This time Kca was used because viral activity was no more of interest. For three weeks, the cell cultures were allowed to grow. Then, cell sorting was applied to the GSE4 transduced cells on a Cell Sorter (BD Influx^{TM}) which carried out a complete selection of these cell cultures based on their fluorescence characteristics. The remaining sorted cells were again subcultured and allowed for further amplification, this time with assured 100% transduction. On the other hand, during this extended period of time, control samples had been kept frozen to guarantee their viability.

2. 3D Co-culture for the Construction of a Bioengineered Corrected Skin Equivalent *(In Vitro Skin)*

Fibrin Scaffold.

The bioengineered skin equivalents would be grown following tissue engineering strategy (Fig 12): the previously transduced keratinocytes would be seeded in a scaffold. The used scaffold was a hydrogel initially composed of fibrin, mimicking the natural skin extracellular matrix. The fibrin in the scaffold would gradually be substituted by collagen fibers, naturally secreted by the fibroblasts which would also be embedded in the grafts.

The different cellular elements required for the construction of the fibrin matrix, which had been kept frozen to guarantee their prolonged viability –except for sorted transduced #157 keratinocytes-, were unfrozen and cultured in T75 flasks. During the unfreezing procedure, the cells, the freezing media (1 mL) and 4 mL of DMEM10 were mixed and then centrifuged for 10 minutes. Then, supernatant was removed by vacuum, thus getting rid of the harmful freezing media which is toxic in liquid conditions. The final compositions of the four different T75 culture flasks are shown in Table 2:

T75 Flask	1	2	3	4
Skin cells	2 ml Healthy Fibroblasts	2 ml #157 Fibroblasts	2 ml Healthy Keratinocytes	2 ml Control #157 Keratinocytes
Feeder	0 ml Feeder	0 ml Feeder	2 ml Feeder (2M)	2 ml Feeder (2M)
Media	12 ml DMEM10	12 ml DMEM10	10 ml Kca	10 ml Kca

Table 2: Composition of the T75 culture flasks after unfreezing the samples, which would be later used for the fibrin scaffold.

On the other hand, the previous feeder cells from the GSE4 #157 keratinocytes culture were removed by partial trypsinization, since fibroblasts would unstick faster than keratinocytes when Trypsin (Sigma Aldrich) was added. Then, the GSE4 #157 keratinocytes were cultured in a fifth T75 flask with Kca and new feeder cells.

The hydrogels for *in vitro* skin developments were made in 6 well plates containing a porous membrane (1µm), since a liquid-air interface was needed (DD Biolabs). First, fibroblasts at a density of $1 \cdot 10^5$ fibroblasts/well coming from the healthy and the DC #157 patients were added to each transwell (1 mL). The hydrogel (Vf=3,5 mL) followed the clotting cascade naturally found during wound healing, *in vitro* reproduced by adding to each transwell:

1) 1,5 ml Kca, in order to get to the desired final volume, 3,5 mL.
2) 30 µl antifibrinolytic Amchafibrin (Fides Ecopharma) which prevented fibrinolysis during fibrin interaction with proteases.
3) 250 µl bovine plasma thrombin (Sigma Aldrich) in $CaCl_2$ 25 mM, since both of them activated the clotting cascade and the reaction of fibrinogen into fibrin.
4) Finally, 750 µl of porcine cryoprecipitate (Sigma Aldrich) was added. This final element contained fibrinogen, thus being the main responsible of creating the skin matrix. Once this element was added, gelation quickly took place. Therefore, this step had to be quickly performed with a 5 ml pipette.

Two hours after the creation of the hydrogel, Kca was added to the top and to the bottom of the transwell. The keratinocytes we worked with during the experimental procedure were epidermal stem cells with the ability to divide unless differentiation took place; this step was performed in order to avoid keratinocytes differentiation during gel creation caused by high concentrations of calcium. Since the added Kca media absorbed calcium, calcium concentration was balanced. Keratinocytes would be allowed to terminally differentiate 17 days after creating the gel, during gel maturation.

As shown in Fig 18, three fibrin gels were made for the control healthy groups, whereas six gels were made with patient #157's fibroblasts – three of which would grow control keratinocytes, and the other three the GSE4 transduced keratinocytes.

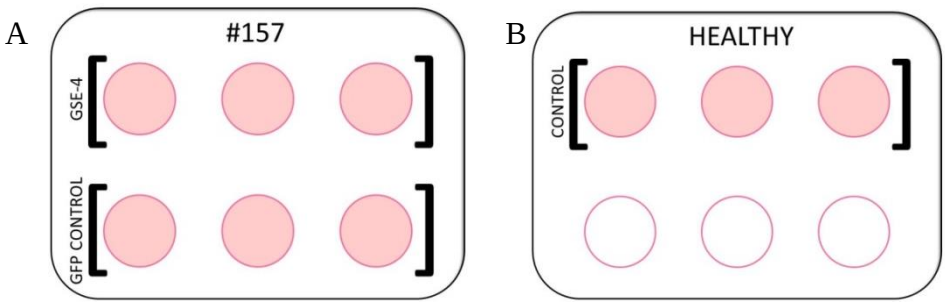

Fig 18: Diagram of the 6 well plates for the fibrin gels. (A) #157 patient, in which all transwells were seeded with Dyskeratosis Congenita #157 patient's fibroblasts. (B) Control sample, grown with healthy fibroblasts.

Cell Seeding.

Two days after the creation of the fibrin gels, keratinocytes were added to the corresponding gels. In order to perform this, the T75 flasks were trypsinized twice: once to remove the feeder cells, since the fibrin gels already had the patient's fibroblasts, and

twice to unstick the keratinocytes. Half of the keratinocytes from GSE4 #157 and Control #157 samples were kept in cryopreservative Dimethyl Sulfoxide [DMSO, (Sigma Aldrich)] for prolonged maintenance.

Keratinocytes on top of the gels were allowed to grow until confluence. Media was changed at the top and the bottom of the transwells every three days.

Keratinocytes Differentiation.

Once fluorescence microscopy inspection showed that top keratinocyte layer was homogeneously fluorescent in the gels, keratinocytes differentiation was initiated. This step took place 8 days after gel creation for #157 samples, and 17 days after in the case of the healthy samples.

In this step, the gels were moved to bigger volume transwells (Fisher Scientific). The top of the gels was dried, which would induce keratinocytes differentiation at the air-liquid interface by the previously explained mechanism of increased calcium concentration. 10 mL of differentiation media (Kca with 0,5% serum) were added to the bottom of the transwells. The big volume of the transwells would avoid the top layer of the gels to get in contact with any media by accidental capillarization.

The organotypic cultures were allowed to further differentiate at 37ºC in a CO_2 incubator during twenty-one days, media being changed twice per week, after which the histological procedure was started.

A timeline of the 3D Co-culture for the Construction of a Bioengineered Treated Skin Equivalent is shown in the diagram below:

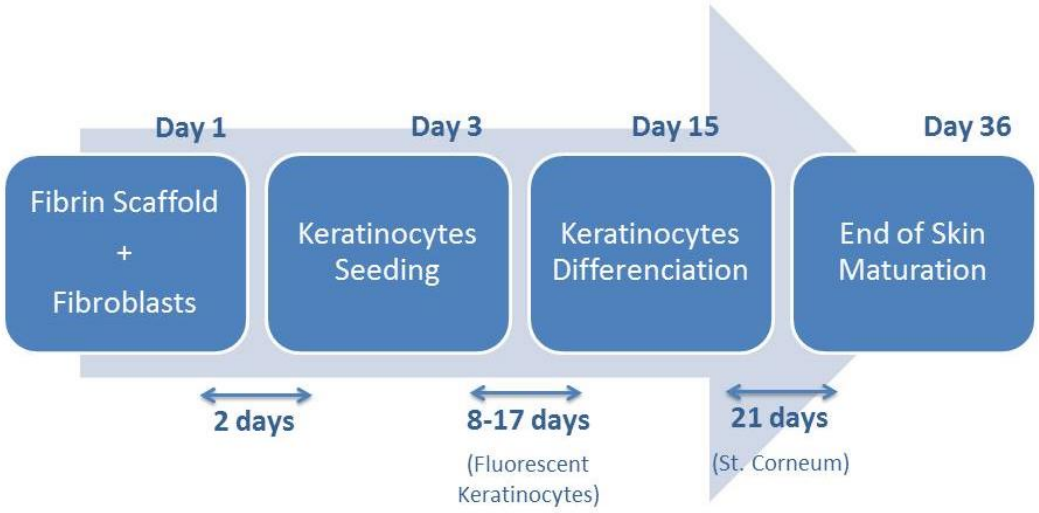

Table 3: Timeline of the second step of the experimental procedure, in vitro skin development.

3. Histology of the Bioengineered Treated Skin Equivalent

Fixation.

The grown skin equivalents were removed twenty-one days after differentiation was started. Every gel was aimed to undergo both paraffin sectioning and cryostat sectioning, therefore every gel was removed from the transwell and cut into two halves. Half of the tissue would be embedded in Tissue-Tek [Optimal Cutting Temperature compound, (Sakura Finetek)], a matrix for cryostat sectioning, and kept at -20ºC. The other half would be immersed in Formaldehyde (Sigma Aldrich) for fixation. Fixation preserved the tissue structure and prevented decay, by stopping enzyme activity. The tissues stayed immersed in formaldehyde for four days, and then further processed.

Processing.

The skin samples were then gradually dehydrated before being embedded in paraffin wax, which would serve as a solid support during sectioning. In a series of consecutive compatible steps (Table 4), the samples were first immersed in hydrophilic 70% ethanol, then in xylene as an intermediate step, and finally in hydrophobic paraffin (liquid at 60ºC). The first step was performed manually, whereas the second last steps were performed by LEICA ASP 3005 ^{TM} tissue processor at CIEMAT research center.

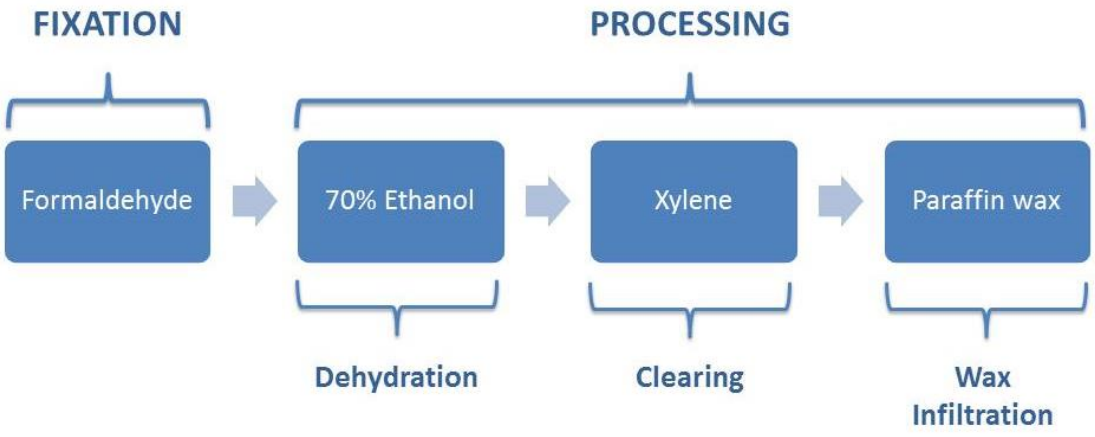

Table 4: Fixation and processing during histological preparation.

Sectioning.

Those samples that would be sectioned with a microtome were first embedded in paraffin wax (solid at 20ºC) in the proper orientation. Fifteen histological sections of 2-

5 µm were obtained from each sample, divided in three groups of five sections obtained at different depths from the sample. Therefore, in total 135 histological slides were obtained. The histological slices were gently placed in 40ºC water right after their sectioning, and then manually adhered on glass slides. 135 sections were also obtained using cryostat sectioning. In this case, the samples were embedded in Tissue-Tek in order to achieve the desired orientation, which hardened at low temperatures (-12ºC) thus allowing cryostat sectioning.

Staining.

Finally, the histological sections were stained with Hematoxylin and Eosin Stain (Sigma Aldrich). Hematoxylin stained acidic nuclear components, whereas eosin stained basic cytoplasmic and extracellular components. In this procedure, gradual rehydration was performed before infiltration in the hydrophilic stains:

1) The histological sections obtained from cryostat sectioning were first immersed in PBS, and then kept for ten minutes in a mixture of methanol and acetone at -20ºC.

2) The paraffin histological sections were immersed in xylene for dissolving paraffin, and then rehydrated by being passed through several changes of alcohol.

3) Every histological section – both from paraffin and from cryostat sectioning – was rinsed in water.

4) Hematoxylin stain was applied 8 minutes

5) The sections were rinsed in water and immersed in a weak alcohol for one minute, which would remove non-specific background staining.

6) The sections are rinsed in water, and then "blued" for one minute with a weakly alkaline solution, which converts hematoxylin into a dark blue color.

7) Eosin stain was applied for one minute.

8) The histological sections were again dehydrated by passing through a series of ethanol infiltrations and final immersion in xylene.

9) Mounting with a layer of polystyrene and a glass cover slip (Fisher Scientific).

Then, the histological slides could finally be observed under the optical microscope. If the first microscopy inspection was favorable, then further immuhistochemistry staining would be carried out with the cryostat histological sections.

Results

In the herein section, the results obtained during the research project will be presented following a chronological order. Results will be separated into the three main stages of the experimental procedure:

First, the different keratinocytes populations cultured and amplified after lentiviral transduction were studied using optical microscopy. The transduction efficiency was evaluated under blue-light fluorescence microscopy.

Next, the 3D culture of transduced keratinocytes and control ones in the bioengineered skin equivalents was observed under optical microscopy.

Finally, the bioengineered skin equivalents were analyzed following histological procedures and later stained with H&E technique. The bioengineered histological samples were analyzed using optical light microscopy.

However, it is important to remark that the amount and quality of the results also followed the above described decreasing order: Lentiviral transduction of the keratinocytes yielded better results than the bioengineered skin equivalent. Therefore, and also considering that the successful transduction was the ultimate goal of our research line, most of the results will be focused on this primary stage of the project.

1. Lentiviral Transduction of Keratinocytes

The GSE4 and Control GFP lentiviruses were created in 293T packaging cells before being isolated for keratinocytes infection. Fig 19 represents the 293T cell culture in which control GFP lentiviruses were grown. On the left, a bright-field microscopy image is displayed. On the right, the superposing fluorescence image is presented. Both images correspond to the same section in the cell culture. The positively fluorescent colonies in Fig 19 B indicate that the lentiviruses had been successfully constructed from the added components, and that they were functional and expressing the GFP gene from the construct.

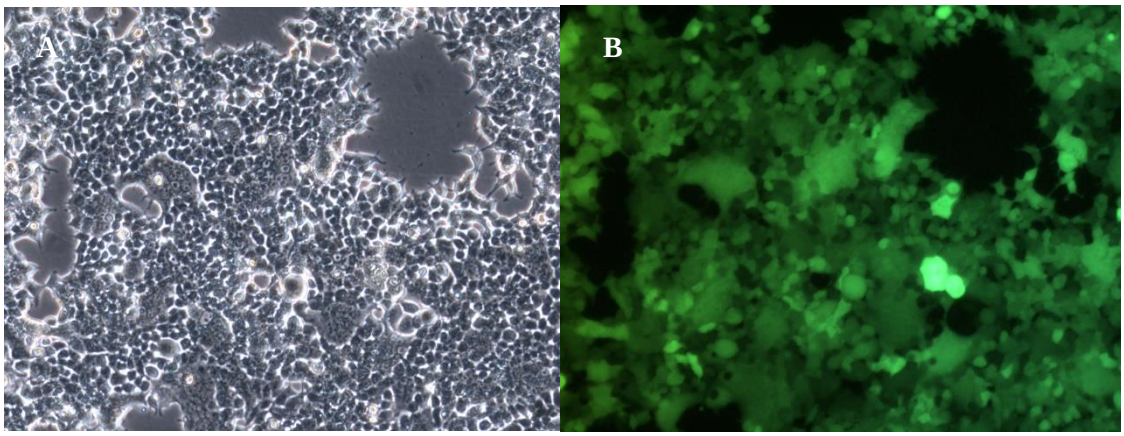

Fig 19: 293T cells under fluorescence microscope, showing that the GFP control constructed lentiviruses were functional. (A) Bright-field microscopy image. (C) Blue-light fluorescence image.

Due to the structure of the genetically engineered lentivirus, fluorescence would serve as a double reporter of successful lentiviral transduction. First, in all cases of lentiviral infection, fluorescence would imply that the lentivirus had successfully integrated its genetic material into the keratinocytes genome, which would be regularly expressing the GFP gene. And secondly, in the particular case of those DC keratinocytes infected with GSE4 + GFP, due to the presence of IRES in the transduced transgene, a fluorescence result would imply that the GSE4 gene was also being coexpressed – that is why GFP is considered to be a double reporter.

The cell culture at Fig 20 below belongs to those keratinocytes from Dyskeratosis Congenita patient #157 five days after being infected with GSE4 + GFP lentivirus. The keratinocytes colony pictured at Fig 20 A was successfully expressing GFP, so it can be inferred that GSE4 was also being expressed.

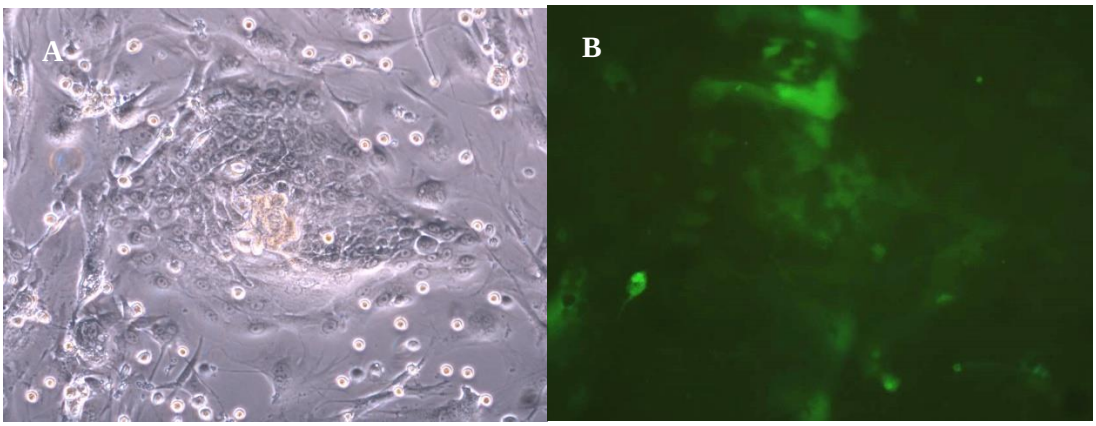

Fig 20: GSE4 #157 Keratinocytes five days after lentiviral transduction. (A) Bright-field image of the cell culture was an indicator of cell viability and proliferation. (B) The superposing fluorescence image of the same capture indicated successful transduction.

The day the pictures above were taken, the different keratinocytes populations were allowed for amplification. We were working with five samples in total: two GSE4 treated DC patients (#157 and #64) and three infected but untreated control samples (#157 and #64 DC patients and one healthy patient). These cell cultures were allowed for proliferation during three weeks, at the end of which the GSE4 transduced keratinocytes were exclusively selected in terms of their fluorescence through cell sorting. Fig 21 shows the percentage of GSE4 #157 keratinocytes that successfully passed the fluorescence sorting. A 97% of the original cells remained after sorting, meaning that up to a 97% of the infected GSE4 #157 keratinocytes had been indeed successfully transduced by the lentivirus.

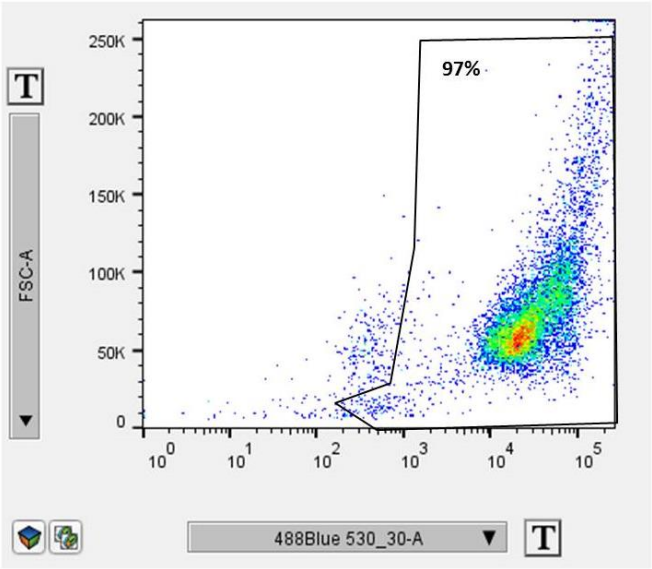

Fig 21: Output from the sorting equipment for cells GSE4 #157. A 97% of the cells remained after selective sorting.

The remaining keratinocytes were again allowed for proliferation, but this time there was a 100% certainty that every cell in the culture had been successfully transduced. The figures below represent the keratinocytes cultures after amplification. Fig 22 represents the same #157 GSE4 line that was already pictured at Fig 22 three weeks after the previous capture. The homogeneous fluorescence along the whole culture proves that sorting had been successful, since all the cells in the culture were successfully expressing GFP.

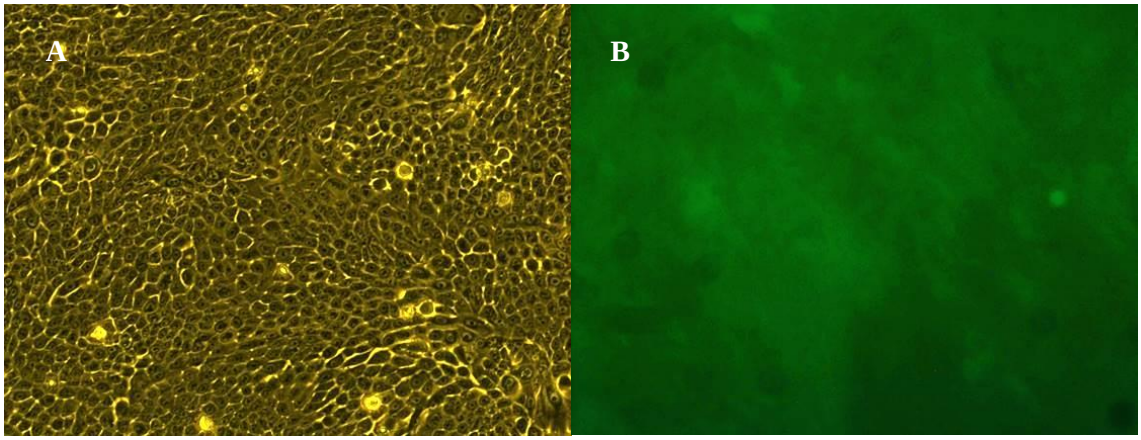

Fig 22: GSE4 #157 Keratinocytes after selective sorting. (A) Optical image of the cell culture shown confluency. (B) The superposing fluorescence image of the same capture indicated the homogeneous succesful lentiviral transduction along the whole cell culture.

Fig 23 below represents those keratinocytes from the same patient, #157, but belonging to the control study: they had been only transduced with GFP gene. Fig 23 B shows how, in contrast with Fig 22, these cells had not been selectively sorted since regions not expressing GFP could be distinguished.

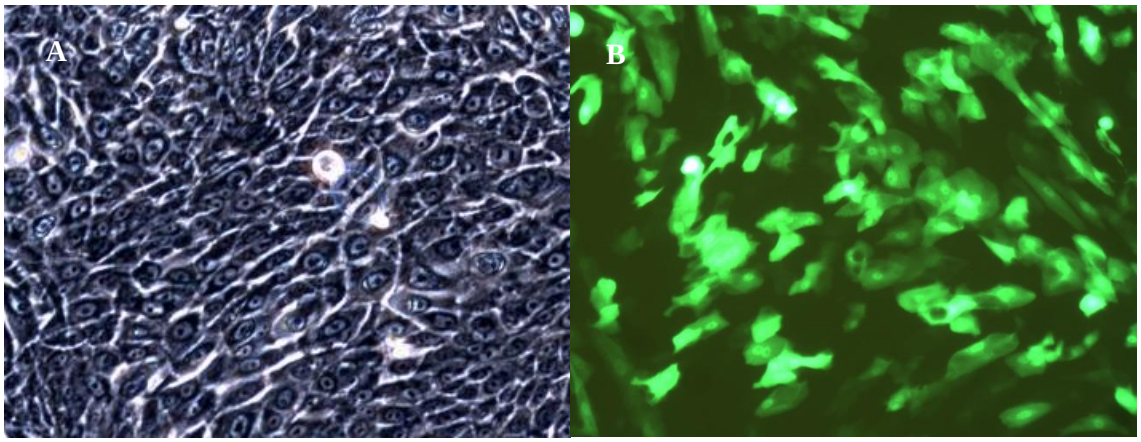

Fig 23: GFP #157 Keratinocytes after amplification, no sorting was applied. (A) Optical image of the control group. (B) Superposing fluorescence image indicating lentiviral insertion in the host's genome, although not with a 100% of efficiency.

On the other hand, the transduced keratinocytes from patient #64 exhibited an abnormal shape, shown in Fig 24 A, which will be later discussed. Since this moment, and even though it exhibited fluorescence (Fig 24 B) and therefore successful transduction, patient #64 was deleted from our experiment.

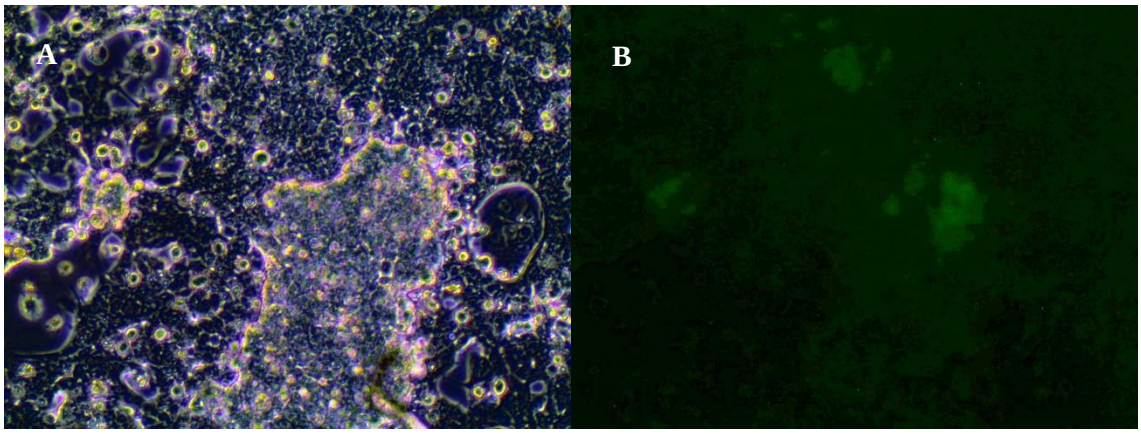

Fig 24: GSE4 #64 Keratinocytes after selective sorting. (A) The optical image shown some abnormal cellular clots. For this reason, patient #64 was not further used along the experimental procedure. (B) Superposing fluorescence image showing successful lentiviral transduction.

In conclusion, results show how lentiviral infection and genetic integration had been successful along all the keratinocytes. Only the successful and reliable results were considered for the next stage of the experimental procedure.

2. 3D Co-culture for the Construction of a Bioengineered Corrected Skin Equivalent

After assuring that gene therapy had been successful, meaning that the prepared lentivirus had transduced the patient's keratinocytes and that the engineered plasmid had inserted in the genome of the epithelial cells, an *in vitro* skin model was constructed using the modified cells as the building blocks. The objective was to assess whether the genetically treated cells grew normally and developed a healthy phenotype.

As expected, the seeded fibroblasts and keratinocytes self-assembled themselves in the natural structure of skin. Fig 25 and Fig 26 were obtained using optical microscopy from the top of the samples. Therefore, the 3D layered structure of the skin could be distinguished in the results: keratinocytes and fibroblast would belong to different planes and would not be able to be focused simultaneously.

Three transwells containing the skin equivalent were prepared from each sample, having in total three samples (and therefore, nine skin equivalents, as represented previously in Fig 18). The three samples to be 3D cultured were:

i. Patient #157 transduced with the GSE4 lentivirus: This sample was genetically treated against DC.

ii. Patient #157 transduced with the GFP lentivirus: This sample was a control for the genetic treatment.

iii. Healthy, non- transduced patient: This sample was a control for the *in vitro* experiment.

Fig 25 and Fig 26 below represent the appearance of the 3D culture four days after seeding the transduced keratinocytes from patient #157 in gels (i) and (ii). Fig 25, belonging to the GSE4 treated sample (i) grew normally along the expected lines of development. In the microscopy pictures, the almost confluent keratinocytes could be distinguished above the underneath layer of fibroblasts. Fig 26 represents the control gel (ii). In this case, the gel had developed some undesired clots of cells, which could be distinguished by their abnormal size and brown color. This result will be later analyzed in the section of discussion.

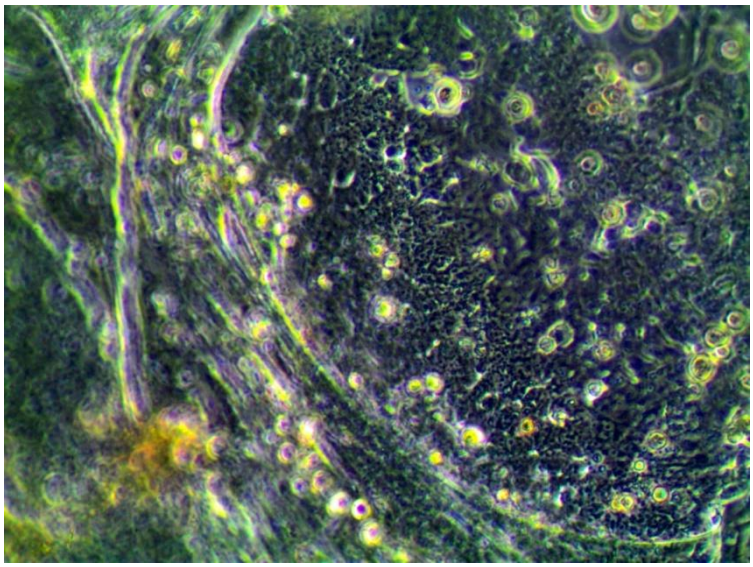

Fig 25: Fibrin hydrogel seeded with GSE4 #157 keratinocytes.

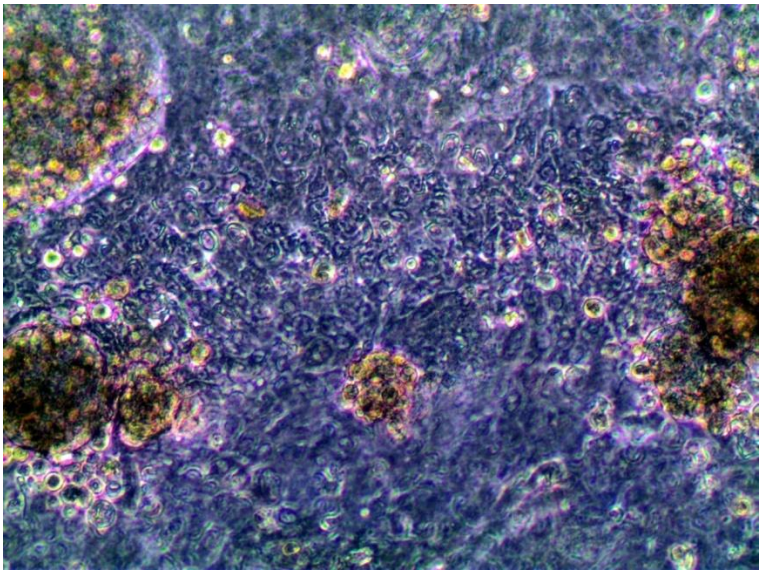

Fig 26: Control fibrin hydrogel seeded with GFP keratinocytes.

3. Histology of the Bioengineered Treated Skin Equivalent

Along preliminary studies, our team has already successfully developed an *in vitro* model of DC skin from patient's keratinocytes and fibroblasts [68]. In Fig 27, a representative image of the obtained result, taken at 10x, is displayed.

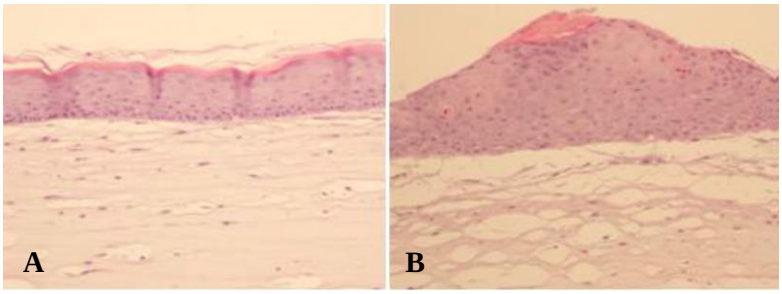

Fig 27: In vitro bioengineered skin from: (A) Healthy cells. (B) DC cells. [68]

In the present study, these preliminary results were corrected since the *in vitro* skin was grown from GSE4 transduced DC cells, whereas the previous study worked with untreated DC cells.

Returning to the last results of our bioengineered skin equivalents described in the previous section, the organotypic culture or 3D co-culture in the fibrin matrix was allowed to mature until successful terminal differentiation was achieved. This event was considered to happen 21 days after keratinocytes differentiation was started, and it could be visually assessed by the formation of a white coat on top of the hydrogel, shown in Fig 28. This layer would correspond to the keratinized Stratum Corneum, indicating that epidermal stratification was completed.

Fig 28: In the completely differentiated *in vitro* skin the stratum corneum can be distinguished.

Once this differentiation was reached, the prepared gels then went through the traditional histological procedure, consisting on fixing, processing, embedding in paraffin and sectioning the sample. They were stained with Hematoxylin and Eosin (H&E staining) and the glass slides were analyzed under optical microscopy and the outcome, which did not yield as expected, will be discussed in the next section.

Discussion

In this section the previously presented results will be further analyzed by comparing them with those obtained during different researches, and a critical discussion on the strong and weak points of our project will be performed.

1. Lentiviral Transduction of Keratinocytes

The main finding of the first section of the project, and moreover of the whole study in itself, is the unprecedented **high efficiency of transduction by the GSE4 vector in the DC keratinocytes**. The keratinocytes obtained from Dyskeratosis Congenita patients were transduced twice with the correcting GSE4 lentiviral vector, and the positive results could be observed significantly early, as shown in Fig 20 five days after transduction. The keratinocytes successfully expressed the GFP protein, encoded in the transgene and connected by coexpression with the therapeutic gene GSE4 through the Internal Ribosome Entry Site (IRES), as Fig 15 previously represented.

Whereas fluorescence was a qualitative way to assess GSE4 transduction, cellular sorting provided a quantitative indicator. Cell sorting provided a means to transduction selection using fluorescence as the discriminant factor: fluorescent cells passed the fluorescent test and were recovered for posterior cell culture, whereas non-fluorescent – and thus, non-corrected- cells were rejected. Fig 21 represented how the **output keratinocytes that remained after cellular sorting included a 97% of the initial input keratinocytes**. This result illustrates how GSE4 transduction was extremely efficient and very close to complete correction.

Moreover, Fig 22 showed how the **GSE4 transduced cells successfully divided** as can be inferred from the expansion of the green fluorescent area (corresponding to the corrected cells) when comparing Fig 20 and Fig 22, which belong to the same GSE4 transduced sample but with three weeks of difference. This cellular proliferation is also an indicator of successful genetic correction against DC since DC cells are characterized for early undergoing cell senescence and quitting cell division [69]. On contrast, our GSE4 keratinocytes behaved as healthy cells in terms of proliferation.

The efficiency in GSE4 transduction was considered to be the most remarkable achievement in the present study not only because it was a pioneer work on gene therapy for Dyskeratosis Congenita, but because it yielded much more positive results than prior studies carried out by our team. Preliminary experiments were performed using the precursor of GSE4: GSE24.2 transgene is equivalent in function to GSE4 but different in structure. Nevertheless, in contrast with the results obtained in this experiment with GSE4 (Fig 15), preliminary infection with GSE24.2 vectors did not yield any successful transduction, as shown in Fig 29 [68].

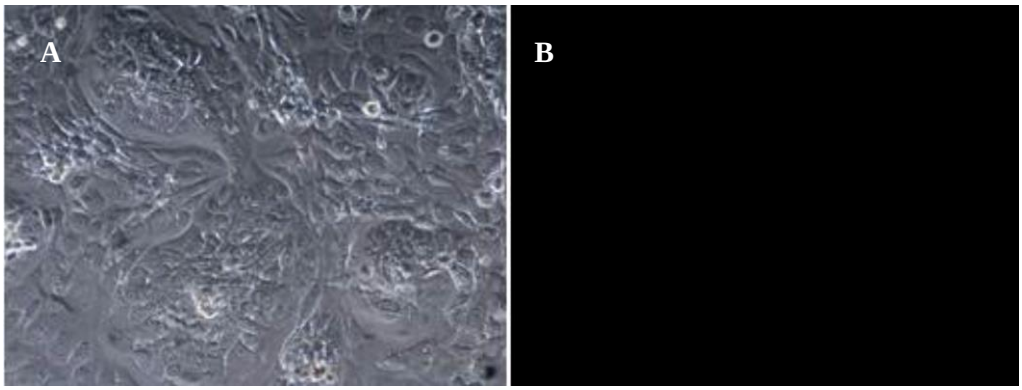

Fig 29: GSE24.2 #157 keratinocytes after lentiviral infection. (A) Bright-field microscope image. (B) Blue-light fluorescence microscopy image for assessing GSE24.2 expression. **[68]**

As previously stated during the review of the state-of-the-art, GSE4 is actually a fragment derived from GSE24.2. However, it yielded positive results in transduction whereas its precursor did not. In order to analyze this difference in transduction, the difference in structure between the two genes will be studied. Comparing their peptide sequence, represented in Fig 30, GSE4 is an 11 amino acids-long peptide corresponding to the N-terminal region of GSE24.2, which has a length of 55 amino acids [65].

GSE24.2: GFINLDKPSNPSSHEVVAWIRRILRVEKTGHSGTLDPKVTGCLIVCIERATRLVK
GSE4: GFINLDKPSNP

Fig 30: Amino acids sequence of GSE24.2 and its 11 amino acids-long derivation, GSE4 [65].

GSE4 is five times shorter than its GSE24.2 peptide and this has most likely made a determining difference in transduction. Its shorter size has allowed it to easily penetrate into the cell during lentiviral infection, whereas GSE24.2 was too big to cross the cellular barriers. This enhanced transduction gave GSE4 a decisive advantage in the development of a gene therapy for DC over GSE24.2, and placed it as the target peptide for further researches along this line.

Regarding these results in transduction, further elements need to be discussed, such as the IRES component in our GSE4 transgene. The **Internal Ribosome Entry Site** (IRES) allows the co-expression of the therapeutic and the reporter gene under the same promoter in order to facilitate a direct, visual result on the gene of interest. During translation, IRES binds to the 40s ribosomal subunit in such a way that gene expression is forced to continue into the next gene [70], as shown in the diagram below.

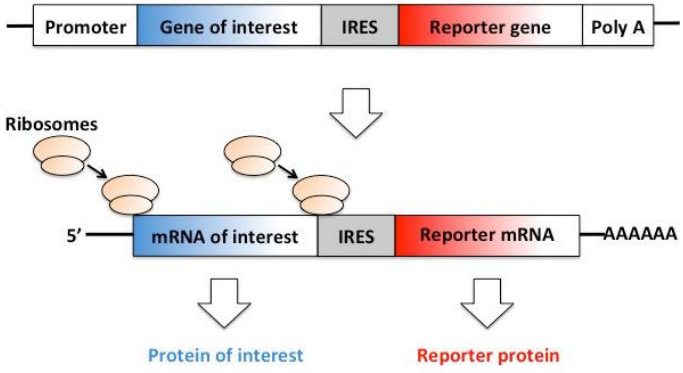

Fig 31: Mechanism of IRES during translation. In our study, the promoter was CMV, the gene of interest was GSE4, and the reporter gene was GFP [70].

IRES is the most common strategy used by researchers to ensure co-expression since it saves time and significantly increases the percentage of double transgenic offspring. Nevertheless, it is important to take into account that IRES also implies some drawbacks and that co-expression of the two genes is not always necessarily equally-balanced, meaning that one gene can be expressed with more efficiency than the other [71]. As a result, in the case of our experiment even though GFP expression did imply GSE4 expression, the relationship in expression did not have to be necessarily directly equivalent. Further experiments on the quantification of GSE4 expression should be performed to evaluate this possibility. On the other hand, in order to avoid this impairment, alternative ways for co-expression could be considered. For example, the synthetic creation of an only polyprotein containing the sequences of several proteins that will be simultaneously co-expressed would solve the unbalanced co-expression while avoiding the use of the large size IRES sequence (~0.5 kB) [72].

Another important point of discussion is the **GSE4 transduction results on patient #64**, previously displayed in Fig 24. Patient #64's keratinocytes were transduced with the GSE4 vector along with patient #157's cells. Nevertheless, GSE4 #64 keratinocytes yielded different results to GSE4 #157 under the same conditions. As Fig 24 B

represents, GSE4 #64 showed also successful transduction. However, the morphology in the #64 cells turned to be abnormal whereas #157 cells grew normally: #64 keratinocytes exhibited abnormally large cellular clusters that could be explained through different hypothesis. On the one hand, the skin cells could have accidentally differentiated therefore creating the characteristic keratinized stratified structure of natural skin [73]. On the other hand, some protoncogenes might have been accidentally activated by the HIV-1 lentivirus promotor, therefore developing abnormal, malignant keratinocytes [74]. A comparison between GSE4 #64 results (Fig 32 A) and representative cell cultures from the two proposed hypotheses is shown in Fig 32, in order to visually facilitate a diagnosis of the abnormalities presented by GSE4 #64 keratinocytes. Further research should be carried out in order to confirm or reject these hypotheses.

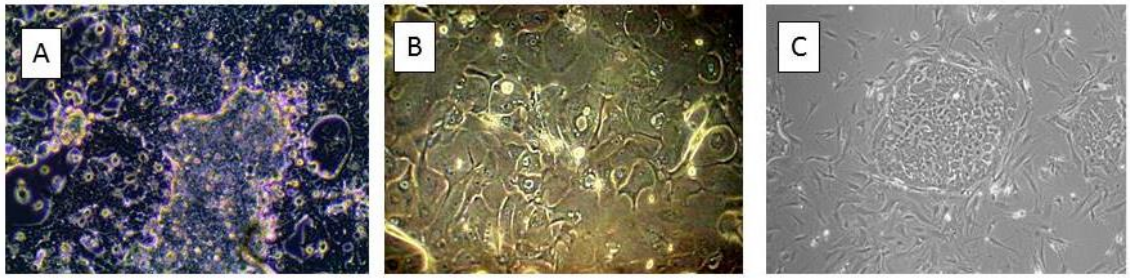

Fig 32: Comparison between abnormal keratinocytes cultures caused by different conditions. (A) GSE4 #64 keratinocytes after sorting and amplification showed an abnormal morphology. (B) Differentiated keratinocytes in cell culture caused by high calcium concentrations **[73]**. (C) Malignant keratinocytes surrounded by fibroblasts, obtained from an epidermal tumour **[74].**

In order to avoid a potential oncogenesis and gene disruption caused by the random insertion of the GSE4 HIV-1 lentivirus in the host's genome, a suicide gene could be added to the vector in order to eliminate the therapy if oncogenic conditions developed. As an example, Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK) has been recently found to be a good candidate for suicide gene therapy [75].

2. 3D Co-culture for the Construction of a Bioengineered Corrected Skin Equivalent

During this section of the experimental procedure, the skin structure was reproduced based on a 3D fibrin scaffold. The dermis was formed from the patient's fibroblasts that had been seeded in this fibrin matrix, which released collagen and formed a mature extracellular matrix that would replace the temporal fibrin scaffold and form the mature dermis. On the other hand, the formation of the epidermis had followed two stages: first, the corrected keratinocytes were seeded in order to form a confluent epidermal monolayer on top of the fibrin matrix; then, this monolayer of keratinocytes was differentiated by calcium gradient, inducing the characteristic epidermal stratification [52]. In the results displayed in Fig 25 and Fig 26, the **different planes** at which the dermis (fibroblasts) and the epidermis (keratinocytes) are located can be distinguished due to the focal differences.

However, the results obtained in Fig 26 need to be further discussed. This sample, which corresponded to GFP #157 control keratinocytes, developed some undesired clots of cells which exhibited a characteristic brown color and abnormal shape. In order to analyze this outcome, two possible causes could be considered. The first hypothesis is that the keratinocytes could have accidentally undergone **premature differentiation** during the experimental procedure. In this case, dead keratinocytes would have accumulated in the top stratum corneum before the complete development of the rest of the layers in the epidermis was fulfilled. A review of the reasons behind keratinocytes premature differentiation was published in 2013 by Borowiec *et al.*, indicating that this complex process requires multifactorial external control [76]. However, this hypothesis would not explain why only the GFP #157 control samples yielded these results.

An alternative explanation would be that these cellular clots were simply the natural phenotype in Dyskeratosis Congenita samples. In this case, this abnormal result would indeed be desired, since the role of the **control sample** is to reproduce the sick DC epidermal manifestations. However, in order to evaluate this hypothesis, further immunohistochemically studies should be performed on this sample and compared with an untreated DC skin model.

3. Histology of the Bioengineered Treated Skin Equivalent

In this final stage of the experimental procedure, the two previous stages would be commonly assessed by histology of the *in vitro* skin prepared from corrected keratinocytes. Unfortunately, when studying the histological slides, the results did not yield as expected. Under optical microscopy, none of the prepared skin equivalents presented the **epidermis layer**. Since the axis of our project was the genetic correction of the keratinocytes, which precisely belong to this undeveloped epidermis layer, and since the phenotype of DC manifests mainly in the epidermis, the obtained results did not represent the object of our study. Therefore, further analysis such as immunohistochemistry in order to more specifically *in vitro* assess the success of the gene therapy could not be performed.

This adverse result was indeed caused by accidental **bacterial contamination** of the bioengineered skin during manipulation and transport. The bacteria had proliferated around the *in vitro* skin samples and released toxins that destroyed the cells on the developing epidermis. The main solution to avoid these unfortunate results would be to **repeat the experimental procedure** and grow new skin equivalents under more controlled sterile conditions.

Theoretically, a similar result should have been obtained throughout the healthy grown skin equivalents and those developed from GSE4 corrected keratinocytes. If gene therapy had been successfully applied – which, theoretically, indeed was due to the results explained in the first section of the present discussion – the DC keratinocytes would have recovered their **healthy function** and normal skin would have developed. Healthy skin, previously represented in Fig 27 A from preliminary studies, exhibits a homogeneous thickness of the epidermal layer and the correct formation of the Stratum Corneum on top of this epidermis. In addition, the epidermal-dermal junction that attaches the two layers of the skin is properly developed in healthy skin, as well as the matrix of dense connective tissue embedding the fibroblasts in the dermis [68].

On contrast, the GFP control skin equivalents should exhibit the characteristic **histological structure of DC**, represented in Fig 27 B. Comparing the histology of healthy and DC skin, the differences characteristic of the disease are easily visible. In DC skin, the Stratum Corneum is abnormally thick and dense in a **hyperkeratotic** behavior, compared with the linear and homogenous St. Corneum in healthy skin. Moreover, the entire epidermis presents a **hyperplasic** phenotype in which cells abnormally grow in number while maintaining a normal morphology, resulting in an

increase in volume of the epithelial tissue. On the other hand, the dermis in diseased skin is formed by **loose connective tissue** with a reduced number of collagen fibers, compared with the dense CT found at the dermis of the healthy skin. This loose dermal conformation reduces the mechanical properties of the whole skin. Finally, the attachment between dermis and epidermis in DC skin is practically null along the **dermal-epidermal junction**, facilitating the detachment of epidermis and reducing the functionality of the skin [10] [68]. All these features of diseased skin are outlined in Fig 33. However, the presence of different protein markers should be assessed by immunohistochemistry in order to quantify these qualitative results.

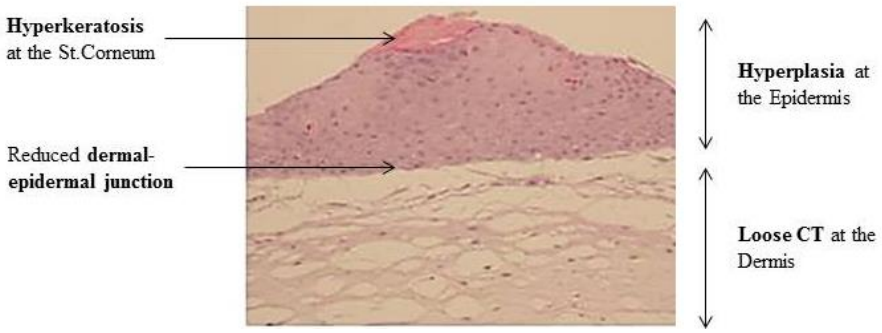

Fig 33: Histological image from *in vitro* DC skin model. The main differences with healthy skin are highlighted.

All these characteristic features of DC skin are correlated between themselves, and fundamentally caused by the impaired function of DC keratinocytes. The hyperplasia at the epidermis might induce the hyperkeratosis at the St. Corneum. The diseased behavior of DC keratinocytes might decrease the attachment at the dermal-epidermal junction, which would influence the composition of the dermal layer. In addition, DC fibroblasts are known to exhibit proliferative defects, as any other cell suffering from telomerase shortage [77]. This reduced number of fibroblasts would explain the loose conformation of DC dermis.

As stated previously, the experimental procedure should be repeated in order to guarantee that these theoretical results indeed coincide with the empirical results. If such an accordance happened, then GSE4 therapy for Dyskeratosis Congenita would be proved to be successful and further clinical trials could be performed in the pursue of a standardized treatment of the disease.

Conclusion

As a conclusion, the present project has achieved **for the first time the genetic correction** of Dyskeratosis Congenita in patients' keratinocytes, since no previous studies on gene therapy for this rare disease have been reported in the literature so far. The obtained positive results have empirically proven that a future treatment based on gene therapy is viable, opening the path to a new therapeutic approach that would address DC at its core and dramatically reduce the cases of this rare disease.

The transgene that was used in the present study was GSE4, a short peptide derived from the mutated DKC1 gene in X-linked recessive Dyskeratosis Congenita. GSE4 was found to be unprecedentedly successful for DC correction, on contrast with its precursor GSE24.2 which was used in preliminary studies but did not yield positive results in DC correction. Even though GSE4 and GSE24.2 share many common features, the **difference in their correction efficiencies lies in the lengths** of the peptides: initial GSE24.2 is 55 amino acids-long, whereas final GSE4 presents a reduced length of 11 amino acids which enhanced lentiviral infection in target cells during gene therapy. This length reduction provided GSE4 with a significant efficiency advantage over alternative transgenes while maintaining the therapeutic effect and functionality.

Moreover, the present project has studied the consequences of premature keratinocyte differentiation on *in vitro* skin equivalents, as well as the effects of HIV-1 vector integration near the transcription start site of a protooncogene during gene therapy. These results were visible during cell culture due to the abnormal morphology of the keratinocytes, and should be avoided in order to achieve reliable *in vitro* models.

Even though the present study gives the first step in the search of a gene therapy for Dyskeratosis Congenita, more steps await before this treatment could become a reality. The next studies to be performed after the herein presented results would be the **development of models using the GSE4 corrected cells**, in order to evaluate whether they indeed behave as healthy tissue. In the present project, an *in vitro* skin equivalent was grown using the GSE4 modified keratinocytes as the building blocks; however, this *in vitro* model should be further analyzed through immunohistochemistry techniques as well as telomere measuring tools. On the other hand, *in vivo* models are also of great importance for determining the viability of gene therapy: the *in vitro* skin would be transplanted as a graft in immunodefficient mice, and the *in vivo* behavior of the graft would be evaluated. *In vivo* studies are fundamental as preclinical models, and if the GSE4 corrected cells yield the expected *in vivo* behavior, a DC treatment will be closer to be real for all those persons suffering from this rare disease.

Alternative Applications and Future Work

The results obtained in the present study have opened a promising path of research in gene therapy for Dyskeratosis Congenita in which the steps to follow are already well-defined. After the successful GSE4 cellular correction, the most coherent study to perform next would be an *in vitro* skin model built from these corrected DC cells to study their behavior. This model was already developed in the present project, although further studies should be performed at this stage. **Immunohistochemical tools** should be applied to the samples in order to visualize the presence or absence of specific DC antigens in the *in vitro* model. For instance, *Filaggrin/Loricrine* could be studied to evaluate terminal differentiation in epidermal layer, or *Prolyl 4-Hydroxilase* for collagen synthesis. Also, the correction of DKC1 gene with GSE4 transgene could be assessed with *α-myc* antibodies, which allow visualizing the **cellular localization** of dyskerin; since dyskerin is a nucleolar protein, if GSE4 gene therapy was successful, α-myc should be found at the nuclei. Finally, **telomere assays** could be carried out in these *in vitro* models since DC is essentially a telomeropathy implying a decrease in telomere length.

The successful development of a gene therapy for Dyskeratosis Congenita would be a milestone in the emerging field of regenerative medicine at its whole, not only for DC patients. Since GSE4 codes for the dyskerin subunit of the telomerase complex, a therapy based on this transgene could be **applied to other telomeropathies** that have their roots on an impaired telomerase activity, such as Aplastic Anemia or Cri Du Chat syndrome.

Regarding alternative designs to address the same goal targeted during this project, many different approaches based on emerging technologies and future medicine could be chosen to study DC other than the 3D *in vitro* models used in the present study. Lately, **organ-on-a-chip** technology has been rapidly developed as a simple, cheap and easily customizable way to create miniaturized models of organs. The main characteristics of the organ of interest are mimicked with equivalent elements in a microfluidic device, and the function of the organ as a whole is reproduced at the micro-scale. Skin-on-a-chip models could be readily customized with DC cells as well as GSE4 corrected cells in order to easily study the characteristic proliferation, telomerase activity or DKC1 expression in these cells.

On the other hand, alternative approaches to treat DC other than the GSE4 lentiviral transduction used in the present study could also be considered within the same framework of gene therapy. **CRISPR/Cas9** is a recently discovered genome editing tool that could replace the HIV-1 lentivirus used as the vector for gene therapy in this project. Theoretically, CRISPR/Cas9 could be customized to delete the mutated DKC1 gene in X-linked recessive DC, as well as to insert the healthy transgene GSE4 in the host's genome. The desired CRISPR/Cas9 complex could be delivered into the target cell through inorganic and organic particles, such as lipoplexes, which would not present the immunogenicity risks inherent in viral vectors. Even though this CRISPR/Cas9 approach is yet to be studied, it stands firmly as a potential alternative to HIV-1 lentivirus in DC gene therapy.

Finally, an alternative approach to diagnose DC instead of the currently used histological tools would be the **genome sequencing** tools, which have rapidly evolved since the recent rise of the field of genomics. Next Generation Sequencing techniques such as *Illumina®* genome analyzer could be used to readily analyze the patient's genome in the search of a mutation in DKC1 gene; also, *Illumina®* could rapidly sequence the telomere in order to diagnose whether abnormal telomere shortening is taking place. Moreover, recently new prototypes of future Next Generation Sequencing platforms have been developed, such as the small *Oxford Nanopores®* which would provide a simple and immediate diagnosis of DC through a new genome sequencing technology based on measuring the current produced by the nitrogenous bases at DNA. Next Generation Sequencing platforms would avoid the time and difficulties implied in histology for current diagnosis.

Dyskeratosis Congenita is a broad pathology, and the more it is studied, the more this broadness increases: New genotypic mutations are found to cause the disease, and more phenotypic manifestations are found to be related with it. Currently, DC comprises a big complex network of genes and characteristics with no apparent linear relationship among them. Taking into account this heterogeneity, it is logical to understand that most probably a combination of different approaches will be determinant for finding a final cure to Dyskeratosis Congenita. In particular, the broad range of phenotypic manifestations in DC patients leads to think of **Systems Biology** as a logical step during the study of the disease. In Dyskeratosis Congenita, the mutation of a single gene within the telomerase complex leads to failures in many different organs throughout the body: skin, lungs, liver, bone marrow,… Initially these organs might appear to be functionally unrelated, but in Dyskeratosis Congenita they all share a common origin of failure. Therefore, thinking of this **multisystem disease as a network**, there is a chance that we

have overlooked at a possible connection between two elements in the network that could be critical for the discovery of a cure for DC. In conclusion, studying Dyskeratosis Congenita from a systemic approach would allow for the comprehensive understanding of the relationships between the simultaneous mutations and malfunctions throughout the whole body during the disease.

In conclusion, there are many different approaches that could be complementarily taken in order to understand Dyskeratosis Congenita, as well as many techniques in order to *in vitro* study the disease. However, once the condition has been understood, the treatment is what, at the end, makes a difference. And in terms of treatment, the only real way to address the problem at its core is to cure the genetic mutation at DKC1. Therefore, **gene therapy stands out as the only real solution** against Dyskeratosis Congenita, targeting the problem at its very root. Even though the most efficient gene therapy technique is yet to be found or perfected, huge improvements are continuously being made in this line and most likely this rare disease will be overcome by future medicine in the next years.

Bibliography

[1] G. Tartora and B. Derrickson, Principles of Anatomy and Physiology, John Wiley & Sons, 2011.

[2] B. Reece. [Online]. Available: <http://www.benjaminreece.com/>.

[3] C. Blanpain and E. Fuchs, "Epidermal Stem Cells of the Skin," *Annual Review of Cell and Developmental Biology*, no. 22, pp. 339-373, 2006.

[4] M. Maggioni and Y. Barrandon, "EuroStemCell: where do they live and what can they do?," 9 Feb 2011. [Online]. Available: http://www.eurostemcell.org/factsheet/skin-stem-cells-where-do-they-live-and-what-can-they-do.

[5] E. Proksch, J. Brandner and J. Jensen, "The skin: an indispensable barrier," *Experimental Dermatology*, vol. 17, no. 12, pp. 1063-1072, 2008.

[6] H. Hennings, D. Michael, C. Cheng, P. Steinert, K. Holbrook and S. H. Yuspa, "Calcium regulation of growth and differentiation of mouse epidermal cells in culture," *Cell*, vol. 19, no. 1, pp. 245-254, 1980.

[7] P. Mason and M. Bessler, "The genetics of dyskeratosis congenita," *Cancer Genet*, vol. 204, no. 12, pp. 635-645, 2011.

[8] I. Dokal, T. Vulliamy, P. Mason and M. Bessler, "Clinical utility gene card for: dyskeratosis congenita.," *EJHG*, vol. 19, no. 11, pp. 1-11, 2011.

[9] «Online "Mendelian Inheritance in Man" (OMIM) 305000,» [En línea]. Available: <http://www.omim.org/entry/305000>.

[10] M. Kirwan and I. Dokal, "Dyskeratosis congenita, stem cells and telomeres," *Biochimica et Biophysica Acta*, vol. 1792, no. 4, pp. 371-379, 2009.

[11] M. S. Fernández García y J. Teruya-Feldstein, «The diagnosis and treatment of dyskeratosis congenita: a review,» *Journal of Blood Medicine*, nº 5, pp. 157-167, 2014.

[12] T. Vulliamy, A. Marrone, F. Goldman y e. al, «The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita,» *Nature*, vol. 413, nº 6854, pp. 432-435, 2001.

[13] C. Greider and E. Blackburn, "Identification of a specific telomere terminal transferase activity in Tetrahymena extracts," *Cell*, vol. 43, no. 2, pp. 405-413, 1985.

[14] S. Cohen, M. Graham, G. Lovrecz, N. Bache, P. Robinson and R. Reddel, "Protein composition of catalytically active human telomerase from immortal cells," *Science*, vol.

315, no. 5820, pp. 1850-1853, 2007.

[15] C. Garcia, W. Wright and J. Shay, "Human diseases of telomerase dysfunction: insights into tissue aging.," *Nucleic Acids Res,* vol. 22, no. 7406-7416, p. 35, 2007.

[16] N. I. o. H. U. N. L. o. Medicine, «Genetics Home Reference: DKC1,» 7 June 2016. [En línea]. Available: <https://ghr.nlm.nih.gov/gene/DKC1#resources>.

[17] S. Knight, T. Vulliamy, A. Copplestone, E. Gluckman, P. Mason and I. Dokal, "yskeratosis Congenita (DC) Registry: identification of new features of DC.," *British Journal of Haematology,* vol. 103, pp. 990-996, 1998.

[18] J. de la Fuente and I. Dokal, "Dyskeratosis congenita: Advances in the understanding of the telomerase defect and the role of stem cell transplantation," *Pediatr Transplantation,* vol. 11, pp. 584-594, 2007.

[19] J. Wong and K. Collins, "Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita," *Genes & Development,* vol. 20, pp. 2848-2858, 2006.

[20] Y.-S. Cong, W. E. Wright and J. W. Shay, "Human Telomerase and Its Regulation," *Microbiology and Molecular Biology Reviews,* vol. 66, no. 3, pp. 407-425, 2002.

[21] A. Islam, S. Rafiq, M. Kirwan and e. al., "Haematological recovery in dyskeratosis congenita patients treated with danzol," *Br J Haematol,* vol. 162, no. 6, pp. 854-856, 2013.

[22] R. Calado and N. Young, "Telomere diseases," *N Engl J Med,* vol. 361, no. 24, p. 2353–2365, 2009.

[23] M. Bessler, P. J. Mason and D. B. Wilson, "National Organization for Rare Disorders: Dyskeratosis Congenita," 2008. [Online]. Available: <http://rarediseases.org/rare-diseases/dyskeratosis-congenita/>.

[24] C. Sieff, Dyskeratosis Congenita. NORD Guide to Rare Disorders., Philadelphia, PA: Lippincott Williams & Wilkins. , 2003.

[25] E. M. Erduran, S. M. Hacisalihoglu and Y. M. Ozoran, "Treatment of Dyskeratosis Congenita With Granulocyte-Macrophage Colony-Stimulating Factor and Erythropoietin," *Journal of Pediatric Hematology/Oncology,* vol. 25, no. 4, pp. 333-335, 2003.

[26] M. Ayas, A. Nassar, A. Hamidieh and e. al, "Reduced intensity conditioning is effective for hematopoietic SCT in dyskeratosis congenita-related BM failure," *Bone Marrow Transplan,* vol. 48, no. 9, pp. 1168-1172, 2013.

[27] H. Sakaguchi, K. Nakanishi y S. Kojima, «Inherited bone marrow failure syndromes in 2012,» *Int J Hematol,* vol. 97, nº 1, pp. 20-29, 2013.

[28] B. Alter, N. Giri, S. Savage and P. Rosenberg, "Cancer in dyskeratosis congenita," *Blood,*

vol. 113, pp. 6549-6459, 2009.

[29] M. Ayas, A. Al-Musa, A. Al-Jefri and e. al., "Allogeneic stem cell trnasplantation in a patient with dyskeratosis congenita after conditioning with low-dose cyclophosphamide and anti-thymocyte globulin," *Pediatr Blood Cancer,* 2005.

[30] F. Ostronoff, M. Ostronoff, R. Calixto, R. Florêncio, M. Domingues, r. A. Souto Maio y e. al., «Fludarabine, cyclophosphamide, and antithymocyte globulin for a patient with dyskeratosis congenita and severe bone marrow failure.,» *Biol Blood Marrow Transplant,* vol. 13, nº 3, pp. 366-368, 2007.

[31] D. T. Robles and e. al, "Medscape: Dyskeratosis Congenita Treatment & Management," 10 July 2014. [Online]. Available: <http://emedicine.medscape.com/article/1110516-treatment#d8>.

[32] The Genetics and Public Policy Center, Johns Hopkins University Berman Institute of Bioethics, «International Law,» 2008.

[33] V. Abbrui, "Wonder Whizkids," [Online]. Available: <http://www.wonderwhizkids.com/>.

[34] D. Bleijs, "Gene Therapy Net," [Online]. Available: <http://www.genetherapynet.com/>.

[35] Science News Staff, "And Science's 2015 Breakthrough of the Year is...," American Association for the Advancement of Science, 17 December 2015. [Online]. Available: <http://www.sciencemag.org/news/2015/12/and-science-s-breakthrough-year>.

[36] H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, "Non-viral vectors for gene-based therapy," *Nature Review Genetics,* vol. 15, pp. 541-555, 2014.

[37] T. A. Ulrich Kuhn†, W. Pfutzner, R. A. Foster and J. C. Vogel, "In Vivo Assessment of Gene Delivery to Keratinocytes by Lentiviral Vectors," *Journal of Virology,* vol. 76, no. 3, pp. 1496-1504, 2002.

[38] W. Xiaolin, Y. L. Bruce Crise and S. M. Burgess, "Transcription start regions in the human genome are favored targets for MLV integration," *Science,* vol. 300, no. 5626, pp. 1749-1751, 2003.

[39] S. Rogers and P. Pfuderer, "Use of viruses as carriers of added genetic information," *Nature,* vol. 219, pp. 749-751, 1968.

[40] S. Rosenberg, P. Aebersold, K. Cornetta and e. al., "Gene transfer into humans--immunotherapy in patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction," *N. Engl. J. Med.,* vol. 323, pp. 570-578, 1990.

[41] W. Anderson and e. al., "T Lymphocyte-Directed gene therapy for ADA-SCID: Initial trial results after 4 years," *Science,* vol. 270, no. 5235, pp. 475-480, 1995.

[42] The Journal of Gene Medicine, "Gene therapy clinical trials worldwide database," Wiley, January 2014. [Online]. Available: <http://www.wiley.com/legacy/wileychi/genmed/clinical/>.

[43] A. Ananthaswamy, "Undercover genes slip into the brain," *New Scientist*, 2010.

[44] Á. González-Murillo, M. Lozano, L. J. A. A. E. Álvarez, S. Navarro, J. Segovia, H. Hanenberg, G. Guenechea, J. Bueren and P. Río, "Development of Lentiviral Vectors with Optimized Transcriptional Activity for the Gene Therapy of Patients with Fanconi Anemia," *Human gene therapy*, vol. 21, no. 5, pp. 623-630, 2010.

[45] S. Pearson, H. Jia and K. Kandachi, "China approves first gene therapy," *Nature Biotechnology*, vol. 22, no. 1, pp. 3-4, 2004.

[46] P. I. A. Shvalb and e. al., "pCMV-vegf165 Intramuscular Gene Transfer is an Effective Method of Treatment for Patients With Chronic Lower Limb Ischemia," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 20, pp. 473-482, 2015.

[47] S. Richards, "Gene Therapy Arrives in Europe," The Scientist, 6 November 2012. [Online]. Available: <http://www.the-scientist.com/?articles.view/articleNo/33166/title/Gene-Therapy-Arrives-in-Europe/>.

[48] A. Coghlan, «Gene Therapy Approved,» The New Scientist, 9 April 2016. [En línea].

[49] S. E. Lynch, L. Wisner-Lynch, M. Nevins and M. L. Nevins, "A new era in periodontal and periimplant regeneration: use of growth-factor enhanced matrices incorporating rhPDGF," *COMPENDIUM-NEWTOWN*, vol. 27, no. 12, p. 672, 2006.

[50] K. Y. Lee and D. J. Mooney, "Hydrogels for Tissue Engineering," *Chemical Reviews*, vol. 101, no. 7, pp. 1869-1879, 2001.

[51] M. J. Escámez, L. Martínez-Santamaría, M. García, S. Guerrero-Aspizua, M. Carretero, F. Larcher, Á. Meana and M. Del Río, "Bioengineered Skin," in *Skin Bopsy - Perspectives*, 2011.

[52] L. Martínez-Santamaría, S. Guerrero-Aspizua and M. Del Río, "Skin Bioengineering: Preclinical and Clinical Applications," *Actas Dermo-Sifiliográficas*, vol. 103, no. 1, pp. 5-11, 2011.

[53] Tissue Engineering and Regenerative Medicine Group (TERMeG), "TERMeG: Regeneración cutánea," [Online]. Available: <http://termeg.uc3m.es/>.

[54] M. Rosdy and L.-C. Clauss, "Terminal Epidermal Differentiation of Human Keratinocytes Grown in Chemically Defined Medium on Inert Filter Substrates at the Air-Liquid Interface," *Journal of Investigate Dermatology*, vol. 95, no. 4, pp. 409-414, 1990.

[55] D. Dyall-Smith, «DermNetz NZ: Dyskeratosis Congenita,» [En línea]. Available:

<http://www.dermnetnz.org/systemic/dyskeratosis-congenita.html>.

[56] A. J. Waine and I. Dokal, "Advances in the understanding of dyskeratosis congenita," *British Journal of Haematology,* vol. 145, no. 2, pp. 164-172, 2009.

[57] D. Ruggero, S. Grisendi, F. Piazza, E. Rego, F. Mari, P. Rao, C. Cordon-Cardo and P. P. Pandolfi, "Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification," *Science,* vol. 299, pp. 259-262, 2003.

[58] D. Hockemeyer, W. Palm, R. Wang, S. Couto and T. De Lange, "Engineered telomere degradation models dyskeratosis congenita," *Genes & Development,* vol. 22, p. 1773–1785, 2008.

[59] S. Agarwal, Y. Loh, E. McLoughlin, J. Huang, I. Park, J. Miller, H. Huo, M. Okuka, R. Dos Reis, S. Loewer and H. Ng, "Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients," *Nature,* vol. 464, no. 7286, pp. 292-296, 2010.

[60] F. Gourronc, m. M. Robertson, A. K. Herrig, P. M. Lansdorp, F. D. Goldman and A. J. Klingelhutz, "Proliferative defects in dyskeratosis congenita skin keratinocytes are corrected by expression of the telomerase reverse transcriptase, TERT, or by activation of endogenous telomerase through expression of papillomavirus E6/E7 or the telomerase RNA compo.," *Experimental Dermatology,* vol. 19, no. 3, pp. 279-288, 2010.

[61] P. Boukamp, "Skin aging: a role for telomerase and telomere dynamics?," *Curr Mol Med,* vol. 5, pp. 171-177, 2005.

[62] R. Machado-Pinilla, I. Sánchez-Pérez, J. R. Murguía, L. Sastre and R. Perona, "A dyskerin motif reactivates telomerase activity in X-linked dyskeratosis congenita and in telomerase-deficient human cells," *Blood,* vol. 111, no. 5, pp. 2608-2614, 2008.

[63] R. Machado-Pinilla, J. Carrillo, C. Manguan-García, L. Sastre, A. Mentzer, B.-W. Gu, P. J. Mason and R. Perona, "Defects in mTR stability and telomerase activity produced by the Dkc1 A353V mutation in dyskeratosis congenita are rescued by a peptide from the dyskerin TruB domain," *Clin Trans Oncol,* vol. 14, no. 10, pp. 755-763, 2012.

[64] A. Hernando, "La doble vida del péptido GSE24-2," SiNC, 25 May 2012. [Online]. Available: <http://www.agenciasinc.es/Reportajes/La-doble-vida-del-peptido-GSE24-2>.

[65] L. Iarricio, C. Manguán-García, L. Pintado-Berninches, J. M. Mancheño, A. Molina, L. Sastre and R. Perona, "GSE4, a Small Dyskerin- and GSE24.2-Related Peptide, Induces Telomerase Activity, Cell Proliferation and Reduces DNA Damage, Oxidative Stress and Cell Senescence in Dyskerin Mutant Cells," *Cells,* vol. 10, no. 11, 2015.

[66] T. Dull, R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono and L. Naldini, "A Third-Generation Lentivirus Vector with a Conditional Packaging System," *Journal of Virology,* vol. 72, no. 11, pp. 8463-8471, 1998.

[67] «Oxford Genetics,» 2015. [En línea]. Available: <http://oxfordgenetics.com/>.

[68] D. Acitores Balboa, *Characterization in a Humanized Context of a Rare Disease: Dyskeratosis Congenita,* Leganes: Bachelor Thesis UC3M, 2015.

[69] J. R. Mitchell, E. Wood and K. Kollins, "A telomerase component is defective in the human disease dyskeratosis congenita," *Nature,* vol. 402, pp. 551-555, 1999.

[70] genoway, "IRES: Co-Express Genes Under One Promoter," [Online]. Available: <https://www.genoway.com/technologies/ires-co-expression.htm>.

[71] E. Wong, S. Ngoi and L. C. , "Improved co-expression of multiple genes in vectors containing internal ribosome entry sites (IRESes) from human genes.," *Gene Therapy,* vol. 9, no. 5, pp. 337-344, 2002.

[72] P. de Felipe, "Skipping the co-expression problem: the new 2A "CHYSEL" technology," *Genetic Vaccines and Therapy,* vol. 2, no. 13, 2004.

[73] M. B. Abdel-Naser, M. Abdallah, H. Larangeira de Almeida Jr and U. Wollina, "Human Skin Cell Culture and its Impact on Dermatology," *Egyptian Dermatology Online Journal,* vol. 2, no. 1, p. 1, 2005.

[74] S. Kazerounian, "Isolation of Mammary Epithelial Cells and Fibroblasts From Mouse Tumor," *Bio-protocol,* vol. 4, no. 4, 2014.

[75] D. Dey and G. R. Evans, "Suicide Gene Therapy by Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK)," in *Targets in Gene Therapy*, 2011.

[76] A.-S. Borowiec, P. Delcourt, E. Dewailly and G. Bidaux, "Optimal Differentiation of In Vitro Keratinocytes Requires Multifactorial External Control," *PloS one,* vol. 8, no. 10, 2013.

[77] E. R. Westin, E. Chavez, K. M. Lee and e. al., "Telomere restoration and extension of proliferative lifespan in dyskeratosis congenita fibroblasts," *Aging Cell,* vol. 6, pp. 383-394, 2007.

[78] S. E. Lynch, L. Wisner-Lynch, M. Nevins and M. L. Nevis, "A new era in periodontal and peri-implant regeneration: use of growth-factor enhanced matrices incorporating rhPDGF," *Compedium-Newtown,* vol. 27, no. 12, p. 672, 2006.

[79] D. Peehl and R. Ham, "Clonal growth of human keratinocytes with small amount of dialysed serum," *In Vitro,* vol. 16, pp. 526-538, 1980.

[80] S. Boyce and R. Ham, "Calcium-regulated differentiation of normal human epidermal keratinocytes in chetrucally defined clonal culture and serum-free serial culture," *J Invest Dermatol,* vol. 81, pp. 335-405, 1983.

Annex I: Legal Regulatory Framework

The present Final Year Project is framed into a biomedical scientific research activity, which is recognized as a fundamental right of maximum protection by the **Article 20.1.b of the Spanish Constitution of 1978**.

Furthermore, the specific legal regulation of biomedical research is stated at **Law 14/2007** of July 3th. However, this law was partially modified by Law 14/2011 of Science, Technology and Research.

At the international level, Law 14/2007 belongs to the **Convention of the European Council** for the protection of human rights and dignity of the human being regarding the application of biology and medicine. This Convention was signed in Oviedo on 14TH April 1997 and was applied in Spain since the 1st January 2000. In this law it is proclaimed that the health, dignity and welfare of the human being participating in biomedical research will prevail over the interest of society or science.

Annex II: Project Planning

The execution of the whole project can be divided into the following five stages, which have been already mentioned throughout the present memory:

1.- **PRELIMINARY STUDY**. First reunion with the project tutor and setting up of the goals. Contextualization of the project, primary planning, and initial literature search…….....*10 hours*

2.- **GENE THERAPY**. Construction of the lentiviral vectors, transduction, and amplification of the corrected cells……*60 hours*

3.- **BIOENGINEERED SKIN**. Development of a fibrin-based *in vitro* skin equivalent from the corrected cells. Terminal keratinocytes differentiation until epidermal stratification is completed…… *50 hours*

4.- **HISTOLOGY**. The *in vitro* skin was processed and sectioned into histological slides, which were later stained for microscopy inspection……. *40 hours*

5.- **DISSERTATION WRITING**. The project was transcribed into the present memory, and several versions were sent for correction to the tutor. Final reunion with the tutor……... *180 hours*

The project was regularly supervised by the tutor, with whom communication and discussion of the obtained results were continuous. Frequent reunions were arranged throughout the whole experimental stages with the tutor.

Table 5 represents the estimated number of hours spent in every stage of the project, whereas the project timeline can be studied in Table 6, in which the relation between the weeks work and the tasks performed are related.

Stage	Hours
1	10
2	60
3	40
4	50
5	180
Total	340

Table 5: Overview of the project in time units.

Table 6: Timeline of the project representing the relation between the weeks of work and the tasks performed

	Stages				
Weeks	**1**	**2**	**3**	**4**	**5**
1					
2		Lentivirus in 293T			
3		Infection of Keratinocytes			
4		Amplification			
5		Amplification			
6		Sorting			
7		Amplification			
8			Fibrin Hydrogel + Seeding		
9			Development		
10			Differentiation		
11			Differentiation		
12			Completed Differentiation		
13				Fixation	
14				Processing	
15				Sectioning	
16				Sectioning	
17				Sectioning	
18				Sectioning	
19				Staining	
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					

Annex III: Budget and Production Cost

A review of the **production cost** of the present project is described in Table 7, which represents the estimated expenses on personnel and on the different laboratory reagents and material consumed throughout the experimental procedure, considering the availability of the information at the user-level. A total amount of 2505,793€ was estimated to be spent in the performed project in reagents and laboratory consumables**.** However, it is important to highlight that not all the materials listed below were used in their totality, but only a part of each unit in most of the cases. This happened because the materials were shared with the whole Cell Culture Bioengineering Laboratory at the University Carlos III of Madrid.

Similarly, the **budget** set aside for the project was included within the global budget of the laboratory. This budget comprises both the materials already mentioned in Table 7 as well as the **specialized equipment**, which require periodic supervision and maintenance, as well as constant electronic supply:

- CO_2 cell incubators: 3517-SHEL LAB
- Blue-light fluorescence microscope: BX53 OLIMPUS
- Thermostatized water bath: BM 302 – NÜVE
- Refrigerated centrifuge: Digtor 21-R ORTOALRESA
- Biosafety cabinet: BIO-IIA-TELSTAR
- Cell Sorter: BD Influx
- Tissue Processor: LEICA ASP 3005

This budget is financed by the research **funds** for University Carlos III of Madrid. In addition, part of these economical funds is designated to instruct every laboratory worker in biological safety. In the Cell Culture Bioengineering Laboratory, as in any other laboratory, the appropriate **security protocol** against potential biological risks was followed.

Table 7: Production costs of the project along the three experimental stages *(continues in next page)*

Consumables

Product Description	*Company*	*Price/unit (€)*	*Amount Consumed*	*Cost (€)*
P100 dishes, 20/bag	Fisher Scientific	134.03	9 units	11.56
75 mL T-Flasks (case 30)	Sigma Aldrich	257.4	10 units	85.8
X50 Falcon Tubes 15 mL	Sigma Aldrich	7.05	150 units	10.575
X50 Falcon Tubes 50 mL	Sigma Aldrich	8.12	70 units	22.736
Electronic Pipettor	DD Biolabs	48.89	1 unit	18.89
X200 Serological Pipet	Fisher Scientific	24.1	400 units	48.2
X500 2 mL Microtube Naturel	Fisher Scientific	7.65	500 units	7.65
Micropipette Tip 200µL	Labbox	36.191	400 units	14.48
Micropipette Tip 10µL	Labbox	6.02	400 units	2.408
X100 Nitrilo Comfort Gloves S6,5	Fisher Scientific	4	200 units	8
			Subtotal	230.299

Reagents: Gene Therapy

DMEM High W/GlutaMax (500 mL)	Fisher Scientific	20.38	3 L	122.28
Keratinocytes Serum Free Medium (10X500 mL)	GIBCO	772	5 L	772
FBS 500 mL	Fisher Scientific	106,4	500 mL	106.4
293T cells	ATCC	358	27 million	358
Feeder cells	ATCC	75	15 million	281.25
Chloroquine	Sigma Aldrich	39.3	3 µL	4.71
Packaging Plasmids	*Donation from Dra Perona*	-	40.656 µL	-
PuraDisc Filter 0.2 µm	Fisher Scientific	66.3	4 units	10.68
Polybrene	Sigma Aldrich	45.3	3.5 µL	7.92
			Subtotal	1663.24

Reagents: Bioengineered Skin

Trypsin 100 mL	Sigma Aldrich	14.9	70 mL	10.48
6 Well Plates with Porous Membrane (1µm)	DD Biolabs	271	2 units	10.84
Amchafibrin	Fides Ecopharma	3.14	30 µL	0.52
Bovine Plasma Thrombin	Sigma Aldrich	277.5	250 µL	277.5
Porcine Fibrinogen	Sigma Aldrich	311.9	750 µL	31.19
Dimethyl Sulfoxide 50mL	Sigma Aldrich	36.7	4 mL	2.936
X50 Falcon Tissue Culture Plate	Fisher Scientific	135.2	2 units	5.408
			Subtotal	338.87

Reagents: Histology

Product Description	*Company*	*Price/unit (€)*	*Amount Consumed*	*Cost (€)*
Formaldehyde (1 L)	Sigma Aldrich	62	1 L	62
Tissue Tek OCT Compound	Sakura Finetek	20,93	2 units	41,86
Hematoxylin Stain (4L)	Sigma Aldrich	238.5	500 mL	29.8
Eosin Stain	Sigma Aldrich	62.4	500 mL	62.4
Xylene (5 L)	Thermo Scientific	105	500 mL	10.5
Ethanol 99.8% (2,5 L)	Thermo Scientific	114	2 L	91.2
Glass Cover Slips (100)	Fisher Scientific	87.44	20 units	17.48
			Subtotal	273.38
			Total Fungible Cost (€)	**2505,793**

Personnel Workhours

Approximate cost per hour: 20€/hr	*Workhours:* 150 hrs		
		Subtotal	3000
		Total Laboratory Cost (€)	**5505.793**

Annex IV: Glossary of Terms

BMF = Bone Marrow Failure

CT = Connective Tissue

DC = Dyskeratosis Congenita

DMEM = Dubbeco's Modified Eagle Medium

DMSO = DiMethyl SulfOxide

ECM = Extracellular Matrix

FBS = Fetal Bovine Serum

GFP = Green Fluorescence Protein

GGT = Germline Gene Therapy

GSE = Genetic Suppressor Element

HIV = Human Immunodeficiency Virus

HSC = Hematopoietic Stem Cell

iPSC = Induced Pluripotent Stem Cell

IRES = Internal Ribosome Entry Site

LV = LentiVirus

SC = Stem Cell

SCNT = Somatic Cell Nuclear Therapy

SCT = Stem Cell Transplantation

TERC = TElomerase RNA Component

TERT = TElomerase Reverse Transcriptase