Universidad Carlos III de Madrid

BACHELOR THESIS

Characterization in a humanized context of a rare disease: Dyskeratosis Congenita

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Bachelor: Biomedical Engineering. Tissue engineering and regenerative medicine.

Acknowledgments

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Abstract

The experimental work of the final degree project was dedicated to apply the knowledge acquired of tissue engineering and regenerative medicine, which are branches of biomedical engineering, focusing on the study of rare skin diseases.

The project was developed at CIEMAT (Centro de Investigaciones MedioAmbientales y Tecnológicas) organization and with support of biomedical laboratories of Universidad Carlos III de Madrid. The project was controlled by Doctor Marta García Díez.

The research was focused in the characterization of Dyskeratosis Congenita, which present different symtomes, including skin defects, due to lack of dyskerin protein. To perform this task *In vitro* and *In vivo* experiments are done for the characterization of diseased skin. In order to obtain a precise result bioengineered skin, histology, antibody detection and other methods are used.

The main objective of this work is the generation and validation of an *In vitro* skin-humanized modelof DC (Dyskeratosis Congenita) of cells patients, recreating the cutaneous phenotype of this disease and then assess therapeutic approaches based on genetically modificated DC cells by lentiviral vectors coding for GSE-24.2.

Motivation and objectives

As the importance of skin, not only being the outermost tissue of the human body but also taking into accounts its social importance. The study of skin diseases is essential for treating millions of people around the world.

With the idea of give a broader view of the characteristics of a diseased skin facilitating a diagnostic, some characterization methods are used. The characterization of skin gives a wide overview of the structure, function and protein expression in Dyskeratosis Congenita skin.

First, the transfection of a lentivirus containing GSE-24.2 gene to treat Dyskeratosis Congenita skin generating and assessing a humanized DC skin model, both *in vitro* and *in vivo* models. *In vitro* studies are developed for modeling the disease, while *in vivo* studies are centered in the correction of the disease using the transfection of specific protein.

Finally some histological studies are done for the comparison of diseased skin with healthy one, also using immunohistochemistry processes; the behavior of the skin cells can be evaluated for the development of new therapies to treat skin diseases.

Planning

PHASE 1 (December 2014) SELECTING A RESEARCH TOPIC AND LITERATURE SEARCH. Selection of the topic and search of information about it, using the knowledge acquired during the university years.

PHASE 2 (December 2014) MENTOR REUNION. Organization and explanation of the project, first steps for investigation.

PHASE 3 (First and second week of February 2015) TRANSFECTION OF LENTIVIRUS CONTAINING GSE-24.2 GENE. A well-known process is used to infect patient cells with the specific protein to treat the disease. Information about the transfection is obtained with microscope images.

PHASE 4 (third week of February 2015) RESULTS ANALSIS AND DISCUSSION WITH THE TUTOR. After obtaining the results, they were analyzed and discussed with the tutor. Following steps for the project are discussed.

PHASE 5 (Fourth week of February and first week of March 2015) PREPARATION OF BIOENGINEERED SKIN. In order to obtain precise models for the characterization of the skin. Cells of patients suffering Dyskeratosis Congenita are used. Also healthy cells are used as control.

PHASE 6 (Second and third week of March 2015) *IN VITRO* AND *IN VIVO* DEVELOPMENT OF BIOENGINEERED SKIN. *In vitro* experiments using a specific culture media for development of skin. *In vivo* experiments performed using mice models for a complex development of skin.

PHASE 7 (First and second weeks of April 2015) HISTOLOGICAL SAMPLES PREPARATION AND IMAGE OBTENTION. Samples are prepared using two different methods for histology. Staining of samples for the improvement of images. Finally a light microscope is used for taking of images

PHASE 8 (Third and fourth week of April 2015) IMMUNOCHEMISTRY METHODS FOR CHARACTERIZATION OF SKIN. Some antibodies are used to detect the concentration of proteins in Dyskeratosis Congenita disease.

PHASE 9 (May, first and second week of June 2015) ELABORATION OF THE FINAL MEMORY. Individual phase for the elaboration of the final document about the project.

PHASE 10 (third week of June 2015) FINAL REUNION WITH THE TUTOR. In order to improve or change some parts of the document.

PHASE 11 (Third week of June 2015) END OF THE MEMORY. After final modifications

Introduction

1. Skin

The skin is the largest organ in the body, comprising 16% of total body weight [1] and covering an area of $2m^2$. The principal function of the skin is to form a physical barrier against external effects, protecting the body from pathogens and also physicochemical changes in the environment. Since is divided in two main layers: Epidermis and dermis [2] and a subcutaneous layer called hypodermis.

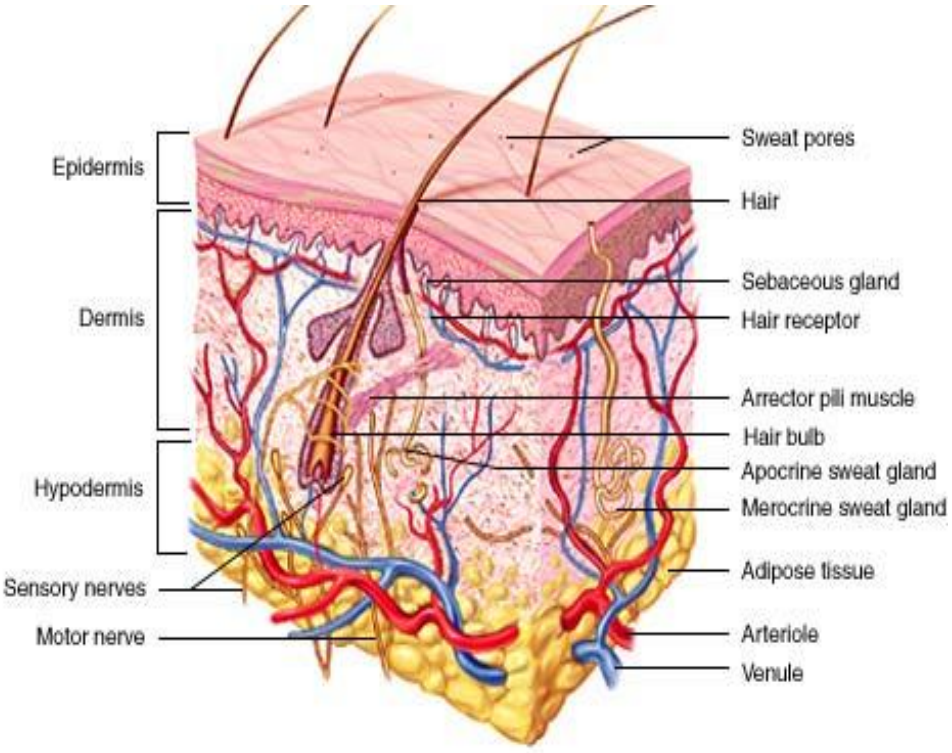

Figure 1. Structure and principal components of skin. The principal components of skin are shown.

The primary function of the epidermis consists on producing a protective, semi-permeable stratum corneum that permits life. The layers produced in the stratum corneum provide a tough and resilient framework for the intercellular lipid lamellae. Lysosomal and other enzymes which are present in the extracellular compartment are responsible for the lipid remodeling necessary to generate a protective barrier [3, 4].

But, functions of skin are not only protective. Skin plays an important role as heat exchanger with the environment. The body maintains its temperature around 37ºC regulating the heat released from the skin. If temperature increases inside the body, capillary vessels of the skin open and heat escapes through the skin surface. When body temperature falls, capillary vessels contract avoiding the expulsion of heat [5]. Moreover, skin works as a sensory system receiving stimuli from the environment. It senses texture, pressure and temperature. Receptor cells inside the skin carry constant information of the central nervous system.

2. Epidermis

Epidermis is a stratified squamous epithelium [6]. The main cells of epidermis are keratinocytes which synthesize keratin. Keratinocytes are connected among them by desmosomes (i.e.: protein bridges). Epidermis is constantly renewing thanks to the stem-cell niche in the basal stratum [7], and then there are constant states of transition from deeper layers to superficial ones. The different layers that can be found in epidermis are formed by different states of keratin maturation. The four layers of epidermis are [6]:

- Stratum basal: It is the innermost layer which lines with dermis, attaching to it by hemidesmosomes. As keratinocytes divide and differentiate, they move from the stratum basal to the surface of the epidermis. Keratinocytes synthesize keratin intermediate filament proteins. These filaments permit the cells to connect with those from the stratum spinosum and to the adjacent cells by desmosomes [8, 9]. In this layer also are produced the melanocytes, which contains a specific pigment called melanin, important to determine the skin color [10]. Melanin accumulates in melanosomes that are transferred to adjacent keratinocytes where they remain as granules. Melanines also provides protection against ultraviolet radiation.

- Stratum spinosum: When cells reproduce and mature, they move from stratum basal through outer layers of epidermis. Cells are connected by intercellular bridges (desmosomes). In this layer it can be found Langerhans cells, which are immunologically active cells derived from the bone marrow.

Then this layer plays an important role in immune reactions, with Langerhans cells acting as antigen-presenting cells [11].

- Stratum granulosum: At this level cells undergo apoptosis (i.e.: programmed cell death), Nuclei and organelles of the cell are degraded, and cells become more flattened. In this layer also lamellar granules are released, which are responsible of the secretion of a lipid-rich substance that accumulates in intercellular space of the stratum [12].

- Stratum lucidum: Is a thin and translucent layer composed by dead skin keratinocytes. It is found, principally, on the palms of the hands and soles of feet [13]. Stratum lucidum is composed by three to five layers of dead keratinocytes. The thickness of the layer depends on the mitosis of epidermal cells; the darkness is determined by melanosomes.

- Stratum corneum: It is the external layer of epidermis. It is composed by hexagonal-shaped, cornified cells known as corneocytes, each corneocyte is surrounded by a protein envelope and filled with water-retaining keratin proteins. Shape and orientation of proteins will determine the strength of the stratum corneum [6]. The structure also facilitates the function of physical and water-retaining barrier of the skin [14]. The movement of epidermal cells from stratum basal to stratum corneum lasts 28 days and it is called epidermal transit time.

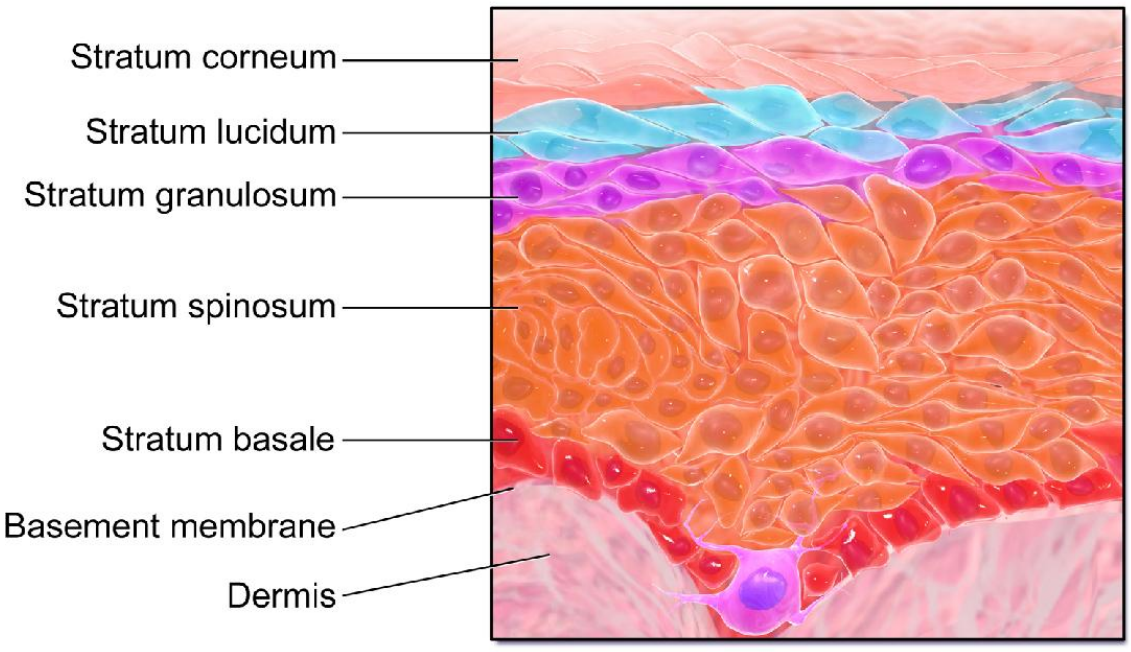

Figure 2. Structure of epidermis and its different layers.

3. Types of epidermal cells

There are four principal types of cells in epidermis:

- Keratinocytes**:** They are the most abundant cells in epidermis (95% of cells). These cells are responsible of the tight-junction formation with the nerves of skin. Moreover, Keratinocytes facilitates the maintenance of Langerhans cells of the epidermis and lymphocytes of dermis in place [15].
In addition to their structural role, keratinoytes take part on immune system protection. They prevent toxins and pathogens from entering in the body and the loss of heat, moisture and other important components. In addition, their principal immune role is immunomodulation, responsible for secreting inhibitory cytokines in absence of injury, but when an injury occur, they stimulate inflammation and activation of langerhans cells.

- Langerhans cells**:** They are members of dendritic cell family and reside near the epidermal basal layer. Their primary function reside in antigen presentation for immune cells, LC acquire antigens in peripheral tissues and

transport then to lymph nodes faciliting the immune response by the organism. Moreover, LC Works as mediator of tolerance [16].

- Melanocytes**:** They are cells of neural crest origin. In epidermis, they form relations with keratinocytes using their dendrites. But, their principal function consist on skin pigmentation by the release and distribution of melanine through though the skin. Melanocytes are regulated by melanocortin peptide alpha-MSH (Melanocyte-Stimulating hormone) [17].

- Merkel cells**:** They are the fewest numerous cells in epidermis. MC is found isolated in the epidermis or associated with epithelial nerve endings. These types of cells are supposed to derive from the neural crest as Melanocytes, which allow living in union with epidermal and neural cells. Principal function of MC consists on a specific, slowly adapting sensory touch receptor [18].

4. The dermal-epidermal junction

The relation and anchoring between dermis and epidermis is an indispensable structure for skin interactions with other tissues and for protection of environment, it is called dermal-epidermal junction. Its principal functions are: bind tightly dermis and epidermis, determine the polarity of basal keratinocytes of epidermis, works as a selective barrier to protect the organism and plays an important role in wound healing [19].

Centering in the structure of DEJ, it can be differentiated in four parts: First, hemidesmosomes of basal keratinocytes in their cell membranes; second, the lamina lucida that anchor filaments transverse and can be seen by electron microscopy; third the lamina densa composed principally by collagen IV and laminin family proteins; and fourth, the sub-basal lamina containing anchoring fibrils [20]. All the parts of DEJ contains ubiquitous basement membrane components, including laminin, collagen type IV, proteoglycans (predominantly in lamina densa) and collagen VII forming anchoring

filaments in the sub-basal lamina densa.

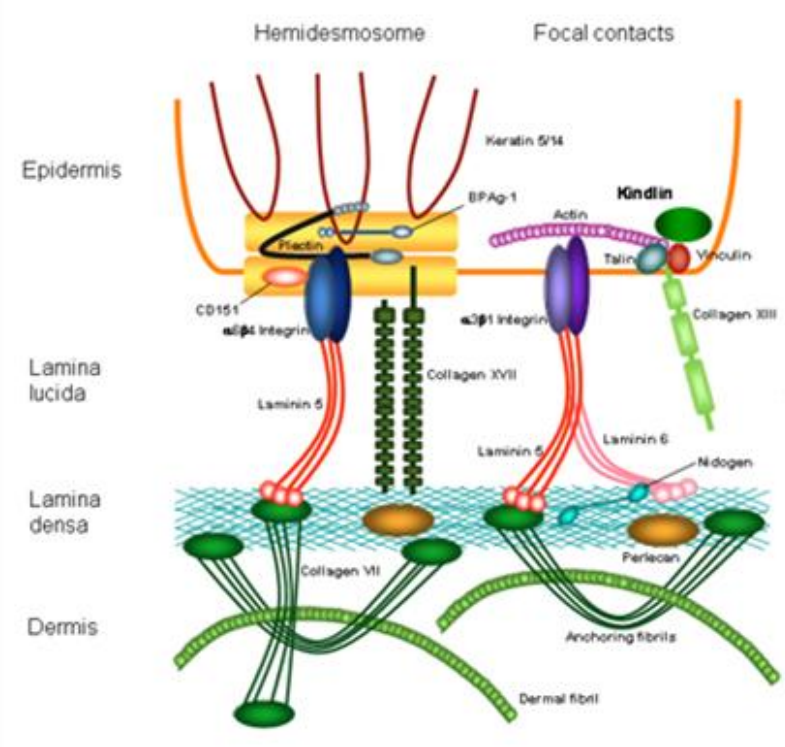

Figure 3. Conformation of dermal-epidermal junction. DEJ is formed by two layers: lamina lucida and lamina densa. The principal components of DEJ are: Laminin 5/6, Collagen XVII/XXIII, α3β1 integrin, etc.

5. Telomerase

Telomerase (or telomere terminal transferase) is a ribonucleoprotein that adds DNA sequence repeats (specifically "TTAGGG") to the 3' end of DNA strands in telomere regions [21]. In linear DNA, there is no place to produce the RNA primer needed to start the final Okazaki fragment on the lagging strand when the replication fork reaches the end of the helix. If telomerase is not present, a single stranded region of DNA would be produced (100 to 200 nucleotides) and DNA polymerase cannot produce a daughter strand. This would produce the shortening of chromosomes each replication cycle. Telomerase is a reverse transcriptase that carries its own RNA molecule, which is used as a template when elongates telomeres.

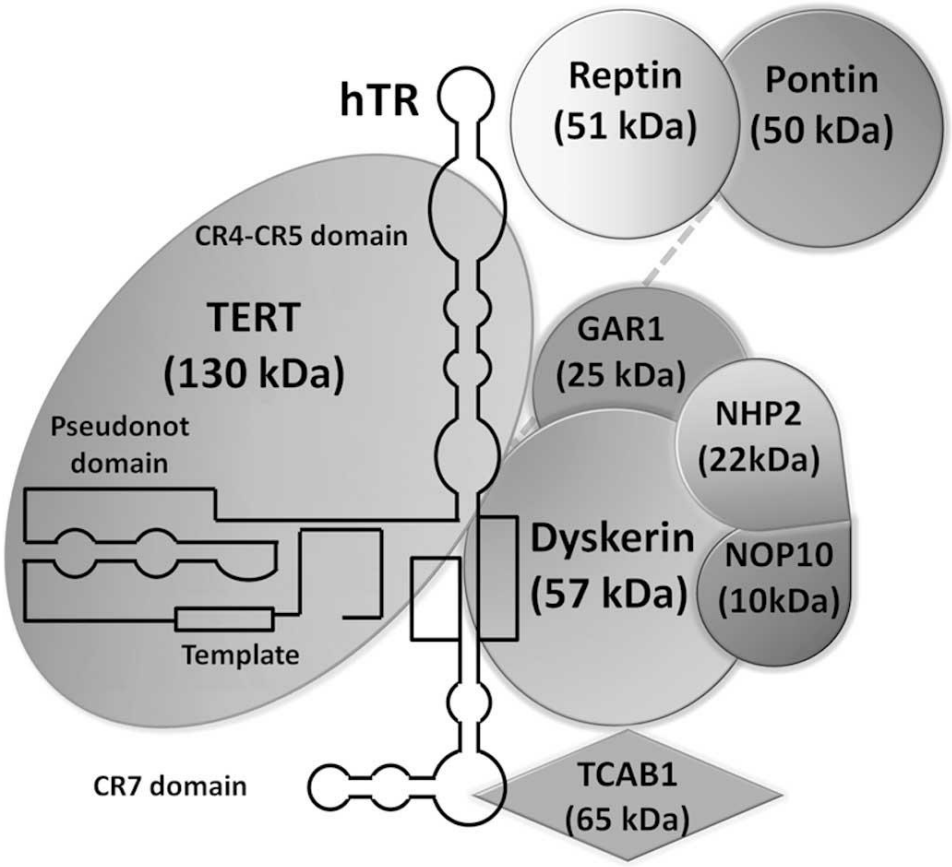

Figure 4. Group of molecules which form human telomerase. The most important parts are: TERT (telomerase reverse transcriptase), TERC or hTR (Telormerase RNA) and dyskerin.

Human telomerase is formed by different molecules: telomerase reverse transcriptase (TERT), telomerase RNA (TERC) and dyskerin (DKC1) [22]. The genes of telomerase molecules are located on different chromosomes in human genome.

TERT is capable to add the six nucleotide repeating sequence (TTAGGG), using TERC, to the 3' strand of the chromosomes. The TTAGGG repeat forms the telomeres. Then, telomerase can add the 6 nucleotides repeat sequence; realign the new 3' end of telomere to the template, and repeat the process to avoid telomere shortening [23].

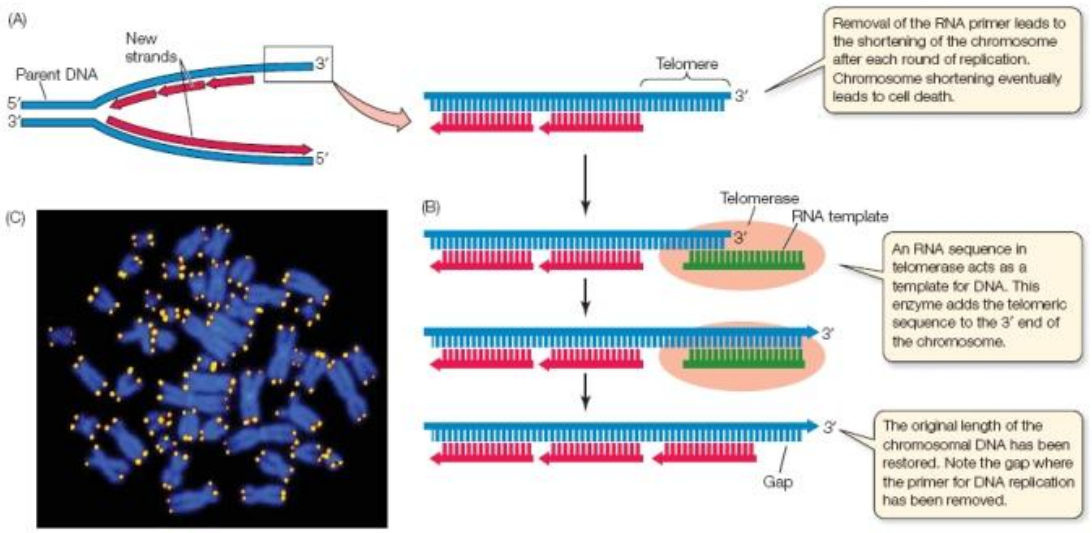

Figure 5.Scheme of telomerase function. (A) Normal replication process, there is a gap in 3' in the lagging strand. (B) Function of telomerase, reducing the gap of 3'. (C) In blue, chromosomes; in yellow, telomerase.

Telomerase is a protein important for clinical studies. It plays a critical role in cell aging and cancer development. Moreover, the lack of telomerase produces several rare diseases as Dyskeratosis Congenita.

6. Dyskeratosis congenita

Dyskeratosis Congenita is an heterogeneous disease which present different symptoms: skin alterations, as nail dystrophy, hyperpigmentation and oral leukoplakia (Fig. 1); pulmonar fibrosis and bone marrow deficiencies [24, 25].

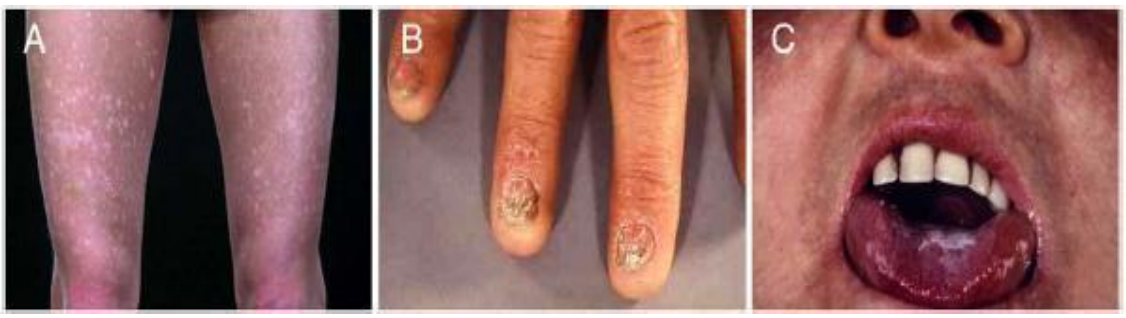

Figure 6. Dyskeratosis congénita symptoms in skin. A) skin hiperpigmentation. B) Nail dystrophy. C) Oral leukoplakia.

DC is caused by deficiencies in telomerase ribonucleoproteic complex, which function is to maintain the length of telomeres in the chromosomes. DC can be cause by mutations that affect to any gene codifying one of the components of Telomerase complex. Depending on the affected component the symptoms, severity and inheritance of the disease change [24, 26].

Recessive Dyskeratosis Congenita linked to X chromosome is caused by mutations in gene DKC1, which codifies for Dyskerina [27]. The most usual mutation that affects this gene is a non-sense mutation that introduces a premature stop codon. The Dyskerina is a protein of telomerase complex which function is matures the telomeric RNA to make its task. In this way, when the mutations affect the DKC1, the telomeric RNA is reduced and the activity of telomerase is reduced and telomeres are shortening [24, 26, 27]. These alterations in the telomere length maintenance drive to aging and cell senescence. The lack in telomerase activity affects to regenerative cells in tissues which are in constant renewal, as in the case of hematopoietic cells and basal epidermal cells. Then, the deficiency in telomerase activity of keratinocytes entails telomere shorten, altering cellular proliferation and perturb the capacity of epidermal renovation [26].

7. Gene therapy

Gene therapy is a therapeutic strategy based on the genetical modification of somatic cells through the addition of nucleic acids with the final proposition of cure inherited and acquired diseases [28].

Gene therapy can be developed using two different strategies:

- *In vivo* strategy is based in local or systemic administration of the vector in the organism. In this way, the vector inserts the transgene in target tissue to create a specific functional gene.

- *Ex vivo* strategies begin with the extraction of patient cells of the

target tissue. Cells are disaggregated and cultured *In vitro*, then they are genetically modified with the transfection vector. Genetically modified cells are selected depending on their capacity to express the exogenous gene. These cells are amplified and recollected to reintroduce in the patient. Gene therapy efficiency is highly superior when cells are treated *ex vivo*, then this strategy is used mostly [29].

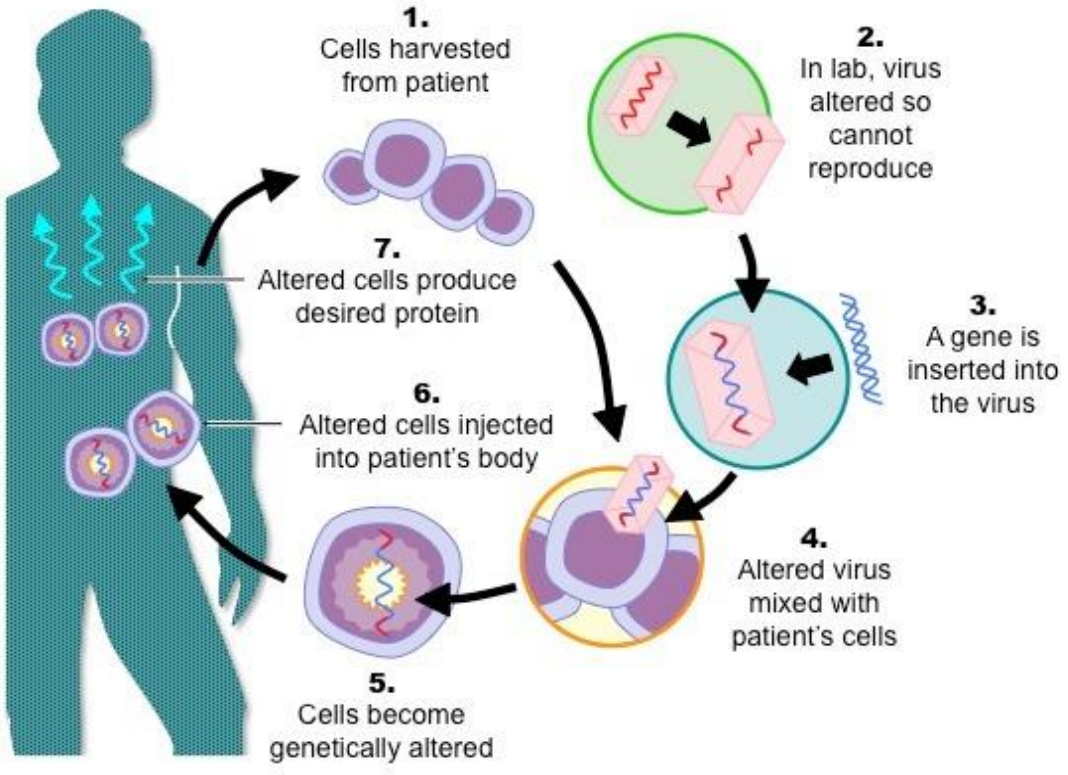

Figure 7. Normal procedure for *ex vivo* gene therapies. Cells are harvested from patient. Trangene vector is developed in laboratory and transfected to the cells. Finally infected cells are reintroduced in the patient.

Depending on the durability of the trasgene therapeutic effect, gene therapy can be transitory or permanent:

- Transient gene therapy: Its main objective is to maximize a transitory physiological process, for example wound healing.

- Stable gene therapy: Its function consists on a permanent correction of a disease. With this objective, the therapy needs to apply two

principal requisites: First, target cells for transgene vector must be stem cells. Secondly, Vector need to be able to integrate the transgene in the genome of stem cell, in such a way, the trangene is transferred to its progeny [30].

Finally, depending on the strategy used for genetic correction in cells two different therapies are used:

- Addition gene therapy: One, or more, copies of the transgene are introduced in the cell and added to the genome in an integrative or non-integrative form.

- Substitution gene therapy: At least a mutated allele of gene is eliminated of the genome; it is replaced by a copy of the transgene using homologous recombination [31].

8. Vectors for gene therapy

Gene therapy systems need to be safe, effective and capable to work in target cell type. Moreover, they need to guarantee the stable expression of transgene. Vectors, which are DNA carrier molecules, are the regular method for transgene transfection. Vectors are systems that facilitate the transference of exogenous gene to target cells. Vectors can be classified in viral and non-viral vectors.

Cells can be genetically modified using different kind of vectors, depending on the selectivity, efficiency and transfer of the trasgene to cells. Non-viral vectors have some limitations in the transfer of the trasgene to cells, due to their low transfection efficiency [32, 33]. In the case of transfection to epidermal cells, lentiviral vectors are widely used.

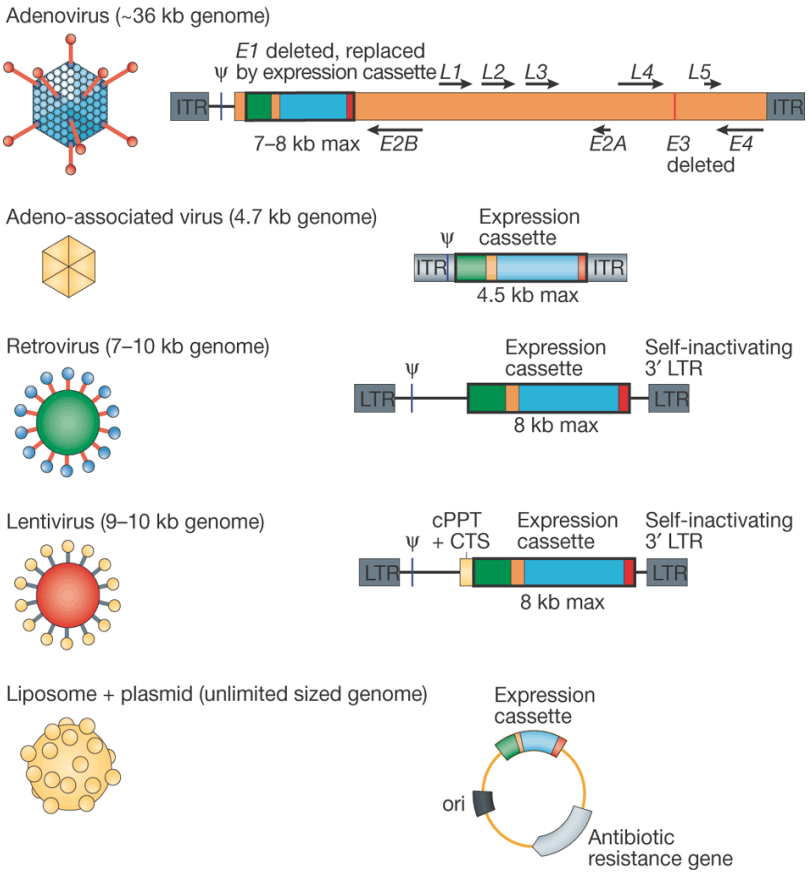

Figure 8. List of the different types of vectors (viral and non-viral) for gene therapy. Lentiviral and retroviral vectors are the most used because they insert in the genome. Adenoviral vectors offer more space for transgene, but they are eliminated by immune system. Adeno-associated viral vectors reduce the immune response with respect to adenoviral vectors. Finally the most usual non-viral vector is a liposome + plasmid complex. It offers, unlimited sized genome and no immune response, but its efficiency is low.

9. Lentiviral vectors

Lentivirus is a subclass of retrovirus. Lentiviral vectors are capable to integrate into the genome of non-dividing cells (while other retrovirus only can infect dividing cells). The viral genome in the form of RNA is reverse-transcribed when the vector is inside the cell to produce DNA. This DNA is inserted at a random position in the genome by viral integrase enzyme. The vector remains in the genome and is passed on to the progeny when cell divides [33].

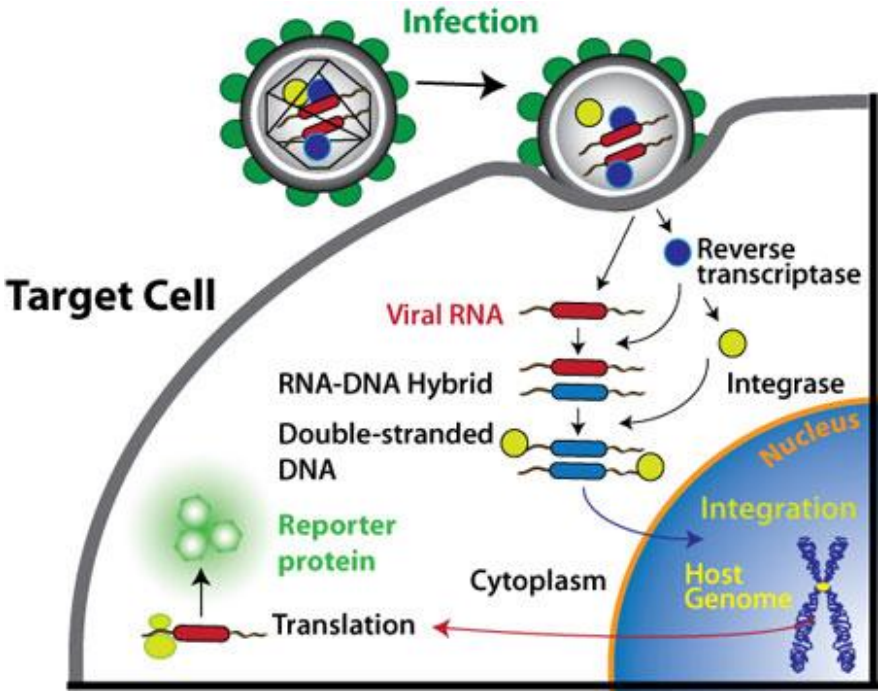

Figure 9. Resume of the lentiviral infection method. The lentiviral vector enters in the cell and converts the viral RNA in viral DNA by the reverse transcriptase. Viral DNA is inserted in cell genome. Transgene carrying the wanted protein is expressed.

The site of integration is unpredictable and can produce some problems. It can disturb the function of cellular genes and lead to the activation of oncogenes promoting the development of tumors. However, some studies have shown that lentiviral vectors have lower tendency to integrate in potential cause cancer than other viral vectors [34].

Lentiviral vectors never carry genes required for their replication for safety reasons. To produce a lentivirus several plasmids are transfected to packaging cell line (normally 293T cells are used). The plasmids used include: virion protein encoding plasmid, as the capsid and the reverse transcriptase; and the specific gene to be delivered by the vector in genome.

Lentivirus contains six accessory genes: *nef*, *vif*, *vpr* and *vpu*, related with virulence proteins; *tat*, which codifies a regulatory protein proviral DNA transcription; and *rev*, which codifies a protein that binds to responsive element RRE (Rev responsive element) situated in the transcription process of *env* gene for the exportation of viral mRNA from nucleus to cytoplasm.

The most common virus used as lentiviral vector is HIV-1 (human immunodefficient virus 1). The own codifying genes of lentivirus are substituted by a plasmid inserting a transgene, necessary genes for vector packaging are introduce in *trans*.

Cellular toxicity caused by several lentiviral proteins, necessary for vector packaging, reduces the possibility of using stable packaging methods. Then, lentiviral vectors are produced in transitory packagning systems. This lentiviral vector is obtained with the transfection of four plasmids: one for the transgene and 3 helper plasmids: for genes *gag/pol*; for gene *rev*; and another for the gene codifying the envelope.

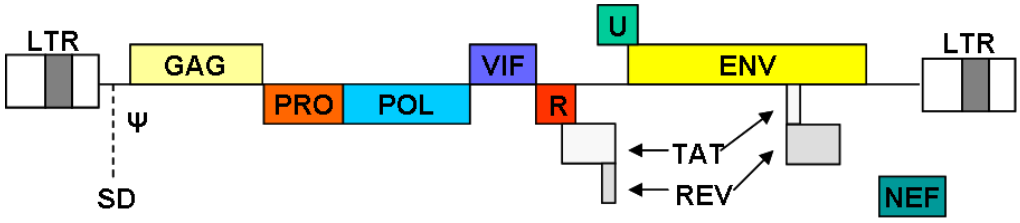

Figure 10. Scheme of lentiviral genome of HIV-1. The lentivirus contains: nef, vif, vpr and vpu related with virulence proteins; tat, related with proviral DNA transcription; and env for exportation of viral mRNA from nucleus to cytoplasm.

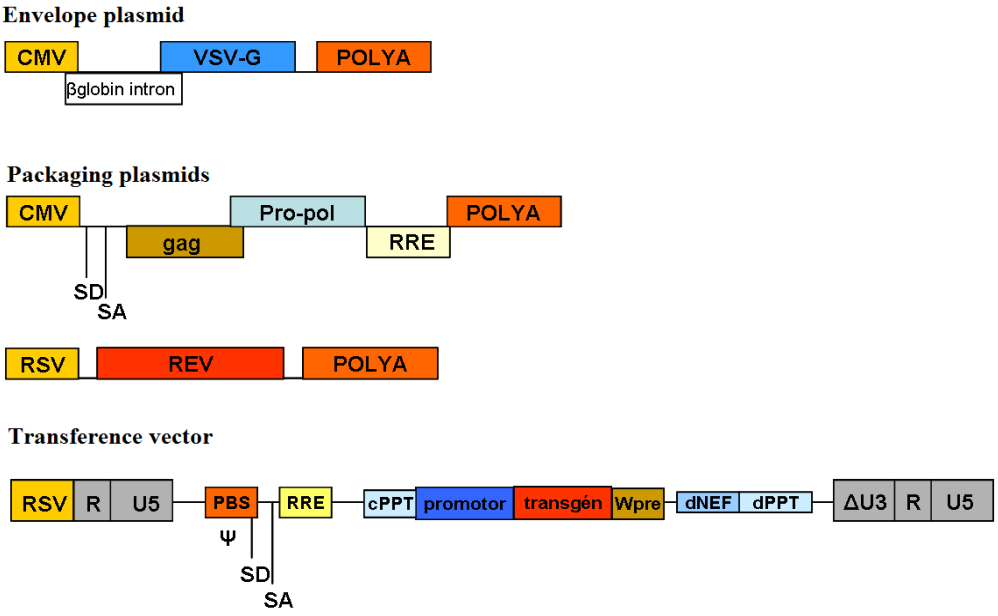

Figure 11. Scheme of the necessary plasmids for packaging of lentiviral vectors. The lentiviral vector is composed by: envelope plasmid, packaging plasmids (pro/pol and REV genes) and the transference vector carrying the transgene.

10. Development of bioengineered skin in animal models and vector transfection

During the last years the field of genetic modification has evolved for fibroblast and keratinocytes, and also for epidermal bioengineered tissues. Skin is a suitable organ for gene therapies: it is accessible, can be modified *in vivo* and *in vitro* and is easy to remove in case of adverse symptoms. The characteristics of keratinocytes allow extracting and isolating from the patient, maintain in a cell culture and be infected *ex vivo.* After infection cells are selected, isolated and expanded in a culture to be introduced again in the patient [35]. Keratinocytes infected *in vitro* maintain their proliferative, maturation and differentiation activities [36]. Nowadays, the strategies for transfection *in vitro* are well-known, in order to introduce a trasgene, with the necessary genetic information to target molecules.

Due to virus in eukariotic cells and, in particular those that affect to humans, have evolved to transfer their genetic material with high efficiency in human cells, the vectors derived from them transduce much more efficiently than non-viral vectors. Retrovirus and lentivirus are very efficient, when it is required a more stable expression of the trasgene. But, when it is wanted a transitory expression, non-integrative vectors, as Adenovirus, are more appropriate [37].

Nowadays, it is possible to infect primary keratinocyte cultures with viral vectors, as retrovirus [38,39] and lentivirus [40]. Lentiviral vectors are capable to infect in vitro keratinocyte cultures with higher efficiency and also promote the stability of trasgene expression, thanks to a specific characteristics: they are integrated, do not possess genetic regulatory U3 region, they integrate in target cell genome using LTR regions through homologous recombination [40]. The integration capacity promotes the infection efficacy and, once integrated, infected cells over express the transcripts respect to other vectors.

Moreover, it has to be taken into account that systems for gene transfer, used to correct genetic abnormalities, must be safe. Then it is necessary to demonstrate the security of gene transfer in infected cells. Lentiviral vectors have less probability to suffer inserccional tumorgenesis [41]: vectors are integrated by LTR regions and its

genetic expression is regulated by cell promoter, reducing the probability to activate the expression of near genes and produce oncogenesis in cells.

In the other hand, epidermal bioengineering is capable to develop in vitro bioengineered skin. The development of differentiated skin is possible thanks to regenerative capacity of epidermal stem cells. Keratinocytes, which constitute the epidermal component of bioengineered tissue, are cultured over a matrix of fibrin, fibroblast and specific growth factors [42]. Fibrin matrix acts as a scaffold and stimulates the proliferation of keratinocyte. This tissue mimics the natural process of wound healing allowing to recreate in vitro the physiological and functional characteristics of natural skin [43]. Moreover, it has been established a humanized mouse model, based on a graft of skin equivalent in a immunodefficient mouse. Then, it is possible to obtain a stratified and differentiated human skin which reproduces the natural characteristics in human epidermis [44].

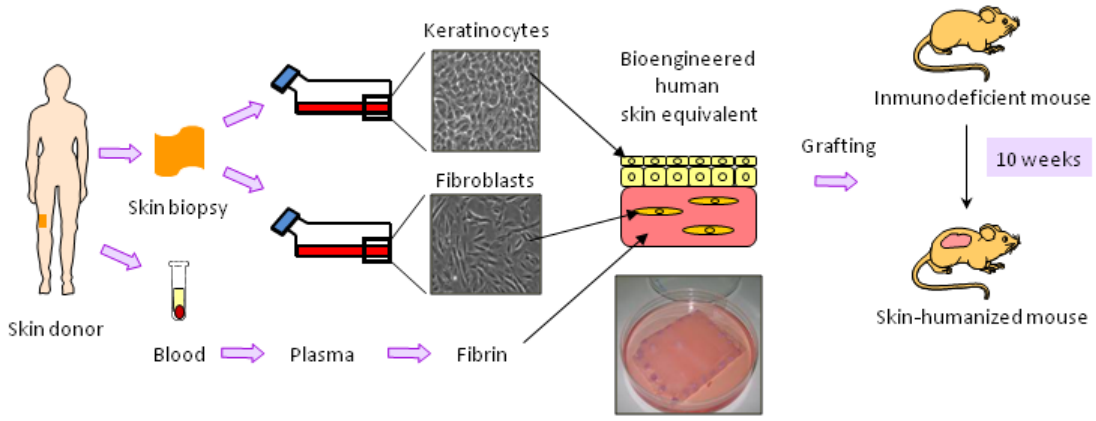

Figure 12. Scheme of the humanized skin animal models. First, bioengineered skin is composed using cells of the patient (keratinocytes and fibroblast) and a fibrin matrix. The bioengineered skin is grafted on immunodefficient mouse for maturation *in vivo*.

This model is not only important for study of physiological dermal processes, but also to generate preclinical models for dermal diseases [44]. The use of diseased keratinocytes as epidermal component of the equivalent skin and the posterior graft in to the immunodefficient mouse allow generating a human epidermis in a mouse that reproduces the pathologic phenotype of the disease [45].

This strategy facilitates the study and preclinical model generation for some skin diseases [45]. This model has been also used for genetic correction of keratinocytes *in vivo*.

Then, the development of skin equivalents, the generation of preclinical animal models and the evolution of gene therapies gives the possibility to characterize the Dyskeratosis Congenita disease. Using a lentiviral vector that includes Dyskerin (GSE-24.2), as approximation of gene therapy of DC, and developing *in vitro* skin equivalents with transfected keratinocytes to generate a humanized mouse model for Dyskeratosis congenita.

Materials and methods

1. Transfection of GSE-24.2 gene

Cell culture. Primary fibroblast and keratinocytes from Dyskeratosis Congenita skin and healthy patients were obtained through skin biopsies of the patients. DC cells were obtained from Dra. Perona.

293T (human embryonic kidney cells) cells are cultured using DMEN (Dulbecco's modified eagle medium) with inactivated serum 10% medium in p100 dishes. Cells were counted to have 3 millions of 293T per dish. Incubate at 37ºC during 24 hours.

Lentivirus production. The preparation of lentiviral vector is composed by different parts:

- Lentiviral plasmid GSE-24.2 gene + GFP, 6.04µL at 1, 49µL/µG (only in DC dish)
- Plasmid-GFP (only GFP), 11, 25µL at 0, 5µg/µL (only in control)

Helper plasmids:

- Genes *gag/pol*: 2, 42µL at 2, 07µg/µL
- Gene *rev*: 1, 73µL at 1, 3µg/µL
- Gene codifying the envelope: 1, 75µL at 1, 8µg/µL

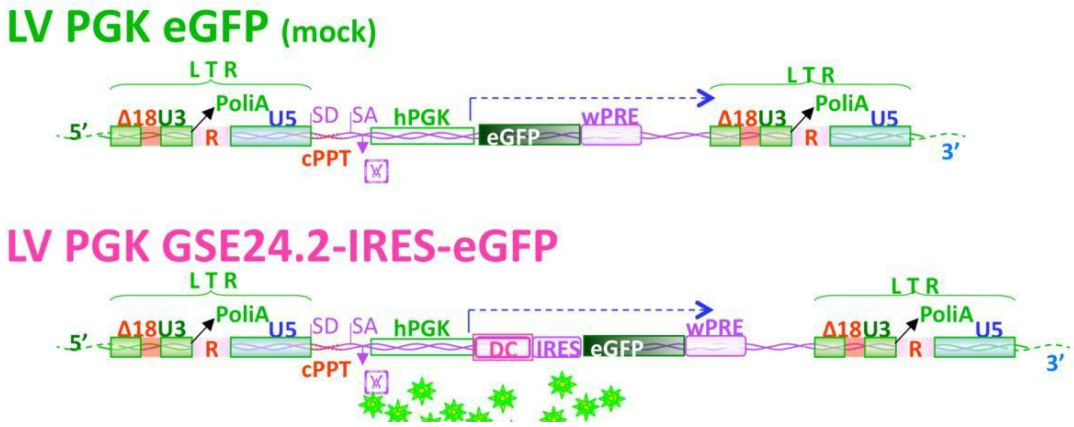

Figure 13. Representation of the structure and compostion of lentiviral vector. First represents the control with GFP. The second also contains the GSE-24.2 protein (DC+IRES). Both vectors are formed by LTR sequences. The regulatory región U3 has been eliminated. Genetic expression is regulated by the promoter of phosphoglycerate kinase and contains a post-transcriptional regulatory sequence, wPRE. GSE-24.2 vector codifies for dyskerin and GFP, it has also incorporated IRES sequence, which facilitates GFP traduction.

When four plasmids are mixed, 61µL of 2, 5 M $CaCl_2$ 2M are added. Then, 1.250 µl of 2X HBS (HEPES 100 mM (GibcoBRL); NaCl 281 mM; Na2HPO4 1,5 mM; are added to the mixture and bubble to homogenize.

Transfection of the vector. First, 293T medium is changed by 9mL of DMEN + 3µL of Cloroquine (specific medium for $CaCl_2$). Lentiviral sample is added to the mixture drop by drop. Incubate the culture at 37ºC during 6 hours. The medium is changed again by fresh DMEM and incubated at 37ºC

After 24 hours cells are observed at the microscope, using fluorescent light to detect GFP concentration. Medium is changed to *kcinact* and incubated at 37ºC during 24 hours.

The final step consists on the use of supernatant of transfected cells to add it to keratinocytes. The medium of transfected cells is retired and fresh *kcinat* medium is added, cells are incubated at 37ºC during 24 hours. Medium of transfected cells is filtered at 0.22. Then the medium is added to DC keratinocytes during 6 hours at 37ºC and changed by fresh medium 24 hours. The process is repeated twice for a higher transfection.

Finally, 293T cells are no more useful. DC Keratinocytes are followed daily changing the medium every 48 hours.

2. Bioengineered skin fabrication

The bioengineered skin is based in a fibrin matrix approach. Fibrinogen, obtained from cryoprecipitate plasma of pig blood, is used as a source for the matrix. Once the plasma is extracted it is treated with three freeze-thaw cycles to obtain the cryoprecipitate, which is centrifuged at 3500 rpm during 15 minutes, 4ºC.

Supernadant is discarded and the pellet is resuspended in NaCl 0, 9% and incubated at 37ºC to dissolve completely the fibrinogen [46]. The production of fibrin gel consist on: mixture 1,5mL of fibrinogen (cryoprecipitate) with 5mL of DMEM supplemented with 10% FBS and 2,5x10e5 human fibroblast, 250 UI of bovine aprotinine are also added. Immediately after, 0,5mL of $CaCl_2$ 0,025 mM and 5, 5 UI of bovine thrombin to a final volume of 3mL.

The mixture was initially deposited in a inserts of 6-well transwell plates (3 with DC patient fibroblast and 3 controls), where it solidifies at 37ºC during 2 hours. Part of the mixture is deposited in 6 well-plates for animal model study (2 for orthotopic method for murine skin transplantation, 2 for Barrandon method). Finally, Keratinocytes are added to the gel. The gel is again incubated at 37ºC, O/N.

3. Growth in vitro and in vivo

The growth and differentiation of skin is performed with two different approaches: *in vitro* and *in vivo* in an immunodefficient mouse model.

In vitro experiment was performed using 6-well transwell plates; DMEN medium is used and changed every 4 days for the growth of the tissue.

When keratinocytes reach confluence, engineered skin equivalents were allowed to differentiate at the air-liquid interface for 17 days at 37ºC in a CO2 incubator. The medium in changed every two days (Figure 14).

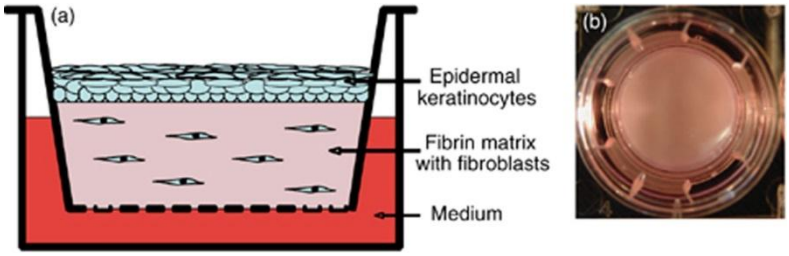

Figure 14: Skin Equivalent structure. (a) Schematic of a skin equivalent in a cell culture insert with a PET membrane (1 μm pores). The surface keratinocytes are exposed to the air, while the construct is fed by medium from below. (b) Skin equivalent after incubation for 17 days at the air/liquid interface showing an intact and dry surface, ready for studies.

In vivo experiment was performed using immunodeficient mice as an animal model. Two approaches were used:

First, orthotopic method for murine skin transplantation. An incision is done in the back of the mouse. The skin is extracted and the bioengineeed skin is set in position (keratinocyte layer is set above). Murine skin pass through a freeze-thaw step 3 times to kill it. The skin is again put in place to fix the bioengineered skin gel and is stitched for a correct fixing. The result of skin growth can be seen in 2 months. (Figure 12)

On the other hand, the second approach is Barrandon method. It consists on doing an incision in murine skin. A sheet of silicone is inserted and bioengineered skin is introduced with keratinocyte layer lying with the silicon sheet. The incision is closed. The results can be seen in 15 days, but it does not recapitulate the morphology of skin as orthotopic skin graft does (Figures 15 & 16).

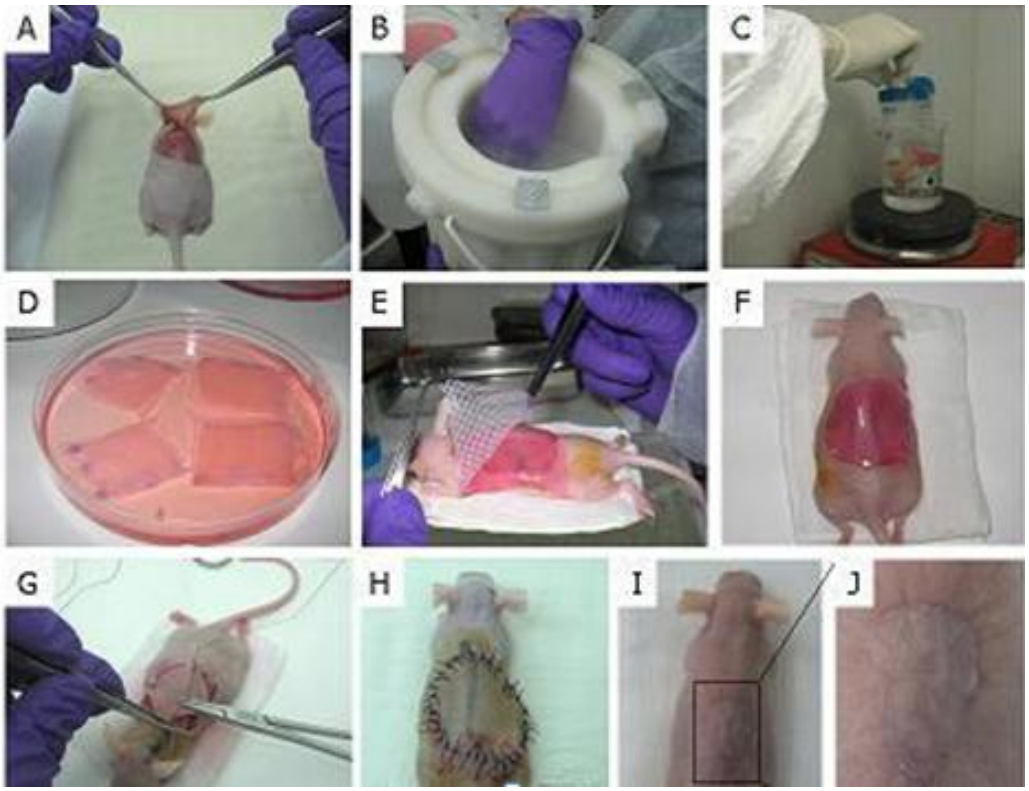

Figure 15. Principal steps for bioengineered skin graft in a murine model. A) Extract the mouse skin at the back. B) & C) Devitalization of skin by 3 thawn freezing cycles. D) Preparation of bioengineered skin. E) & F) Transplant of bioengineered skin in the mouse. G) & H) Suture the desvitalized skin over the bioengineered equivalent. I) & J) Regeneration of human skin in mouse.

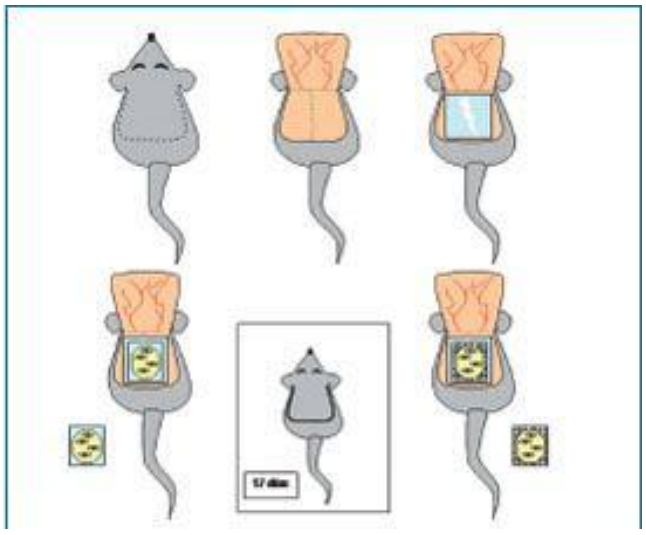

Figure 16. Graphic representation of the technique described by Barrandon et al. for dorsal graft implantation in athymic mice.

4. Histology & immunohistochemistry

Bioengineered skin growth *in vitro* and *in vivo* are prepared for histology and future immunohistochemistry studies. Two approaches are used for histology preparation: paraffin and frozen sectioning.

Paraffin proccesing of tissue and sectioning. First of all, tissue fixation using formalin and ethanol as principal agents is used. When the tissue is fixated paraffin infiltration is produced. In this procedure, tissue is dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with paraffin wax. Tissues processed into paraffin will have wax in the cassettes; in order to create wax blocks, the wax is melted away placing the entire cassette in 58ºC paraffin bath for 15 minutes.

Once the sample is embedded in paraffin wax, it is freeze to make the paraffin wax easily cut. A microtome is used for sectioning the sample. The size of the slices is fixed in 3µm. The slices are rapidly set in water at 40ºC, and taken by glass slides for posterior staining.

Frozen sectioning. The tissue is embedded in a gel like medium called OCT (optimal cutting temperature compound), which contains ethilene glycol and polyvinil alcohol. The temperature is reduced to -12ºC (specifically for skin). When the OCT with tissue inside hardens, the cryostat is used. The size of slicing sections is 5µm and they are directly set in glass slides for a future treatment. The quality of the slides produced by frozen section is lower than paraffin embedded tissue processing, but it is less time requiring.

Hematoxilin & Eosin staining. Paraffin samples are processed using the following proccedure for staining tissues:

1. Deparaffinize and hydrate to water
2. Introduce in hematoxylin for 15 minutes

3. Wash in running tap water for 20 minutes
4. Counterstain with eosin for 2 minutes.
5. Dehydrate in 95% and 100% alcohol, two changes of 2 minutes each or until excess eosin is removed. Check under microscope
6. Clear in xylene, two changes of 2 minutes each
7. Mount and analyze with microscope.

Antibody detection. Deparaffinize Samples are washed in PBS and it is blocked in PBS + BSA (bovine serum albumin) 3% for 15 minutes. Antibodies are added, in this study we use five different antibodies: Involucrin (1:100 in PBS with 3% BSA, mouse antibody), GFP (1:200 in PBS with 3% BSA, rabbit antibody), k14 (1:100 in PBS with 3% BSA, rabbit antibody), ki67 (ready to use) and Vimentin (1:100 in PBS with 3% BSA, mouse antibody). Samples are stored 20 minutes at 37ºC. Again wash of PBS and the second antibody is added: Anti-rabbit (1:25 in PBS) for GFP, ki67 and k14; and Anti-mouse (1:250 in PBS) for vimentin and involucrin. Again, samples are stored 20 minutes at 37ºC. Finally, another fluorescent staining called DAPI is used to label the nuclei of cells. Ready for taking images under fluorescent light microscope.

Results & Discussion

In this section all the results obtained will be evaluated. The first part consists on the efficacy of the transfection lentiviral vector. Secondly, the generation of *In vitro* and *In vivo* models for the comparison between DC skin and control morphology with hematoxylin and eosin staining. Finally, immunohistochemistry results for the different protein expression in DC skin and control.

1. Transfection of lentiviral vector

The objective of this study consist on quantify the efficacy of infection with lentiviral vectors, by the level of expression of GFP in infected keratinocytes. As it is said before, two cultures of 293T cells were developed. One with the vector carrying the GSE-24.2 + GFP genes and another one only with GFP to serve as a control. The supernatant of 293T cells containing the viral vectors are used to infect the keratinocytes that will form the bioengineered skin. It is important to remark that keratinocytes infected with GSE-24.2 and control (only GFP) are from Dyskeratosis Congenita patients.

Using a blue-light microscope, the results of the vector transfection can be evaluated. First, images without fluorescence are taken to see the development of keratinocyte cell culture. (Figure 17 & 18)

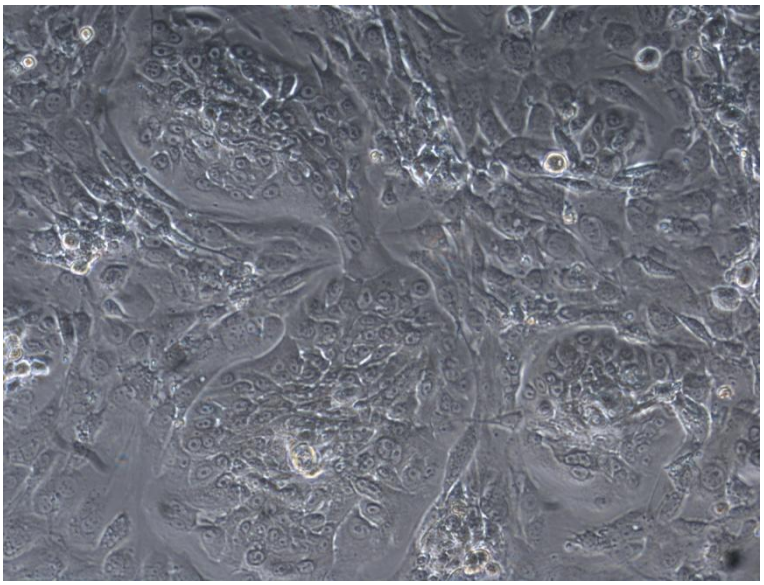

Figure 17. DC keratinocytes transfected with GSE24.2 + GFP vector.

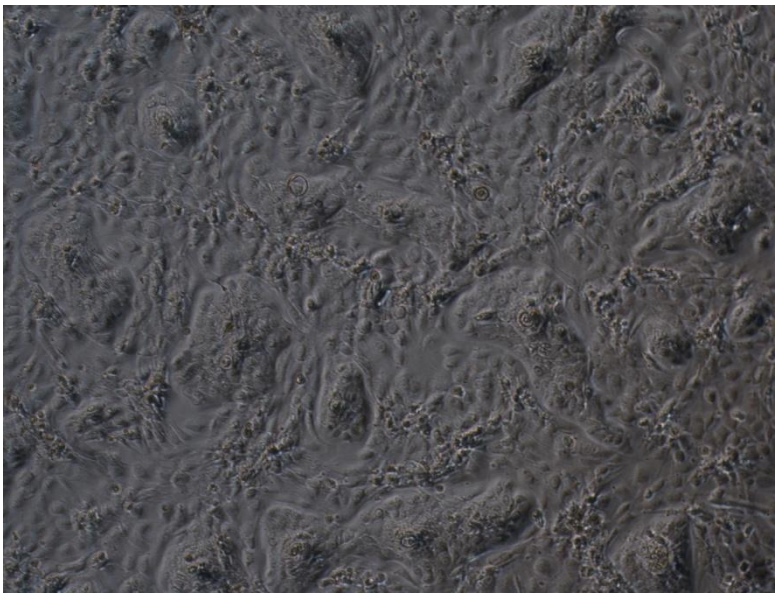

Figure 18. DC keratinocytes infected with GFP vector.

In both images, it can be seen the two keratinocyte cultures. The first one represents the culture infected with GSE-24.2 lentiviral vector and the second represents the control (DC keratinocytes + GFP). The two cultures show normal growth of cells.

Using blue-light microscope, the expression of GFP can be easily quantified. First for the GSE-24.2 culture the following images are obtained. (Figure 19)

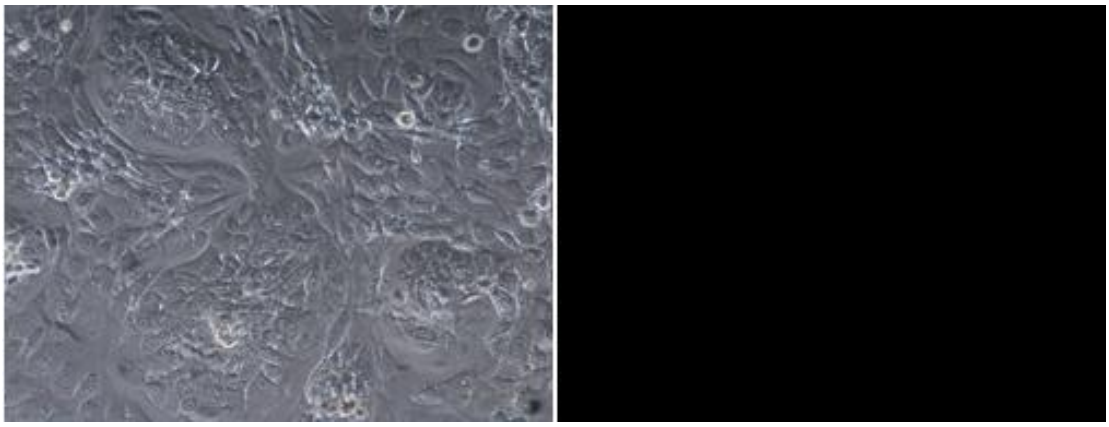

Figure 19. DC keratinocytes transfected with GSE-24.2 + GFP (Left) and expression of GSE-24.2 + GFP by DC keratinocytes (right). DC keratinocyte culture is shown normal growth of cells in culture (left). But, using blue-light microscope, no fluorescence can be seen in the image (right). This means that there is no presence of GSE-24.2 + GFP vector. So, the transfection has not worked as supposed.

The left image is taken using bright field light. The right one only uses fluorescent light to see GFP. As can be seen in right image, no GFP is present. This can be caused by several reasons: incorrect infection, problems with GFP sample, problems with the miscroscope, etc.

As no presence of GFP in the first culture, the study of the control culture is necessary to evaluate the possible problems. The following image shows the representative case (Figure 20):

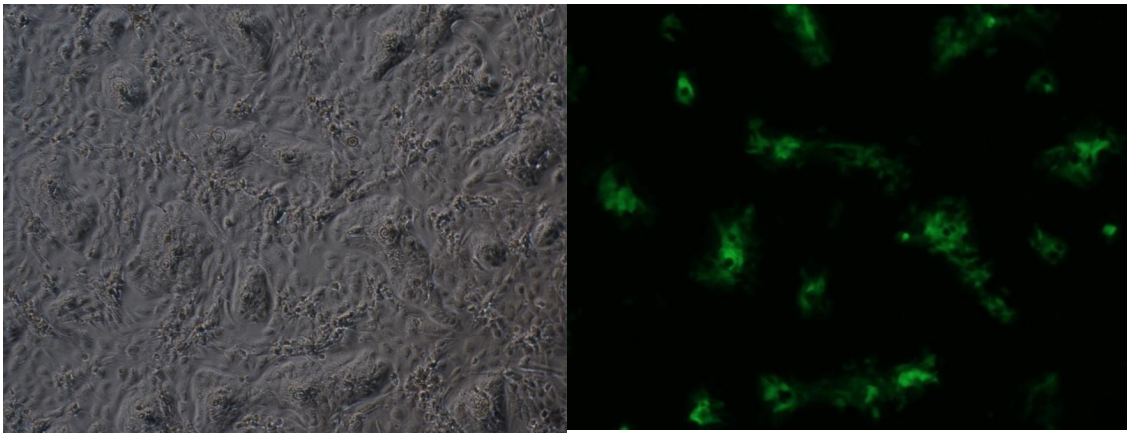

Figure 20. DC keratinocyte transfected with GFP vector (left) and expression of GFP in DC keratinocytes (right). DC keratinocytes culture shows normal growth of cells (left). Using blue-light microscope some green fluorescence is represented, which means that GFP is expressing in the culture. So, the transfection has worked as supposed.

In this case, it can be easily seen the GFP presence. The GFP has been dispersed by all the culture as we were trying to achieve.

So, we can conclude that the lack of presence of GFP in the GSE-24.2 culture must be related with the plasmid containing the therapeutic gene plus the GFP or with errors in the protocol process in that culture, because the control culture (DC keratinocytes + GFP) shows a correct infection by the cells.

The experiment was repeated again, in order to infect diseased cells. Even so, the result obtained was the same. Then the final conclusion about the lack of expression of GFP in the DC keratinocyte culture can be produced by: the bad quality of the sample containing the protein plus the GFP or incorrect infection with the viral vector to the cells. Due to the infection problems with the virus containing the therapeutic gene, the work will focus on the development of a model for DC.

2. *In vitro* model of DC: Phenotypic and biochemical characteristics

Hematoxyilin and eosin staining is the standard method for histological tissue stain to study the architecture and phenotypic characteristics of the samples. Hematoxyilin color the nuclei of cells with a dark blue color. Eosin stains the rest of the tissue with different shades of pink, red and orange.

The staining of tissue samples is very important for the characterization of the tissue. The obtained results will be useful for knowledge about the differences between Dyskeratosis congenital skin and normal one.

For this experiment, 6 different samples are used: 3 *in vitro* bioengineered skin controls, 3 *in vitro* bioengineered DC skin. In Figure 21, it is shown one representative experiment.

Images are taken at 10x, to study deeply the characteristics of different tissues.

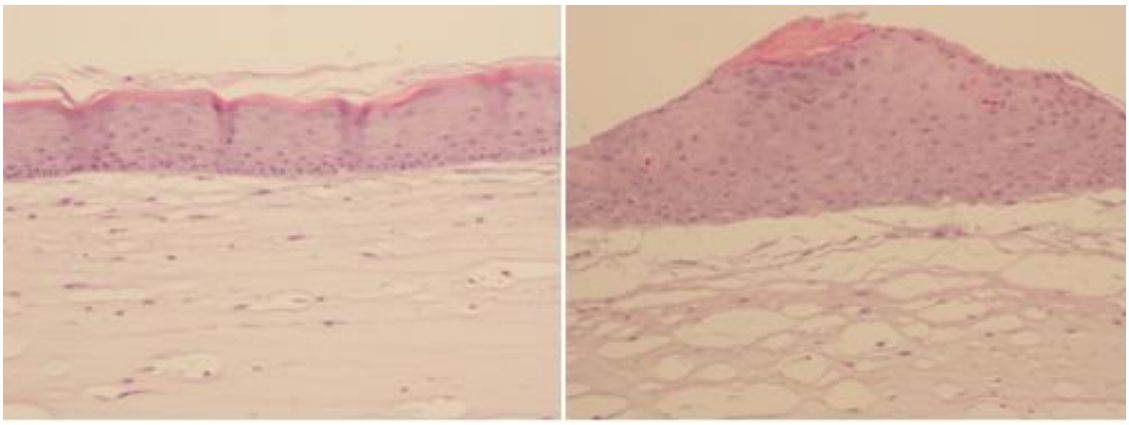

Figure 21. In vitro bioengineered skin from: Healthy cells (left) and DC cells (right). The differences between healthy and DC skin are easily appreciable. Healthy skin shows homogeneous structure in all layers of skin, while DC skin shows a hyperplasic behavior of the epidermal layer. The distribution of stratum corneum in DC skin is also heterogeneous, and it can be explained by the hyperplasia of epidermis. Finally, dermal layer also present some differences, as the dermal-epidermal junction and the loose conformation of fibroblast in DC skin

The control sample is *in vitro* bioengineered skin with healthy keratinocytes and fibroblast. *In vitro* model can be useful for a comparison with diseased skin, in order to determine the principal differences in the global characteristics of dermis and epidermis between DC and healthy skin.

The principal characteristics of skin control showed above are: homogeneous thickness of epidermal layer; correct formation of the stratum corneum in the outermost part of epidermis; correct attachment between layers in the epidermal-dermal junction and a relatively well-formed matrix among fibroblast in the dermis.

The differences in the development of skin between DC and healthy are easily visible. In diseased skin, Stratum corneum is hyperkeratotic (a thickening of the outer layer of the skin and more compact) compared with the "normal basket-weave structure" in normal skin.

Moreover, the entire epidermal layer presents a hyperplasia. This condition increases the number of normal cells in a normal arrangement in an organ or tissue, which increase the volume, compared with skin generated by healthy keratinocytes. Then, the structure of epidermis in *in vitro* DC skin doesn't allow the correct functionalization of epidermal layer.

The attachment between layers in DC skin is practically null along the entire dermal-epidermal junction. As in the case of heterogeneous epidermal layer, the reduced dermal-epidermal junction reduces the function of skin as a whole. This facilitates the detachment of epidermis producing problems for normal development of skin. To confirm this conclusion more studies with different attachment markers must be done. For example markers for Collagen VII/XVII, integrins, laminins, etc.

Finally, the dermis formed by fibroblast presents a loose conformation in comparison with healthy skin. The loose structure of this layer has influence in the whole function of skin reducing the mechanical properties of it.

Two principal conclusions can be obtained from the comparison between both kinds of skin: First, DC bioengineered skin shows hyperkeratosis and hyperplasic characteristics, due to the incorrect function of diseased cells. Instead of we are obtaining an overall view about the structure of skin, it is easily seen the problems that present the diseased skin.

Secondly, that once we have an overall vision about the *in vitro* skin, it is necessary a precise study to a better understanding of the difference between healthy and diseased skin. Then, with this purpose, immunohistochemistry processes are used to see the difference in protein expression between normal and DC skin.

3. Immunohistochemistry

Immunohistochemistry refers to the process of detecting antigens (i.e.: proteins) in cells of a tissue section by using antibodies that specifically binds to antigens in biological tissues. As we are treating to modeling a skin disease, the antibodies used are those that have significance in skin development, epidermal differentiation and growth, dermal structure, etc.

GFP

GFP is used as a reporter gene of expression. In this case, GFP was used as a control in transfection to determine if keratinocytes were infected by the lentiviral vector.

In Figure 22, the expression of GFP is shown:

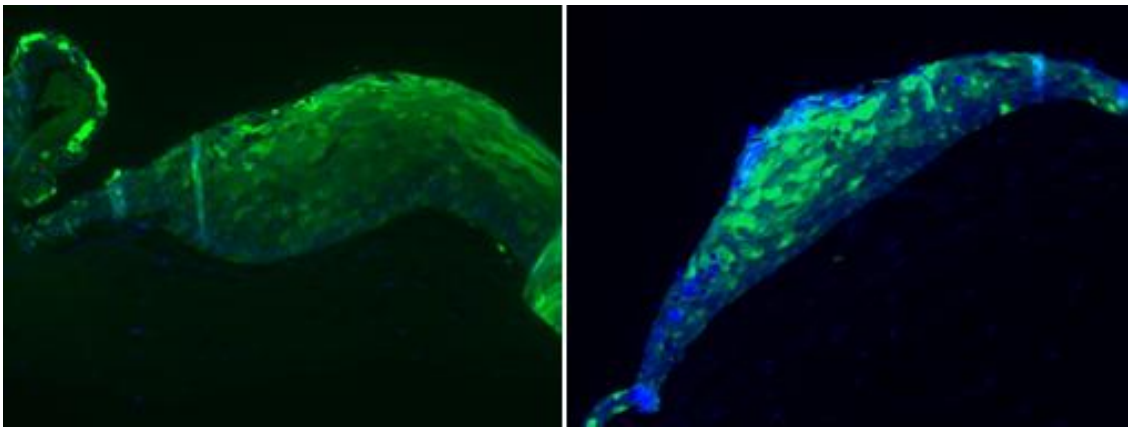

Figure 22. GFP expression in control (left) and DC (right) keratinocytes. The image shows the transfection of the GFP vector in both, control and DC sample. DAPI is used to stain cell nucleus in blue.

Images obtained under fluorescent microscope demonstrate that keratinocytes (control and DC) were infected correctly and express GFP proteins properly. The idea is to achieve the same result but with the GSE-24.2 lentiviral vector.

Involucrin

Involucrin is a protein that works as a structural component in mature squamous epithelial cells. Then, it is a marker used for terminal differentiation in epidermal layer (Figure 23).

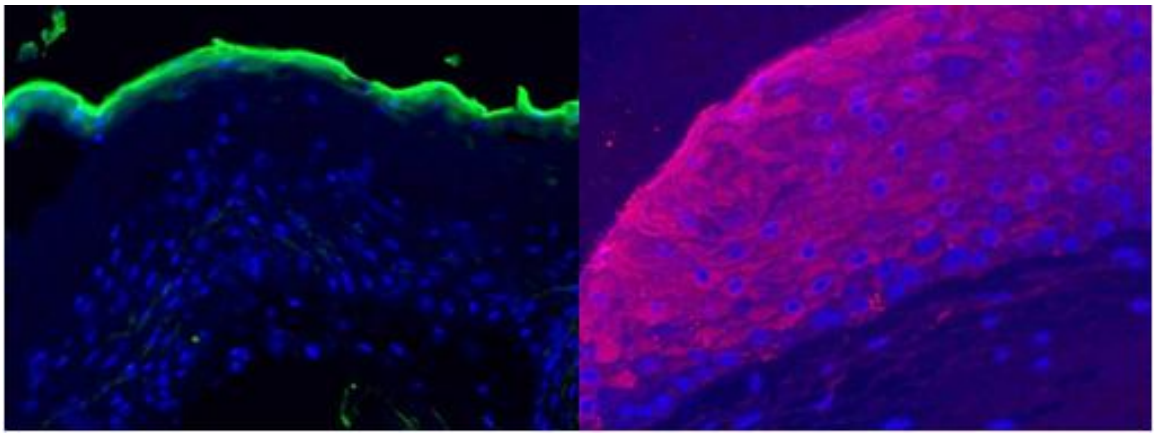

Figure 23. Expression of involucrin in control (left, green) and DC (right, red) in epidermal layer. Involucrin is used as a marker for terminal differentiation. Then, the expected result is to find it at the outermost part of epidermal layer. The control sample shows involucrin expression in the expected place (left, green). But in DC skin, it can be seen expression of involucrin in internal parts of epidermis (right, red). This means abnormal maturation and differentiation of keratinocytes in DC skin.

The green fluorescence represents the involucrin in control sample in the outermost part of epidermal layer, which means that cells are correctly differentiating and maturating from stratum basal to stratum corneum through the epidermis.

For DC skin, two important conclusions can be obtained: First, the expression of involucrin in the outermost part of epidermal layer is reduced with respect to healthy skin. Second, In this case the presence of involucrin is not reduced to the external part of epidermis, it is also found inside the epidermal layer. This means an abnormal maturation and differentiation of keratinocytes in DC skin. It is also related with the migration of cells through the epidermal layer.

Ki67

Ki67 is a protein strictly associated with cell proliferation. The protein is present during all active phases of the cell cycle (G1, S, G2 and mitosis), but absent in resting cells (G0). This characteristics makes ki67 a good marker about the growth fraction of a given cell population.

The results obtained from control and DC skin are:

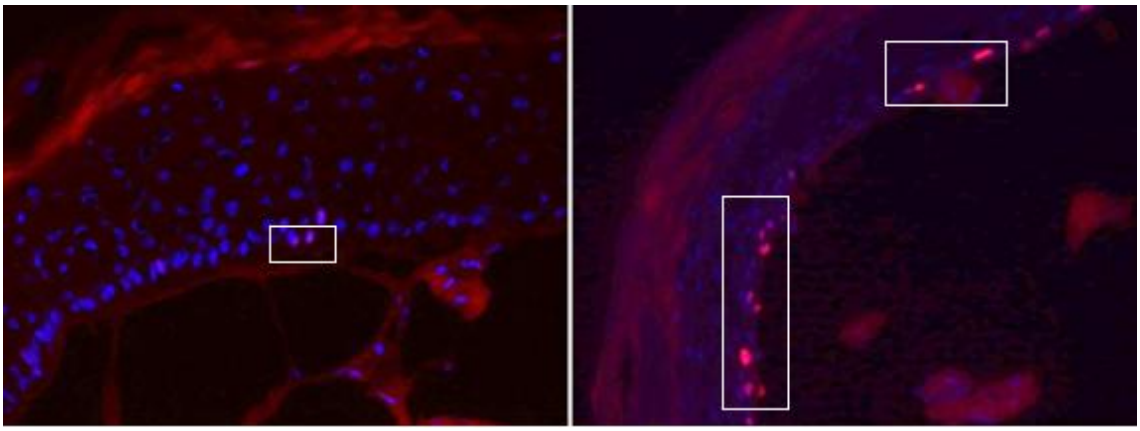

Figure 22. Expression of ki67 in control (left) and DC (right) skin. Ki67 is used as a marker of cell proliferation. The comparison between ki67 in control and DC skin shows that it is higher in DC skin. But, it is supposed that DC cells show less proliferation level due to lack of telomerase. Then, the result is unclear.

Instead of the expression of ki67 is low, it is present in cells. This means that cells are proliferating and generating new cells for epidermal layer. The proliferation of epidermis is very important for maintain homeostasis of the tissue.

The comparison in the level of proliferation (Ki67 positive cells) is higher in the DC skin than in the control one. This is in agreement with the hyperplasic shown by results obtained in DC skin (Figure 23). However, if DC patients are characterized by a low proliferation rate due to the lack of telomerase, these results are surprising and more studies will have to be made for a better explanation.

Keratin 14

K14 is expressed in mitotically active basal layer cells in epidermis. The expression of k14 is down-regulated as cells differentiate. Then, it is used as a marker of level of differentiation in epidermal keratinocytes (Figure 23).

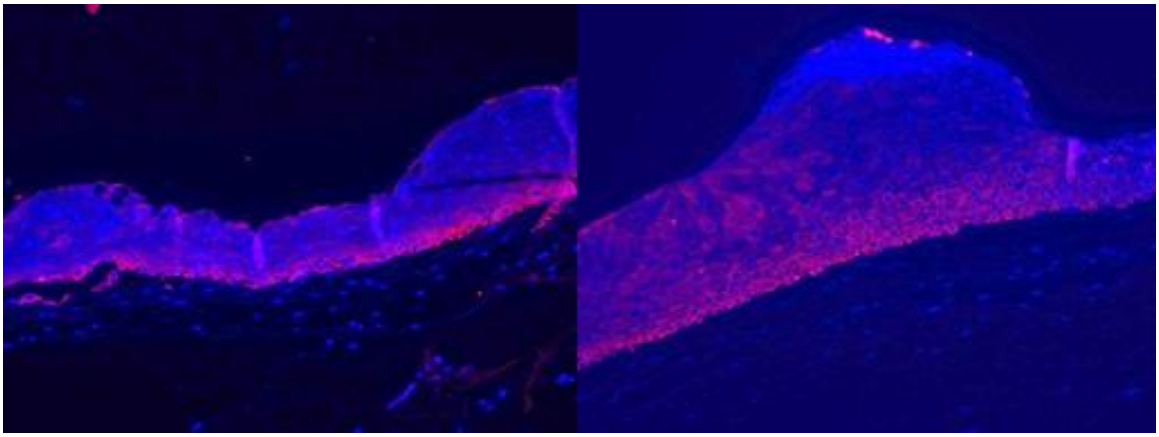

Figure 23.Expression of k14 in control (left) and DC (right) epidermis. K14 is used as a marker of level of differentiation. Then, it is highly present in basal layer of epidermis. In control sample the result is the expected one, but in DC skin the k14 is expressed over the whole epidermis. This can be the explanation for the hyperplasic behavior of DC skin.

Both samples present a similar expression of k14 in basal layer of the epidermis, which allow concluding that basal layer is composed by low differentiated cells. The interesting result obtained in the experiment is the expression of k14 in the suprabasal layer of the epidermis in DC skin. The result shows problems in the proliferation and differentiation of skin. The hyperplasic phenotype of DC skin can explain the abnormal expression of k14 out of the basal layer.

Vimentin

Vimentin is a protein which forms the type III intermediate filaments. This type of filaments is expressed by fibroblast in dermal layer. Then, it is used as a marker for fibroblast concentration; it gives an overview of structure of dermis (Figure 24).

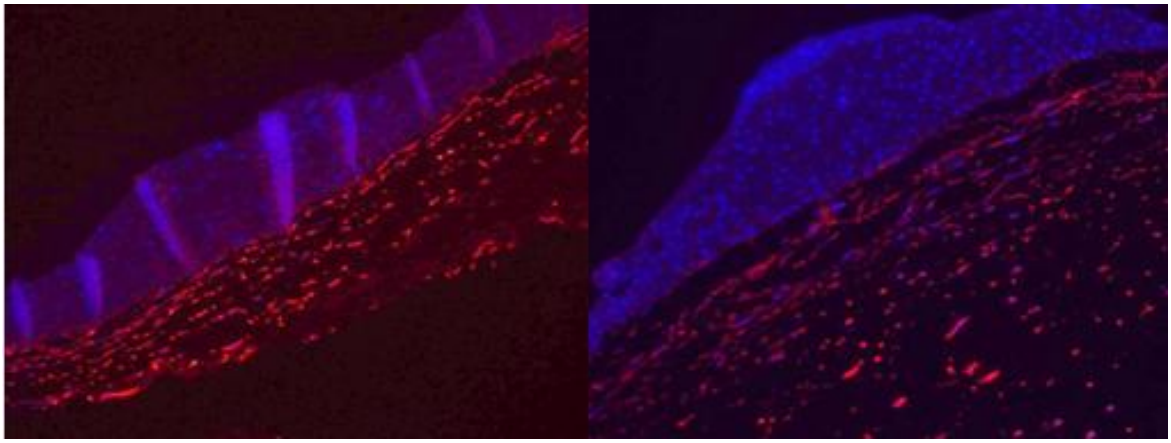

Figure 24. Expression of vimentin in control (left) and DC (right) skin. Vimentin is used as a marker of fibroblast concentration in the dermal layer. In this case, both (control and DC skin) shows similar expression of vimentin in dermal layer. As a conclusion, DC has no effect over vimentin expression in fibroblast. Other markers could be use to better understand the behavior of dermal layer and dermal-epidermal junction.

The images obtained in, both control and DC skin shows a similar expression of vimentin in dermal layer. Then, we can conclude that DC has no effect over vimentin in fibroblast.

It would be necessary realize more immunohistochemical processes using fibroblast and ECM markers to determine the functionalization of dermal layer in diseased versus healthy skin.

Some examples about possible markers for dermal layer could be: Desmin, another protein related with intermediate filaments; or Prolyl 4-hydroxilase, related with collagen synthesis.

4. *In vivo* development of skin-humanized mouse models for DC

Two approaches were done for *in vivo* studies: Barrandon method and orthotopic graft.

For both methods, bioengineered DC skin was used.

The skin obtained by Barrandon method was analyzed using a blue-light microscope. (Figure 25):

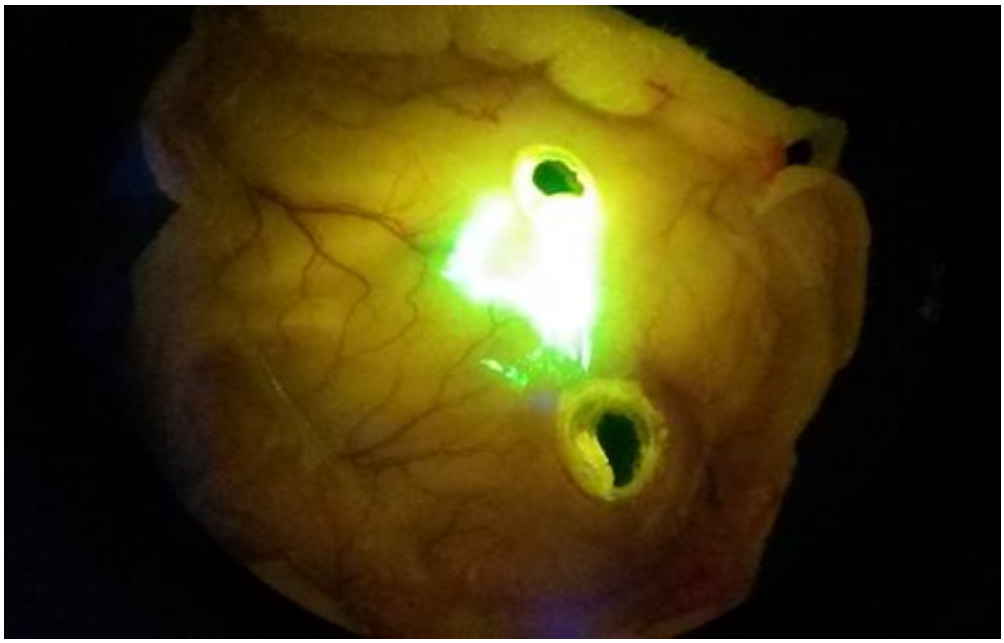

Figure 25. Image obtained with fluorescent microscope expressing GFP. The skin extracted from the mouse had been previously treated using Barrandon method to introduce bioengineered skin. Bioengineered skin needs to last for 15 days to mature inside the mouse. The extracted skin is studied at fluorescent microscope. The GFP present in bioengineered skin keratinocytes is shown in fluorescent microscope. Then, we can conclude that human skin has matured in mouse model.

The fluorescence produced by GFP in skin graft shows that bioengineered human skin is maturating inside the murine model. With this assumption, skin showing the fluorescence was taken and processed for histological studies.

After H&E staining, the following image was obtained:

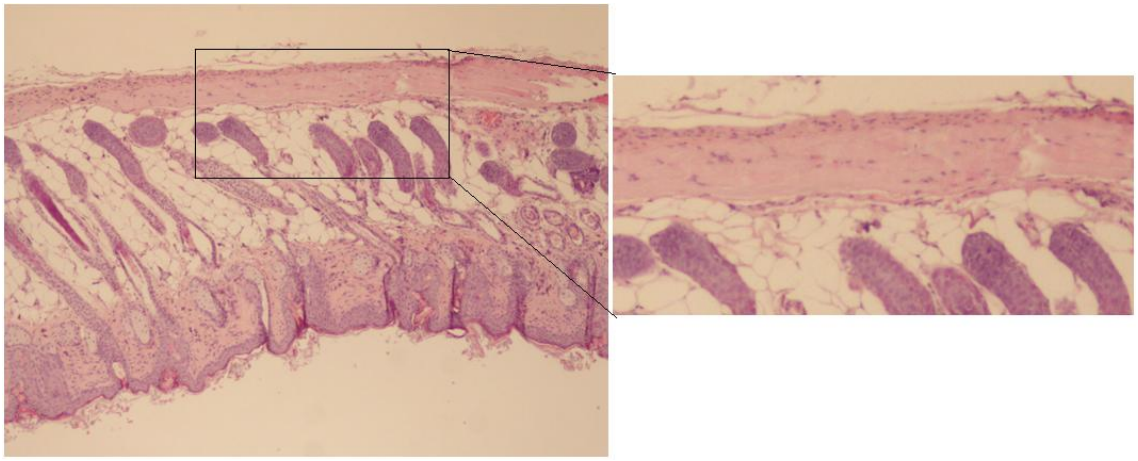

Figure 26. Bioengineered skin developed *In vivo* using Barrandon method. Skin obtained in figure 25, is paraffinize , treated with H&E staining and observed at microscope. The upper part of the image represents the human skin growth in mouse model. The zoom allows to see and differentiate among dermis, epidermis and stratum corneum.

In the upper part of the image, it can be seen the regenerated human skin, where the dermis and different layers of keratinocytes, including the stratum corneum, can be differentiated. Although human skin is clearly visible different markers for human skin has to be made.

The orthotopic graft was done, but due to it requires between two and three months to take and mature, the extraction of human skin has not been performed yet. But, it will be performed a posteriori.

In the following image can be seen the integration of human skin in mouse:

Figure 27. Development of bioengineered skin *In vivo* using orthotopic method. The steps required for orthotopic method of murine skin transplantation are developed in figure 14. Instead of skin cannot be extracted until 2-3 months after introduction of bioengineered skin, an evaluation of the integration of human skin in mouse model can be realized. The graft (black square) has been taken by the mouse and it is maturating.

Conclusion

As a conclusion, the obtained results allow to explain briefly the functionalization of cells with Dyskeratosis Congenita disease. It has been generated an *In vitro* skin model able to recapitulate, at least in part, the pathological skin features of patients with DC.

An *In vivo* skin model of DC has been also acquired. Those approaches allow creating feasible human skin models, which recapitulates better the structure and function of human skin as can be seen in Barrandon method model. Next result, with the orthotopic model, will give us more data about the physiopathology of this disease.

It is important to remark that DC affect to telomeres which are related with cell aging. If a new experiment, over a longer period of time, is realized, some conclusions about the effect of aging in epidermal cells could be obtained. Moreover, the use of other markers can facilitate a better explanation of the diseased skin behavior.

Alternative applications and future lines

Instead of the obtained results, more studies must be done to really understand the behavior of diseased skin over the normal one.

The principal lines to improve actual techniques consist on the use of new models that mimic the human skin in the body. Nowadays animal models, specifically mice, are used for this kind of test. The idea is to use new animals that improve the similarity with respect to humans or the use of bioreactors controlling the necessary conditions to differentiate the skin, producing a good model of human skin.

Another approach could be the use of "organ-on-a-chip", in this case "skin-on-a-chip", that can be useful not only for the development of the skin but also for drug screening in unhealthy skin.

The characterization of a disease can be done, in principle, for any tissue in the body (Instead some organs are much difficult to mimic). Then this process can facilitate the study of different diseases through the whole body.

Legal regulatory framework

The final project is framed into a biomedical scientific research activity, which is recognized as a fundamental right of maximum protection in the article 20.1.b of the Spanish Constitution of 1978.

Moreover, the specific legal regulation of biomedical investigation is present in law 14/2007 of July 3th. However, this law has been partially modified by the law 14/2011 of science, technology and investigation.

At international level, the law 14/2007 takes part of the Convention of the European Council for the protection of human rights and dignity of the human being with regard to the application of biology and medicine. In this law it is proclaimed the health, dignity and welfare of the human being who participates in the biomedical investigation, will prevail the interest of the society or the science.

Glossary of terms

DC: Dyskeratosis congenital

LC: Langerhans cells

MSH: Melanocyte-stimulated hormone

MC: Merkel cells

DEJ: Dermal-epidermal junction

TERT: Telomerase reverse transcriptase

TERC: Telomerase RNA component

DKC1: Dyskeratosis congénita 1, dyskeryn

HIV-1: Human immunodefficiency virus

LTR: Long terminal regions

DMEM: Dulbbeco's modified eagle medium

GFP: Green fluorescent protein

HBS: Hepes buffered saline

DAPI: 4',6-diamidino-2-phenylindole

K14: Keratin 14

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