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Epithelial stem cell characterization aimed at an efficient ex-vivo gene therapy approach.

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ABSTRACT

EB is a family of rare inherited blistering skin disorders due to mutations in genes coding several adhesion proteins. Nowadays, there is no cure for EB, all the treatments are palliative and focused on the relief of the EB symptoms. Recent advancement in molecular biology, stem cell biology and regenerative medicine have fostered new therapeutic approach such as the one that lead to the successful ex vivo gene therapy phase I clinical trial on Laminin 332-dependent JEB in 2006. This approach has demonstrated that the long-term restoration of JEB epidermis requires a defined number of transduced epidermal holoclones [1]. However, no cell membrane epithelial stem cell markers have been identified that allow the prospective isolation and transduction of stem cell in culture. For this reason the current gene therapy approach is to obtain a close to 100% of transduction efficiency of an heterogeneous keratinocyte culture in order to be sure to transduce keratinocyte stem cells.

We developed the same approach to genetically correct epidermal stem cells from COL17-dependent JEB, envisaging MLV-derived retroviral vectors (MLV-RV) and Self Inactivated-RV (SIN-RV). For MLV-RV strategy, we generated Am12-Col17 packaging cells, clones were selected and used to transduce keratinocytes using a co-culture procedure, which allowed to achieve a 95% transduction efficiency. Transduced keratinocytes were maintained in long-term culture and their Colony Forming Ability was measured to evaluate cell toxicity. Epidermal stem cell transduction was demonstrated with clonal analysis. In parallel, we performed similar experiments using SIN-RV viral constructs that differ in endogenous promoters and vector backbones.

We have selected the best vector to efficiently correct the genetic defect and actually we are testing the transduction efficiency on keratinocyte culture of different SIN packaging cell line clones.

In both strategies, genotoxicity assays are ongoing to show whether SIN-RV and MLV-RV have a different safety profile in terms of potential insertional mutagenesis. In particular we are testing transduced cultures in soft agar assay, serial cultivation and growth factor dependence assay.

In parallel we tried to identify a keratinocyte stem cell marker that can be used in clinic in order to allow the identification and -eventually- the selection of a pure population of stem cells for gene correction. To this end we analyse the transcriptional profile of keratinocyte stem cells (Holoclones) compared to transcriptional profile of the transient amplifying cells (Meroclones and Paraclones). Bioinformatical analysis of microarray data allowed to identify differentially expressed genes and delineate a molecular signature of each class of clones. Moreover data were analysed by the network-based Ingenuity Pathways Analysis tool, to search for the most relevant molecular interactions, functions and pathways differentially expressed in Holoclones vs Meroclones and Paraclones.

Genes that resulted upregulated in stem cells were confirmed by real time PCR, while protein expression relative to upregulated genes was investigated by western blot and immunofluorescence analysis. Moreover gain and loss of function studies are ongoing to investigate the role of selected genes in keratinocytes homeostasis. Consistent with this preliminary data we can confirmed that Holoclones Meroclones and Paraclones have a quite different gene expression profile that makes possible to define a specific gene signature of human KSCs.

Molecular characterization of keratinocyte stem cell has an important impact on gene therapy approaches since it would improve the efficiency of stem cell transduction assuring the long-term restoration of corrected-EB epidermis.

INTRODUCTION

THE EPIDERMIS

The epidermis is a stratified keratinised epithelium mainly composed of keratinocytes organized in four different cellular layers: basal (which contains proliferative cells), spinous, granular and stratum corneum. Being the first barrier of our body, the epidermis provides us a protection from infectious agents, harmful microbes, UV radiation, mechanical stress and also prevent from dehydration.

The human skin epithelium is regenerated continuously through the periodic proliferation of keratinocyte stem cells (KSCs) and an intricate balance between growth and differentiation.

KSCs reside in the basal layer, that is the only proliferative compartment of the skin. Basal cells adhere to an underlying basement membrane rich in extracellular matrix ad growth factors. [2,3]

Each stem cell can generate transient amplifing cells (TACs) that reside in the basal layer and divide to initiate the process of terminal differentiation. The first step of keratinocyte differentiation consists on the transition from basal to spinous layer with a change in specific protein expression. Cytokeratins K14 and K5 identify the stratified squamous epithelial cells that possess proliferative potential and reside in the basal compartment. Spinous cells instead switch on the expression of K10 and K1 to form a robust intermediate filaments network linked to cell–cell junctions known as desmosomes. [3] This expansive cytoskeleton reinforces cell-cell junctions and provides resistance against mechanical stresses at the body surface.

When spinous cells progress to the granular layer, they produce electrondense keratohyalin granules packed with the protein profilaggrin. Morover the cornified envelope proteins (Lce), which are rich in glutamine and lysine residues, are synthesized and deposited under the plasma membrane of the granular cells. At the granular/stratum corneum transition occur other important changes in transcription, morphology and function such that differentiated cells reaching the skin surface are enucleated cellular skeletons packed with cables of keratin filaments encased by γ-glutamyl ε-lysine cross-linked cornified envelope of proteins. The final step of terminal keratinocytes differentiation involve the extrusion of lipid bilayer packaged in lamellar granules, onto the scaffold of the cornified envelope. With

this continuous process the keratinocyte cells move from the basal layer to the skin surface where the dead stratum corneum cells create an impenetrable layer.

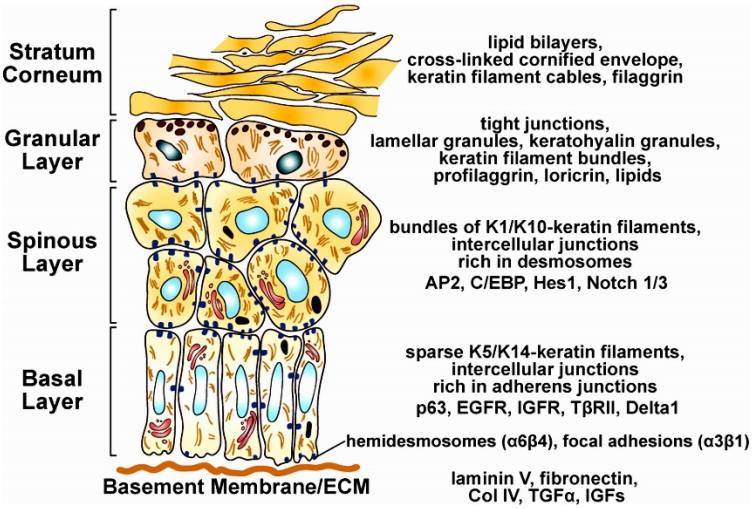


Figure 1. Schematic representation of epidermal layers with specific key markers. [3]

Holoclone forming cell as epidermal stem cell

Epidermal stem cells are defined by their capacity to self-renew and to generate the entire tissue all life long. Lineage tracing experiments and functional skin reconstitution assays in mouse model demonstrated the presence of cells, with stem cell properties, in the interfollicular epidermis[4], the upper constant region of pelage hair follicles and the ducts of sweat glands [5,6]. These stem cells are multipotent and clonogenic, slow-cycling and express cell surface proteins like Lgr5 and Lgr6 , CD34 , Plet1 and Lrig1 [7,8].

Stem cell markers identified in mouse do not select for human KSCs and label retaining, lineage tracing or genetic manipulation experiments cannot be performed in human for obvious ethical reasons. Actually the only way to capture and identify human epidermal stem cells is a clonogenic assay.

Human keratinocytes are defined and characterized in relationship to their clonogenic capacity and growth potential. Clonogenic capacity indicates the capacity of a basal cell to generate a colony, instead the growth potential deals with the self-renewal potential during the lifetime. Although many of the basal cells are capable of multiplication (clonogenic capacity) few of them are self-renewing stem cells. [9]

In the 1985 Barrandonn and Green demonstrated that the clone-forming ability of a human keratinocyte in culture can be estimated from its size: when keratinocytes are 11 μm or smaller they give rise to clones with high frequency (clonogenic colonies), this frequency diminished or is completely abolished with larger keratinocytes[10]. Once a colony has formed, its growth potential is not specified by the size of the founding cell so they developed a new method of analysis,the clonogenic assay, that reveals the growth potential of individual clones and that actually is the only way to define an epidermal stem cell in culture[9]. Single keratinocytes are cultured on irradiated feeder layer and after 7 days the progeny of each clone is subcultured onto indicator dishes while they are still growing esponentially. After 12 days, indicator dishes are fixed and stained with Rodhamine B. According to the growth in the indicator dishes it is possible to define the clonal type and so classify the original clone. Epidermal basal layer epithelial cells can give rise to three clonal types: holoclone, meroclone and paraclone. [9]

Holoclone forming cells have self-renewal capacity, telomerase activity and a great proliferative potential, so they represent the phenotype of human keratinocyte stem cells in culture [11]. Holoclones have a growth capacity up to 180 divisions and can generate enough cultured epidermal graft to permanently cover the entire body of an adult human. Holoclones are the only cells capable of maintains the epithelium for the entire life of the individual and their presence in culture is fundamental to permanent restore epithelial defects. [12]

Meroclones derive from holoclones and generate a mixture of progressively growing colonies and terminal colonies. Paraclones are transient amplifying cells committed to a small number of divisions, that generate only terminal aborted colonies expressing terminal differentiation markers like involucrin. The conversion of holoclones to meroclones and paraclones, termed clonal conversion, is an irreversible phenomenon under normal circumstances and result in progressive restricted growth potential. [9,13]

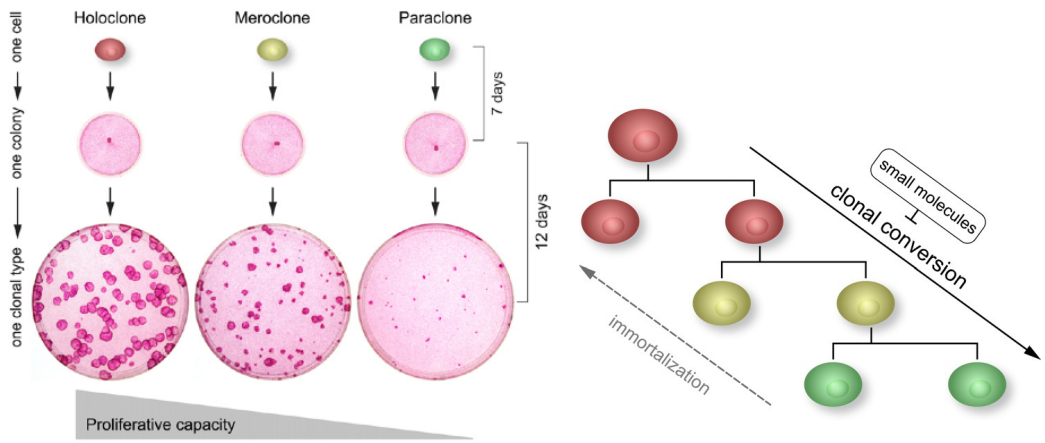


Figure 2. [13] **Clonal analysis.** A holoclone (red) generates a progeny that forms large progressively growing colonies and less than 5% of terminal colonies. A meroclone (yellow) forms both large progressively growing and terminal colonies. A paraclone (green) generates only terminal colonies.

Clonal conversion. In the lifespan of human keratinocyte stem cells in culture there is a progressive loss of growth potential caused by clonal conversion. A holoclone (red) with extensive growth potential converts into a meroclone (yellow) that in turn converts into a paraclone with restricted growth potential (green). Clonal conversion results from microenvironmental insults and is irreversible under normal conditions.

The derma-epidermal junction

Epidermal homeostasis is maintained by a hierarchy of proliferative cells within the basal layer and the adhesive properties of basal keratinocytes are closely linked to the regulation of keratinocyte proliferation and differentiation [14].

The epidermis is non-vascularized and receives nutrients from blood vessels in the underlying dermis. The epidermis and dermis are separated by a basement membrane that is composed of extracellular matrix proteins, including fibronectin, Collagen IV and Laminin 5.

The synthesis of basement membrane components is guaranteed from both the epidermis and the dermis, but only the basal layer of epidermis contribute to the production of Collagen IV and Laminin 5. The basal layer of epidermal cells both synthesizes these components and adheres to them, and also polymerizes and organizes them into the basement membrane.

Epidermal cells adhere to the basament membrane via adhesion molecules known as integrins, that mediate cell-extracellular matrix adhesion and are composed by an α and β subunit. Their expression is restricted to the basal layer of the epidermis and the predominant ones are $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$. [14]

β1-integrin partners with α2-integrin to make a receptor and organizer for collagen, and with α3-integrin to make a receptor and organizer for fibronectin. So the absence of β1-integrin cause the greater severity of basement membrane synthesis or assembly defects.

The attachment of basal keratinocytes to the basement membrane is mediated by specialized adhesion structures termed hemidesmosomes (HD), wich are based on protein-protein interactions. [15]

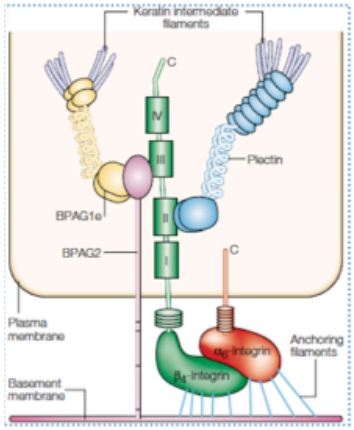


Figure 3. The hemidesmosomal structure [15]. Schematic representation of protein-protein interactions in the hemidesmosome. The involved proteins are a6b4 integrin, HD1/Plectin and the bullous pemphigoid (BP) antigens BP180 and BP230.

The α6β4 integrin heterodimers form the core of the hemidesmosome binding laminin-5 in the basement membrane. [14] Other important hemidesmosomal proteins are Collagen 17 (also known as BP180 or BPAG2), BPAG1 and plectin.

Collagen 17 is a homotrimer consisting of three identical chains and contains a cytosolic NH2 terminus ad an extracellular COOH domain. [16] Within the hemidesmosomal structure it maintains a linkage between the intracellular and extracellular elements. At extracellular levels it binds α6 integrin and laminin 5, whereas intracellular ligands include β4 integrin, plectin and BP230. [16-18] Collagen 17 occurs in two different forms, as a full length transmembrane protein and a soluble ectodomain, a specific proteolytic cleavage product generated by ADAMs. [19,20] In skin wound healing it was demonstrated a diffuse cytoplasmic expression of Col17 at the edge of the migration sheet, while it is located along the basal membrane of the cells in stationary epithelium. It was also observed that increased shedding of the ectodomain of Col17 lead to a decreased cell motility and migration. [21] All these observations suggest that the proteolytic cleavage of COL17

regulates the adhesiveness of HDs and that Col17 plays a role in the inhibition of keratinocyte migration.

Mutations in genes that encode for integrins and hemidesmosome associated proteins caused several aggressive deseases. [15] Defects in the genes that encode the Lam5 chains cause the devastating human blistering skin disorder junctional epidermolysis bullosa (JEB). Mutations in the genes that encode α6- and β4-integrins, and Collagen17 are all known to result in the loss of hemidesmosomes, which causes the epidermis and the underlying basement membrane to separate, as in JEB.

EPIDERMOLYSIS BULLOSA

Inherited Epidermolysis Bullosa (EB) constitutes a group of phenotipically diverse genodermatoses which share as a common feature: mechanical fragility of the skin. A characteristic manifestation of all types of EB is the presence of recurrent blistering or erosions of the skin and mucous membrane, as result of even minor traction to these tissues.

EB is classified as a rare disease and it affects 500.000 people in the world, most patients are diagnosable at birth or shortly thereafter, whereas mild forms may not become apparent until late childhood or early adulthood. Many of the more severe types and subtypes of inherited EB are usually accompanied by complications within other organs. Many of these complications cause a very poor quality of life and a significant morbidity, leading for the most severe form to premature death. In addition to mechanically fragile skin and easy induction of blisters or erosions, inherited EB shows several common features like mila (tiny firm white papules, resembling cysts or pustules), nail dystrophy or absence, and scarring. Additional useful findings include aberrant granulation tissue, localized or confluent keratoderma of the palms and soles, and dyspigmentation.

EB is caused by mutations in genes encoding for proteins located at dermo-epidermal junction and it is classified into three main types based on the level of tissue separation within the cutaneous basement membrane zone. Tissue separation occur in the basal keratinocytes, dermis and lamina lucida of the basement membrane in EB Simplex (EBS), Dystrophic EB (DEB) and Junctional EB (JEB) respectively. [22-24]

Major type	Major subtype	Protein/gene
Simplex (EBS)	Dowling–Meara EBS Koebner EBS Weber–Cockayne EBS	Keratin 5, 14
	EBS with muscular dystrophy EBS with pyloric atresia	Plectin
Junctional (JEB)	Herlitz JEB Non-Herlitz JEB JEB with pyloric atresia	Laminin 332 BP180, laminin 332 α6β4 integrins
Dystrophic (DEB)	Dominant DEB Hallopeau–Siemens Recessive DEB Non-Hallopeau–Siemens Recessive DEB	Type VII collagen

Figure 4. Schematic table of EB subtypes [22]

EBS

In all forms of EBS, blister formation first occurs within the basal keratinocytes. Dominantly inherited EBS is caused by mutations in keratin 5 and 14 genes and is divided into three subtypes: the Weber-Cockayne, Koebner and Dowling-Meara. The most severe subtype is Dowling-Meara EBS, Weber-Cockayne EBS has a milder phenotype than that of Koebner EBS. Recessively inherited EBS is caused by mutations in the plectin gene. [22]

All EBS forms are characterized by generalized blistering and muscular dystrophy. Only EBS-DM presents a markedly increased risk of developing basal cell carcinoma by mild-adulthood. [23]

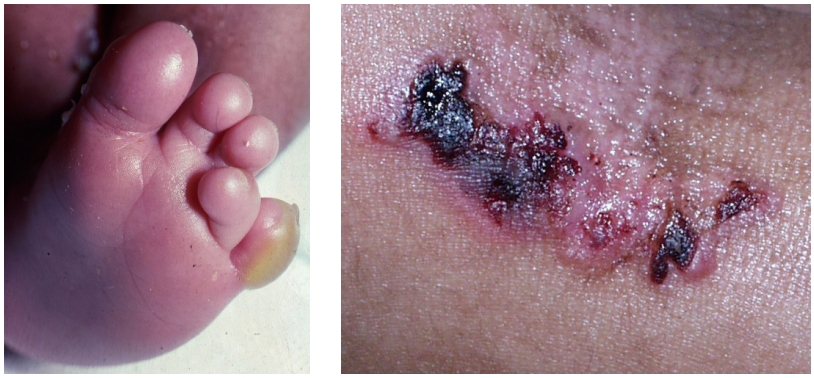


Figure 5. Blister development and hypercheratosis in EBS patient [22]

JEB

All JEB subtypes are inherited in an autosomal recessive manner and are characterized by blister formation in the lamina lucida. JEB is divided into three subtypes: the Herlitz subtype, the non-Herlitz subtype and the subtype with pyloric atresia. [22]

JEB-Herlitz is the more severe one because involves all skin surfaces and it is characterized by exuberant granulation tissue that involves not only the skin but also the upper airway. JEB-H patients are also prone to develop squamous cell carcinomas and are exposed to the highest risk of infant mortality as a result of sepsis, failure to thrive, or tracheolaryngeal obstruction.

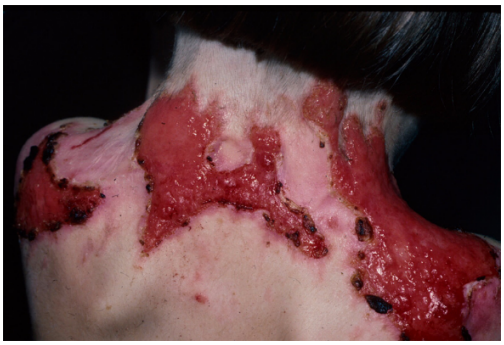


Figure 6. Exuberant granulation in the skin of JEB-H patient represent a typical feature of this severe EB subtype[22].

The most common JEB subtype is non-Herlitz JEB, a generalized disorder characterized by the presence of blistering, atrophic scarring, and nail dystrophy or absence. Many patients affected by the non-Herlitz subtype survive to adulthood. [23]

JEB-H is caused by mutations in any of the three genes encoding Laminin 5, these mutations are homozygous or compound heterozygous premature termination codon (PTC) mutations. Other types of mutations, (as missense mutations) in one or both allels of the gene encoding Laminin 5 are usually associated with non-Herlitz JEB.

Non-Herlitz JEB is also caused by mutations in the *BPAG2* gene which encode the Col17 protein. The majority of *COL17* mutations are homozygous or heterozygous non-sense mutations, or small insertions or deletions, resulting in premature termination codon (PTC) and absence of Collagen17 in the skin. The *COL17* null mutations and ectodomain deletion lead to severe epidermal dysadhesion and

missense mutations in *COL17* domain cause faulty folding and degradation of the protein, whereas a large intracellular deletion is associated with mild JEB phenotype. [25]

Finally JEB with pyloric atresia is due to mutations in the genes encoding the b4 integrin and a6 integrin chains.

DEB

Dystrophic EB, in which tissue separation occurs in the dermis, manifests as blistering, scarring and milia formation and it has both autosomal dominant and autosomal recessive forms. [22]

The prototypic Dominant DEB (DDEB) patient has generalized blistering at birth which is associated with milia, atrophic scarring and nail dystrophy. Recurring esophageal blistering and erosions, leading to progressive dysphagia, is common among these patients. [23] In general, the phenotype of dominant DEB is milder than that of recessive DEB. There are three main subtypes of RDEB: Hallopeau-Siemens RDEB, non Hallopeaus-Siemens RDEB and inverse RDEB. [23]

All DEB forms are caused by mutations in *COL7A1*, which encodes typeVII Collagen, a major component of anchoring fibrils. DEB dominant forms are due to a glycine substitution mutation whereas recessive forms, like the most severe one, are caused by premature termination codons present on both allels of the *COL7A1*. Therefore, no or low expression of type VII collagen is observed in the basement membrane in these patients. Only the milder form of recessive DEB, non Hallopeau-Siemens recessive DEB, expresses low levels of type VII collagen and is caused by PTC mutations in one allele and a missense mutation or in-frame mutations in the other allele. [22]

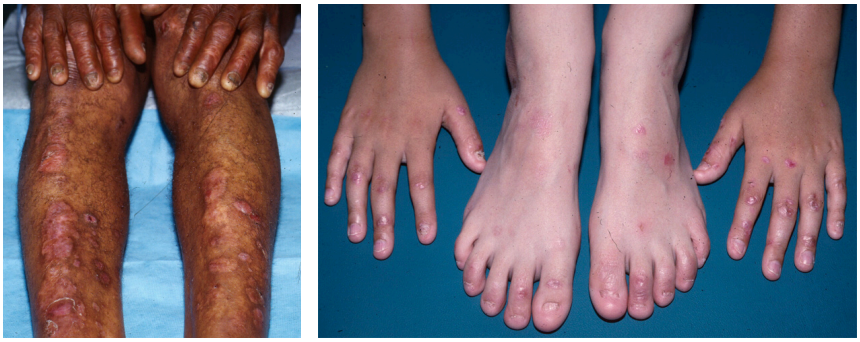


Figure 7. Hypertrophic scars and finger dystrophism in DDEB patients [22].

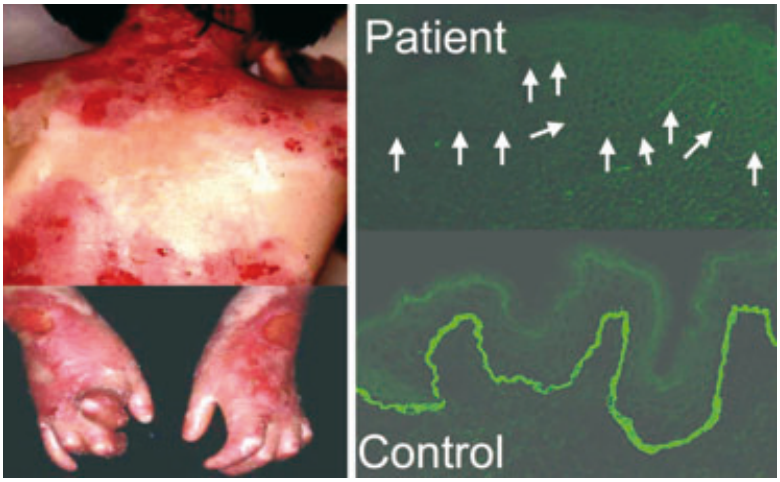


Figure 8. Blister and erosions development in a patient affected by a severe phorm of RDEB. In the right panel it's shown the complete absence of Col7 by imunofluorescence[22].

EB gene therapy

Up to now no definitive cures for EB have been developed. All the treatments are palliative like bendages or topical antibiotics to avoid skin traumas and infections. All these treatments are only meant to improve the overall quality of life of patients and not to cure the symptoms. Moreover, these palliative cures cost 10.000 euros every month for each patient. [26]

Different therapeutic approaches were developed to treat EB defects:

- substitution of the damaged protein using topical application or local injection[27];
- cellular therapy using autologous fibroblast or bone-marrow derived hematopoietic stem cells [28,29];
- gene therapy [1].

The only way to obtain a permanent correction of EB defects is a gene therapy approach, which consists in a genetic correction of epidermal stem cells, expansion of keratinocyte culture and transplantation of epidermal sheets on the patient's body. Stem cells correction assures a functional renewal of epidermis during the all life of the patient.

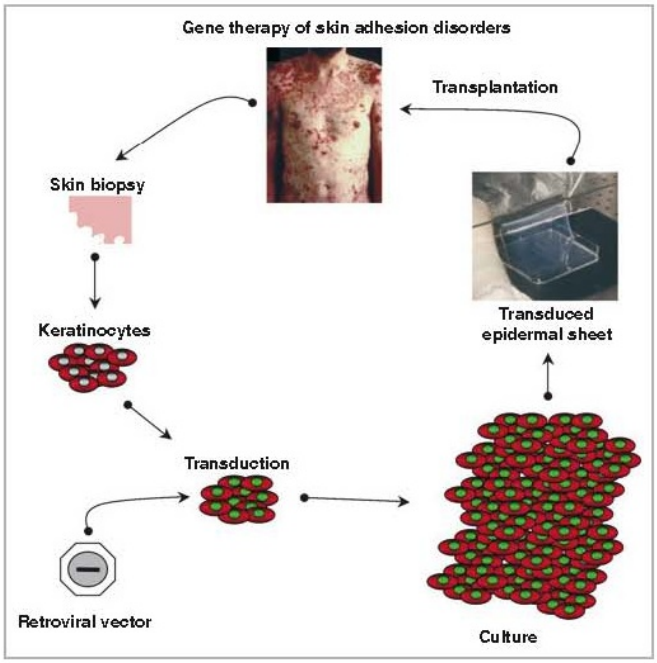


Figure 9. Ex-vivo gene therapy approach for epidermis genetic defects [26].

To achieve an efficient gene therapy it was demonstrated the importance to achieve several objectives: (a) target the right cells (b) choice the correct gene transfer agent (c) assure a long term expression of the transgene (d) develop surgical procedures for the transplant of genetically modified epidermal grafts. [26,30]

a) *Target the right cells*

Human epidermis renewal throughout adult life is maintained by the proliferation of keratinocytes stem cells, characterized by an high proliferation capacity and the ability to generate a differentiated progeny. Over 20 years of successfull keratinocyte mediated cell therapy confirmed the importance of Holoclones in maintaining the integrity of epidermis. Several clinical trials were developed using autologous cultured keratinocytes to treat different defects like massive full-thickness burns, damaged corneal surfaces in patients with severe alkali burns, vitiligo or piebaldism and to reconstruct the antherior urethra. [31-33]
The use of keratinocytes in gene therapy for skin disorders such as EB therefore requires the stable integration of the transgene in the genome of epidermal stem cell, that is, the holoclone forming cell. [26,30]

b) *Choice the correct gene transfer agent*

Gene delivery can be performed in two different strategies: *in vivo or ex-vivo*.

In vivo strategy consist in the direct introduction of the genetic material into the skin by injection, electroporation, "gene gun" or topical application. In contrast, *ex vivo* gene delivery involves removal of a skin sample from the patient followed by in vitro propagation of epidermal keratinocytes, gene transfer and return of the genetically engineered cells in the form of a skin graft back to the patient. Clinical trials proved that ex-vivo gene approach could be clinically more relevant in term of stem cell transduction, expansion of corrected population prior to grafting and persistence of skin grafts in long term follow-up.

Features of an ideal keratinocyte gene delivery platform include the ability to target stem cells (Holoclones forming cells) efficiently without inducing cell death or apoptosis, the ability to integrate in the host genome without activating potential oncogenes or being silenced, and not being immunogenic. Several approaches were developed to target keratinocytes: non viral methods like spliceosome-mediated RNA trans-splicing (SMaRT), ϕC31 bacteriophage integrase, and the Sleeping Beauty Transposon system. [34-37] or viral systems such as adenoviral, retroviral and lentiviral based vectors.

Murine-based retrovirus (RV) vectors remain today the most used integrating viral vectors for efficient epidermal stem cell transduction. Also HIV-1 LV based have shown a very high efficiency of transduction and unlike RVs they infect both proliferative and non-proliferative keratinocytes. RVs and LVs shown also a different integration pattern: the first integrate preferentially near the start site of the transcription unit, whereas the latter prefer integrate anywhere in the transcriptional unit.

c) *Assure a long term expression of the transgene.*

To achieve an efficient gene therapy is necessary to avoid a transient expression of the transgene and this is possible only transducing keratinocyte stem cells.

The transient amplifying cell population proliferates only for a limited period of time and represents the largest group of dividing cells. For this reason, ex vivo transduction of human keratinocytes likely favours this subpopulation but the transgene expression gradually disappear with the differentiation process.

Transduction of stem cells would instead result in sustained and permanent level of transgene expression. [26]

d) *Develop surgical procedures for the transplant of genetically modified epidermal grafts.*

Surgical procedures were developed for grafting large skin areas with a relatively noninvasive procedure. For gene therapy purposes, however, such an invasive surgical procedure would leave unacceptable scars on patient's body. The development of a new noninvasive timed surgery followed by transplantation of autologous epidermal sheets has allowed the replacement of natural epidermis without generating scars. Timed surgery allows selective removal of large areas of the epidermis from the underlying dermis while maintaining the integrity of the dermal papillae. For this reason it appears to be particularly suitable for replacement of JEB epidermis with autologous grafts of genetically corrected keartinocytes. [30]

Phase I/II clinical trial

In 2006 started the first worldwide clinical trial for EB envisaging gene therapy strategy with MLV retroviral vector. The phase I/II clinical trial involved a patient with a double heterozygous mutation on LAMβ3 gene. Skin stem cells were recovered only in palms byopsies that contained a sufficient number of holoclones despite of the majority of biopsies from others body's areas. [38,39]

Primary keratinocytes obtained from two palm biopsies were transduced by a retroviral vector expressing the full length LAMβ3 cDNA under the control of the Moloney leukemia virus (MLV) long terminal repeat (LTR). Clonogenic cells were transduced at >95% efficiency leading to full restoration of Laminin5 synthesis and expression.

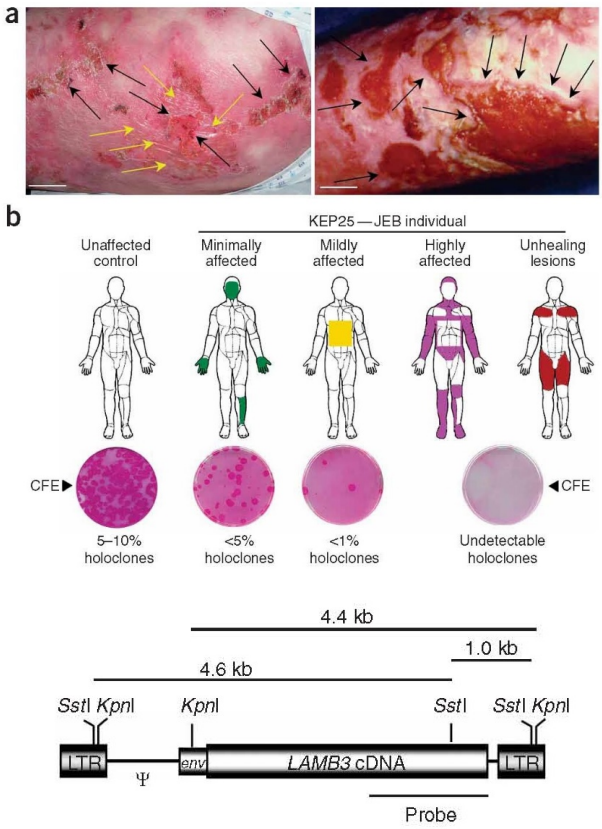


Figure 10. [1]

a) Blister development in the JEB patient enrolled for the phase I/II clinical tria

b) Clonal analysis from cells dervider from different body sytes. Only cells derived from palms give rise to holoclones in culture

c) MLV-derived retroviral vector carrying the *LAMβ3* cDNA

Transduced epidermal sheets were then grafted on both upper legs and on clinical examination no blisters were observed in the transplanted area throughout all these years of follow up. The regenerating skin is stable, robust and normal looking while the usual chronic blistering lesions characterize the surrounding skin. [39] After 4 months from transplantation, the epidermis resulted normal and fully differentiate on histological analysis, with a normal dermal-epidermal junction. Immunofluorescence staining also revealed a correct expression and location of Lam5-β3, Lam5-γ2 and α6β4 integrin. [1]

The long term follow-up (6.5 years) also shown a transplanted epidermis normal looking, pigmented and robust. It did not form blisters, that instead were observed around the transplantated area. It was performed also an in situ hybridization using vector-specific Laminin5-β3 probes which confirmed that the regenerated epidermis consisted only of transgenic keratinocytes. Immunofluorecence analysis

showed the maintainance of Lam5β3 expression after 6.5 years of follow up and also the expression of Lam5-γ2, Lam5-α3, α6 integrin, and β4 integrin were identical in transgenic, and normal epidermis.

This long term follow-up demonstrated the initial transduction of epiderml stem cells because of the monthly renewal of human epidermis. Six different integrations in 10 mm^2 of transgenic epidermis were identified by linker-mediated PCR and this means that the regenerated epidermis is sustained only by these few engrafted and transduced stem cells.[40]

Safety of retroviral vectors in gene therapy clinical trials

Retroviral vectors (RVs) have been widely used to deliver therapeutic genes in the context of gene therapy clinical applications for monogenic disorders, cancer and infectious diseases providing stable and efficient expression of the transgene to patients. Clinical trials for primary immunodeficiencies have clearly demonstrated the therapeutic benefit of retroviral-based approaches, but the field of gene therapy was significantly impacted by the occurrence of severe adverse events linked to insertional mutagenesis due to aberrant vector-on-host interactions. [41,42] Thus, insertional profiling, aimed at identifying vector integration sites and studying their potential impact in preclinical and clinical samples, has become an important tool to evaluate the global safety profile of clinical trials. The occurrence of severe adverse event in two clinical trials for X-linked severe combined immunodeficiency (SCID-X1) revealed the potential genotoxicity of RV.

However, it's really improbable that these adverse events occur in clinical trials for EB with integrating retroviral vectors. First because the mechanism that is emerging suggests a potential collaboration between the *IL2gR* transgene (used in SCID-X1 gene therapy trial), insertional activation of LMO2 protooncongene and additional genetic defects. Second, the number of transduced epidermal stem cells used for transplantation is lower than the number of gene modified hematopoietic stem cells in the X-SCID patients. Third, as >80% of EB patients develop aggressive squamous cell carcinomas, probably due to the continuous wound healing, the benefits of a gene therapy approach are still reasonably acceptable compared to the associated risks and the lack of alternative treatments. [38,43]

Morover the ADA-SCID clinical trial demonstrated the safety of MLV-derived

retroviral vectors in gene therapy strategy with a 100% survival rate at three years post treatment for all children in the pivotal study. Actually the European Commission has approved Strimvelis, the first ex-vivo stem cell gene therapy to treat ADA-SCID patients. [44]

For these reasons a clinical trial for EB patients using MLV-derived retroviral vectors it was approved in Austria. It would be anyway desirable to develop safer, self inactivating (SIN) retroviral vectors. SIN vectors carry a safer profile compared to γ-RVs based on their integration preferences, they lack the strong viral enhancers in the LTR, and have a lower propensity to generate tumors in preclinical animal models. [45,46]

The risk due to insertional mutagenesis can be diminuished by modifying the vector's enhancer-promoter elements. It was demonstrated, in hematopoietic cells that the cellular enhancer-promoters derived from human genes, elongation factor-1a (EF1a) and phosphoglycerate kinase (PGK), are much less likely to activate potential oncogenes than are viral promoters.

In this way SIN vectors containing the EF1α promoter greatly decrease the risk of insertional transformation. [47]

CAPTURING STEMNESS FOR CELL AND GENE THERAPY

Epithelial stem cells in vivo represent a minor subpopulation of basal cells and give rise to TA cells that proliferate and finally differentiate. This hierarchical structure is present in other tissues like the hematopoietic system, where it is possible to selectively identify and isolate the pool of stem cells and progenitors by the expression of specific surface proteins.

It was demonstrated that epidermal stem cells express high levels of β1 integrin and α6 integrin and low levels of the transferring receptor (CD71) whereas TA cells express high levels of CD71. Also expression of other cell surface proteins like α1 integrin, α3 integrin, Lrig1 or ABCG2 can capture stemness. [48]

Until now the more promising factor used to identify keratinocyte stem cells was p63 transcription factor. It was demonstrated that the p63 null mice lack the epidermis and its appendages and all the tissues derived from the same stem cells

that give rise to the epidermis, including mammary, sebaceous, lacrymal and salivary glands

These findings report an essential role of p63 in epithelial progenitor and also its requirement in the normal development of squamous stratified epithelia. [49] Also in human epidermis it was demonstrated that p63 has a basal epidermal localization and its expression decreases during clonal conversion. Definetively p63 is expressed in cells that are proliferating or posses a great proliferative potential [12]. However none of these proteins is stem cell specific and the identification of specific cell marker for epidermal stem cell isolation remains an unresolved issue, especially because it is the prerequisite for the maintenance of an epidermal graft. [13,14]

FoxM1 – a plausible marker for KSCs

FOXM1 is an activating transcription factor

FOXM1 is a member of the FOX (forkhead Box) transcription factor family characterized by a conserved DBD, the forkhead domain of forkhead box. The canonical forkhead domain contains a compact α/β structure consisting of three α-helices (H1, H2, H3), three β-strands (S1,S2,S3), and two loops or wings (W1,W2) arranged in the order H1-S1-H2-turn-H3-S2-W1-S3-W2. [50]

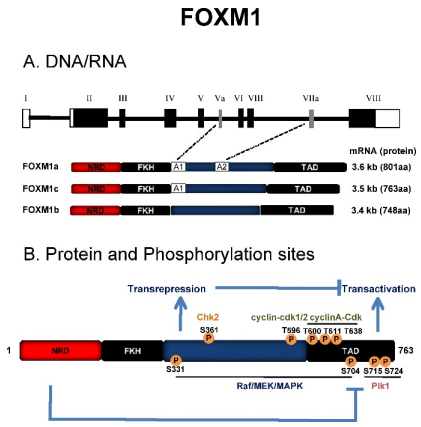


Figure 11. FoxM1 DNA with the three splice variants (FoxM1a, FoxM1b, FoxM1c) and its protein with phosphorylation sites. [51]

The tree human or murine splice variants *FOXM1A, FOXM1B* and *FOXM1C*, display the same DNA-binding specificity and bind to DNA binding sites with the consensus sequence 5'-A-C/T-AAA-C/T-AA-3'. Both FoxM1B and FoxM1C are expressed in various tissues and cell lines and they are biologically active, whereas FoxM1A biological relevance in vivo is unclear. FoxM1 is a strong transactivator and can acts as transcription factor binding DNA with its DBD or via TATA-BOXES and also in a DNA-binding indipendent manner trough protein-protein interactions with DNA-binding transcription factors. [50]

FoxM1 plays a central role in the embrionic and fetal development, as well as during tissue homeostasis and regeneration in adults. [52]

In particular it's involved in cell cycle regulation and progression, regulation of VEGF and VEGFR in vasculogenesis process [53,54], cell migration and proliferation [51], stem cell self renewal [55] and in DNA damage repair response.

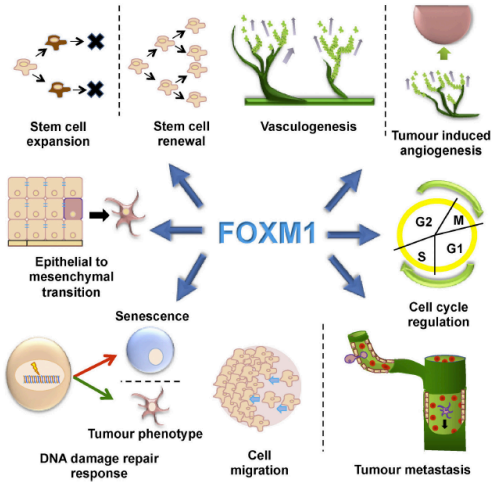


Figure 12. FoxM1 biological roles [52]

FoxM1 role in DNA replication and mitosis

FoxM1 is frequently related to proliferation especially in cell lines and hematopoietic system and it exibits a strictly proliferation-specific expression pattern.

It is expressed in all proliferating cells but not in resting cells and its expression is strongly induced upon cell cycle entry from quiescence, whereas it ceases upon cell cycle exit into quiescence, senescence, or terminal differentiation. [56]

In the cell cycle program it was shown that FoxM1 transactivate many important cell-cycle regulatory proteins like Cyclin B1 and CENP-F and it's involved in

G1/S and G2/M progression. FoxM1 expression is also ageing related and its downregulation coincides with an increase in the proportion of cells with 4n DNA content and polyploidy and leading to mitotic misregulation. FoxM1 deficient cells show chromosome instability and several additional mitotic defects like the failure of cytokinesis. [54]

FoxM1 in carcinogenesis

FoxM1 expression is frequently related to benign and malignant transformation and microarray analysis of human solid tumors have demonstrated that it is one of the most common overexpressed genes.

It is overexpressed in human non-small lung cancers (NSCLC), head and neck squamous carcinomas, hepatocellular carcinomas (HCC), intrahepatic cholangiocarcinomas, colon carcinomas, basal cell carcinomas infiltrating ductal breast carcinomas, anaplastic astrocytomas, glioblastomas, pancreatic carcinomas, gastric cancer, acute myeloid leukemia and other human tumors and human neoplastic cell lines. [57]

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women worldwide. Several pathways have been implicated in the progression of this cancer, including p53, epidermal growth factor receptor (EGFR), PDGF/PDGFR, PI3K/AKT, and mammalian target of rapamycin (mTOR). In particular AKT pathway is strongly activated during breast cancer development and progression, and the activation of AKT is observed in approximately 80% of breast cancer patients and it is associated with poor disease-free survival. FoxM1 expression levels are elevated in 87% of breast cancer patients and are correlated with breast cancer development. It was demonstrated that FoxM1 has a critical role in regulating breast cell tumorigenicity by directly binding PDGF-A promoter which in turn leads to the activation of the AKT pathway. Activating the PDGF/AKT pathway FoxM1 leads to the survival and growth of human breast cancer cells and with a regulatory feedback also PDGF-A elevates FoxM1 expression.[58]

This regulatory feedback between FoxM1 and PDGF/AKT pathway may represent a critical mechanism for the proliferation and tumorigenesis of human breast cancer and a possible therapeutic target to block breast cancer progression. [58]

Altough FoxM1 at physiological levels has been reported as a regulator of DNA repair, its upregulation is likely to interfere with the normal DNA repair mechanism, leading to enhanced genomic instability rather than enhanced DNA repair. This instability could be reach trough a program of malignant transformation in which the upregulation of FoxM1 lead to deregulation of cyotokinesis and epigenetic modifications whereby genomically unstable cells acquire oncogenic survival advantage. [59]

Regarding FoxM1 role in carcinogenesis it was shown a strong correlation of this transcription factor and skin cancer initiation, because FoxM1 expression is upregulated in Basal Cell Carcinoma (BCC).The effects of FoxM1 upregulation on human keratinocytes were investigated in response to UVB, a major aetiological factor in BCC and upon UVB irradiation. It was found that UVB dose-dependently increased the expression of FoxM1B and its upregulation leads to increased level of various pro-apoptotic/stress-response factors such as p21, p38, p53 and poly(ADP-RIBOSE) polymerase (PARP). In this way it is accelerated the selection of genetically unstable cells and it represent the mechanism by which FoxM1 promote carcinogenesis. [60]

Given that protein levels of this transcription factor are upregulated in most human cancers, it was investigated the possibility that acquisition of aberrant FoxM1 expression in clonogenic stem/progenitor cells might be a fundamental oncogenic initiation mechanism which in turn contributes to its upregulation. This hypotesis was confirmed in human oral mucosa cancer. In normal situation FoxM1 protein is found mainly in the proliferative epibasal layers of the oral epithelium with an heterogeneous expression pattern and it is significantly upregulated in $p75NTR^{hi}$ cells, instead its expression is diminished in the differentiating $p75NTR^{lo}$ cells.[61] The aberrant upregulation of FoxM1 in p75NTRhi cells lead to an enhanced proliferative capacity and to a hyperproliferative state with a perturbed balance between stem/progenitor cell renewal and terminal differentiation process. This finding suggests that the perturbation of differentiation program could enhance FoxM1 expression during the tumor progression and that a premalignant progenitor with high levels of FoxM1 lead to a tumor with a hallmark of elevated FoxM1. [61]

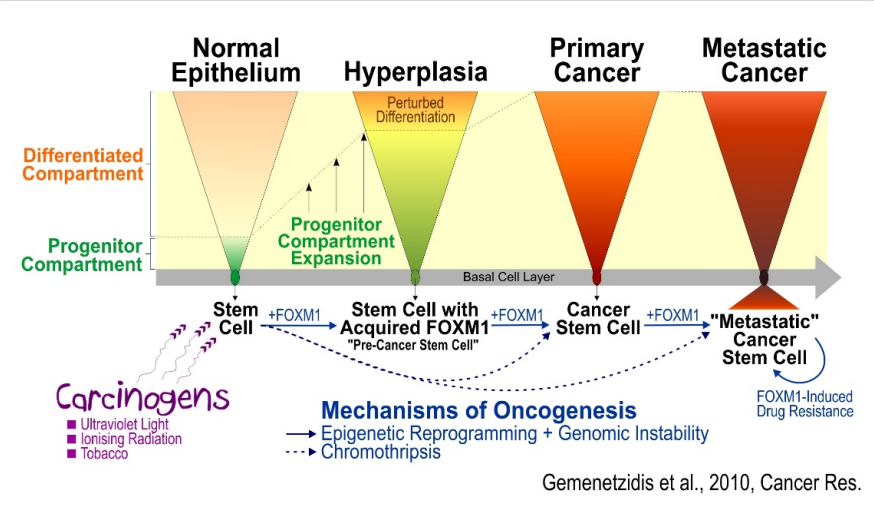


Figure 13. Scheme of tumor progression from normal epithelium to metastatic cancer with progressive increase of FoxM1 expression

FoxM1 protein is also involved in the development of soft-tissue sarcomas. In these case however it is not required for tumor initiation but is necessary for growth and progression.

It was demonstrated a strong correlation of FoxM1 with the Hippo pathway, which is fundamental in the regulation of cell proliferation. Hippo pathway results deregulated in >25% of sarcomas and of consequence the effector protein Yes-Associated protein (YAP) is overexpressed with an increase in proliferation levels. It was demonstrated that YAP, via TEAD, directly bind FoxM1 resulting in coregulation and overexpression of pro-proliferative targets. In this way YAP/TEAD/FOXM1 complex facilitate proliferation and promotes sarcomagenesis, making it an interesting target for therapeutic intervention. [62]

Indeed FoxM1 is studied as an indirect target of several widely used cancer drugs because of its central role in initiating or promoting cancer. For example, tyrosin kinase inhibitors (TKIs) and monoclonal antibodies, that target ERBB2 and/or epidermal growth factor (EGFR) have ben shown to function by repressing PI3K-AKT signalling and consequently leading to FoxM1 downregulation. Morover some conventional cytotoxic chemotherapeutic drugs, such as doxorubicin, epirubicin and cisplatin, also mediate their cytotoxic effects partly trough targeting the P3K-AKT-FoxO-FoxM1 axis. Doxorubicin in particular activate MAPKs pathaway leading to FoxO3A phosphorylation and nuclear accumulation, which in turn antagonizes FoxM1 activity.

FoxO and FoxM1 have a central role in mediating the cytostatic and cytotoxic effects of chemoterapeutic drugs and this can lead to drug resistance. FoxM1 indeed acts by regulating DNA damaged repair genes and its upregulation render cancer cells resistant to a range of diverse anticancer therapeutics. Congruosly, silencing of FoxM1 re-sensitizes drug-resistant cells to chemotherapy, partly by inhibiting the induction of DNA repair genes. [63]

Another anticancer strategy is the use of miRNAs when coupled with a good delivery system. FoxM1 is indeed regulated by miRNAs, and potentially their expression can be targeted by RNA interference to specifically and effectively eliminate cancer cells or to impair their growth.

FoxM1 mouse models

To better understand the role of FoxM1 in the organs development and in the different tissues several research groups have studied general and conditional knockout mouse models.

The complete knockout of FoxM1 is embrionically lethal with defective development of heart, lung and liver. FoxM1 -/- embryos also displayed a gelatin-like consistency of the skin evidentiating defects in skin development. [64]

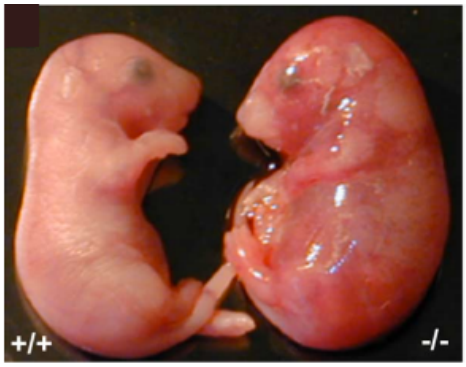


Figure 14. Wild Type and FoxM1 -/- mice. The KO mouse display a gelatin like consistency of the skin[64]

FoxM1 also plays an important role in lung morphogenesis and it is required for perinatal lung function and for proper development of lung epithelial cells. Its conditional deletion in the developing pulmonary epithelium cause death in 24 hours from birth showing that it is critical for adaptation to air breathing immediately after birth. [65]

FoxM1 and stem cell self-renewal

FoxM1 may be important for stem cell self-renewal because it exerts a positive effect on the self-renewal capacity of stem and cancer cells. [55,65,66]

In hematopoietic system it was demonstrated that FoxM1 is essential for maintenance of the quiescence and self renewal of hematopoietic stem cells (HSCs) in mice. FoxM1 downregulation lead HSCs to exit from the quiescence and quickly proliferate, reducing self renewal capacity of either HSCs and progenitors. The absence of FoxM1 in HSCs is also correlated with Nurr1 downregulation, wich is a key gene for the manteinance of self renewal capacity in hematopoietic system. [67]

FoxM1 is also important during development of conducting airways because it is required for proliferation and differentiation of Clara cells as well as for the proper epithelial differentiation. The conditional deletion of FoxM1 from airway Clara cells leads to disruption of normal patterning of epithelial cell differentiation in the bronchioles of the developing mouse lung, causing squamous and goblet cell metaplasia, and the loss of Clara cells. FoxM1 is so a key transcription factor for long-term maintenance of bronchiolar epithelium and airway structure. [68]

The importance of FoxM1 was investigated also in the mammary tissue of adult mice where conditional deletion of FoxM1 resulted in an expansion of differentiated luminal cells, with a concomitant loss of the mammary stem cell and luminal progenitor populations. [69]

Regarding human system: it has been demonstrated that in glioma cells FoxM1 is expressed only in culture condition that preserve stemness and its overexpression augmented the formation of neurosphere. It was also demonstrated that FoxM1–expressing cells decrease the differentiation markers and upregulate Sox2 expression, that is typically associated with stem status. [70] The same role was described in human oral mucosa where FoxM1 favour the clonal expansion of epithelial stem/progenitor cells in contrast with differentiated keratinocytes. [61]

AIMS OF THE THESIS

Based on the successful results obtained in the Lamβ3 gene therapy clinical trial we want to develop an ex vivo gene therapy strategy with an MLV-derived retroviral vector carrying the *COL17* cDNA, to treat JEB patients with a mutation on Collagen17. In addition, because the MLV approach has raised safety concerns associated to the insertion of LTRs in human chromosomes resulting in endogenous gene dysregulation we also decide to develop a gene therapy approach using SIN γ-retroviral vector, which have a safer integration profile, carrying *COL17* gene.

In both viral strategies, we need an high efficiency of transduction, low proviral integrations, to limit the potential insertional mutagenesis risks, and the correction of stem cells in culture.

Although in these years, great progresses has been made in stem cell research, epithelial stem cell markers, that allow the prospective isolation and transduction of stem cell in culture, have not been identified. For this reason, together with the development of gene therapy protocols for the treatment of Col17 JEB, we aimed to identify keratinocyte stem cell markers that can be used in clinic, together with p63, for the identification and -eventually- the selection of a pure population of stem cells. The transduction of an enriched epithelial stem cell culture would assure the generation of a long-lasting corrected epithelia graft and epidermal restoration.

MATERIALS AND METHODS

Cell cultures

Human primary keratinocytes were obtained from abdomen and breast biopsies of healthy and JEB adult donors (20-52 years old) and expanded by cultivation onto lethally irradiated 3T3-J2 cells (a gentle gift from H. Green's lab) in growth KNO (DMEM and Ham's F12 media mixture (2:1) containing FBS (10%), penicillin-streptomycin (1%), glutamine (2%), insulin, adenine, hydrocortisone, cholera toxin, triiodotyronine medium. After 3 days KNO medium was replaced and KC medium (KNO medium containing 10ng/ml EGF) was added to the culture. Keratinocytes were trypsinized at sub-confluence and replated onto a new feeder-layer. Mouse NIH/3T3 fibroblast cell line was maintained in Dulbecco's Modified Eagle's medium (Euroclone), supplemented with 10% fetal calf serum.

Clonal analysis

Skin biopsy was subjected to standard trypnization protocol. Cell suspension was serially diluited until to obtain a final concentration of 1 cell/ 0,1 ml. Single cells were isolated using limiting dilution (0,5 cell / well) into a multi 96 well plate already containing irradiated 3T3 cells and KNO medium. After 3 days in culture, cells were feeded with KC supplemental medium. At the 7th day of cultivation, clones are identified under an inverted microscope. Each clone was photographed and then it was dissociated with trypsin. After centrifugation, a colony quarter (1/4 cell suspension in the falcon) is plated in a 100 mm dish containing lethally irradiated 3T3 cells (indicator dishes) and the remaining colony (3/4 cell suspension in the falcon) in a 35 mm dish containing feeder layer (clone progeny flasks). For the indicator dishes, the cells were cultivated for 12 days before the cultures were fixed and stained with rodamine B. The clonal type was determined by the percentage of aborted colonies (scored as in Barrandon and Green, 1987) formed by the progeny of the founding cell. When 0–5% of colonies were terminal the clone was scored as holoclone. When all colonies formed were terminal (or when no colonies formed), the clone was classified as paraclone. When > 5% but < 100% of the colonies were terminal, the clone was

classified as a meroclone [9]. Selected clones were serially propagated to determine the number of cell generations. Cells plated in the second dish were cultivated for 4–5 days checking day by day under an inverted microscope that the colonies did not interact each other to promote clonal conversion and then used to prepare cell extracts destined to Western analysis.

Determination of n° of cell doublings (cell generations)

The number of cell generation was calculated using the following formula: x=3,322 log N/No. The number of cells (No) was the actual number of colony-forming cells plated, since they were the only cells capable of dividing. CFE data, determined separately at the time of cultures were passaged, were used for the calculation. N was the total number of cells obtained at passage.

Western blot analysis

All samples destined to Western analysis were depleted of feeder layer. Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% deoxycolate, 1% Triton X-100, 0,1% SDS, 0,2% sodium azide, pH 7,5 containing protease inhibitors) for 30' on ice, and protein amount determined by Bradford assay (Thermoscientific). Cell lysate samples (7-20 μg) were run on a 6-10% SDS-PAGE (100 V, 1-2 hrs) gel, transferred 100 V at 4°C for 2 hours onto nitrocellulose membrane (Biorad) and immunoblotted using mouse monoclonal antibody against p63α protein (1:10.000, Primm), Col17A1 protein (1:500, Sigma), FoxM1 protein (1:1000, Santa Cruz C-20), GAPDH (1:10000, AbCam). Protein detection was carried out using a chemiluminescent labelling detection reagent (ECL, Thermoscientific).

Immunohistochemistry

IF was performed on skin sections and on keratinocyte cultured on glass coverslips. For skin sections, samples were embedded in optimal cutting temperature compound frozen (OCT) and sectioned (7 μm). Immunofluorescence was performed using these antibodies: Col17 (1:100, Abcam), Keratin 14 (1:10000, Covance), FoxM1 (1:2000, SantaCruz), rabbit purified anti-p63α immunoglobulin G (IgG; PRIMM) (Di Iorio

et al., 2005). Alexa Fluor 488 goat or Alexa Fluor 568 goat anti-mouse or anti rabbit (Life Technologies) conjugated secondary antibodies were used for detection. Cell nuclei were stained with DAPI. Fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar × 40/1.3 oil immersion objective, and analyses were done with the LSM510 Confocal Analyzer (Zeiss).

Generation of MLV-col17 retroviral vector and primary human keratinocytes transduction

A retroviral vector expressing the 4-kb full-length Collagen 17 cDNA under the control of the MLV LTR was constructed by cloning in the MFG-LacZ backbone and integrated by transinfection in the amphotropic Gp+*env* Am12 packaging cell line.
Briefly, plasmid DNA was transfected into the GP+E-86 ecotropic packaging cell line (28) by standard calcium phosphate coprecipitation. Forty-eight hours after transfection, supernatant was harvested and used to infect the amphotropic packaging cell line GP+env Aml2 for 16 hr in the presence of 8 ug/ml Polybrene. Infected Aml2 cells were clonally selected in HXM medium (GIBCO) supplemented with 10% FCS (HyClone), and containing 0.8 mg/ml G418 and 0.2 mg/ml hygromycin B (Sigma). Single colonies were screened for Col17 expression by immunofluorescence assay. JEB patient, were enrolled under informed consent. Keratinocytes were obtained from full-thickness skin biopsies and cultivated as described. Subconfluent primary cells were plated (6×10^3 cells/cm^2) onto a feeder layer (8×10^4 cells/cm^2) of lethally irradiated 3T3-J2 and Am12-Col17 cells in a 1:2 ratio. After 3 d, cells were transferred onto a 3T3-J2 feeder layer.

Southern Blot analysis

DNA was extracted from $1–5 \times 10^6$ cells, digested (10 μg) with different restriction enzymes, run on a 0.8% agarose gel, transferred to a nylon membrane (Duralon, Stratagene), and hybridized to a [^{32}P]-labeled Col17–specific probe.

Organotypic culture

Deepidermized dermis was obtained from skin biopsy of healthy donors. Human skin flaps were maintained in PBS at 37°C for 10 days ntil the detachment was complete. After 10 days, epidermis and dermis were totally separated. The dermis was then placed in a 24 well support in Kno medium and keratinocytes were plated at primary density as immersed culture. After 3 days the medium was replaced with Kc and at day 7 keratinocytes were exposed to air.
After 20 days of culture the organotypic culture was embedded in OCT then sectioned (7 μm) and used for immunofluorescence analysis.

Titration of viral vector on ht1080 cell line

HT1080 cells were plated at 50.000 cells for a 12 well support. The next day viral supernatant (froma AM12-Col17 clones and SIN-retroviral vector) was distributed on plated cells at different concentrations (range from 1 ml to 0.01 ml). HT1080 cells were counted at the time of infection and change of medium occurs 12 hours post infection.
48 hours post infection cells were detached and plated on cover slips to perform immunofluorescence against Col17 and determine the viral titer.
Viral titer is calculated by immunofluorescence analysis performed with anti-collagen 17 antibody, and according to the following formula:
(% of Col17+cells n° of 3T3 cells at the moment of infection) / ml of virus.

Primary human keratinocytes transduction with SIN-retroviral vector

Retronectin® was plated at 5 $\mu g/cm^2$ concentration on 24 well support and conserved one night at 4°C. wells were washed once in PBS then incubated 30' at room temperature with PBS/BSA 2%. After incubation were washed with PBS and viral supernatant was distributed at desired concentration and centrifuged at 1500 g for 60' at 32°C. Viral supernatant was then removed and lethally irradiated 3T3-J2 were plated with keratinocytes at standard concentration. After 3d keratinocytes were replated onto new 3T3 J2 feeder layer.

Bioinformatical analysis

The byoinformatical analysis were performed by a pair-wise comparison of two groups of arrays (1) H versus P 2) H versus M. We estimated the number of differentially-expressed genes (DEGs) as those whose difference in log2-based expression values exceeded a custom threshold of expression fold-change; the software calculates the absolute difference between the two group means and test whether the mean difference is equal to zero by the unpaired t-test, with a p-value threshold set to $p< 0,01$. Since tens of thousands of genes are compared, many genes can be false positives; False Discovery Rate (FDR) for multiple-comparison adjusted p-values, which can be estimated by performing random permutations of group labels.

We performed the supervised analysis based on different criteria, in order to obtain a list of DEGs in a more/less stringent way; arrays were analysed with Affymetrix, using crescent fold-change thresholds ($FC >1,5 < -1,5$) and stringent p-value thresholds, retaining only DEG lists with $FDR < 0,05$. The striking huge number of DEGs obtained even at higher fold changes and stringent p-values confirmed what already assessed by the unsupervised clustering.

RESULTS

MLV-RETROVIRAL VECTOR STRATEGY

Am12 Col17 packaging cell line construction and selection

The first strategy that we have developed for EB gene therapy involved a gamma-retroviral vector (AM12 MCB), carrying the *COL17* cDNA. This MLV vector was already successfully used in the phase I/II clinical trial carrying the *LAMβ3* gene for two single patient treatments.

As shown in Figure 15A the backbone of the Moloney Murine Leukemia Vector (MMLV) MFG-LacZ [71] was used to generate the construct termed MFG-Col17 (12,7 Kb). The MFG-LacZ vector was digested with BamHI and NcoI giving a fragment of 8Kb, whereas the *COL17* fragment was obtained from the digestion of PLZR-*COL17* with XbaI and SalI and subsequently filled in with Klenow. The *COL17* insert was cloned into MLV backbone and the resulting product was EcoRI digested to verify the *COL17* cDNA orientation. We performed the analytical digestion to confirm the correct orientation of the insert, obtaining the final plasmid MFGCol17.

In this vector, *COL17* gene is expressed under the control of the viral long terminal repeats (LTR) promoter, which ensures persistent and high level gene expression in human keratinocytes.

The construct was transfected in Phoenix Ecotrophic cell line using $CaPO_4$ transfection, and the supernatant was spinocultaed on Am12 packaging cell line, which does not produce a recombinant helper virus and which has been used many times in clinical studies since 1991. Am12 packaging cell line were infected with a 100% efficiency of transduction measured by immunofluorescence analysis (Figure 15B) and stable clones were isolated through a clonal diluition assay. Progeny of single Am12 colonies was screened for the expression of the transgene by immunofluorescence and western blot analysis.

In order to choose one single Am12 clone feasible for clinical applied gene therapy, we performed a series of preliminary experiments on primary JEB keratinocytes with five selected Am12 clones. The transduction protocol on JEB

keratinocytes was performed with the co-culture strategy successfully used for a phase I/II clinical trial for Lamβ3 patients. Keratinocytes were plated on a co-culture of irradiated murine feeder layer and Am12 clone at 1:2 ratio for three days, then cells are re-plated on a new murine feeder layer until reached sub-confluence.

Immunofluorescence (Figure 15C) and western blot (Figure 15D) analysis revealed the ability, of all AM12Col17 clones tested, to transduce keratinocytes with a high efficiency.

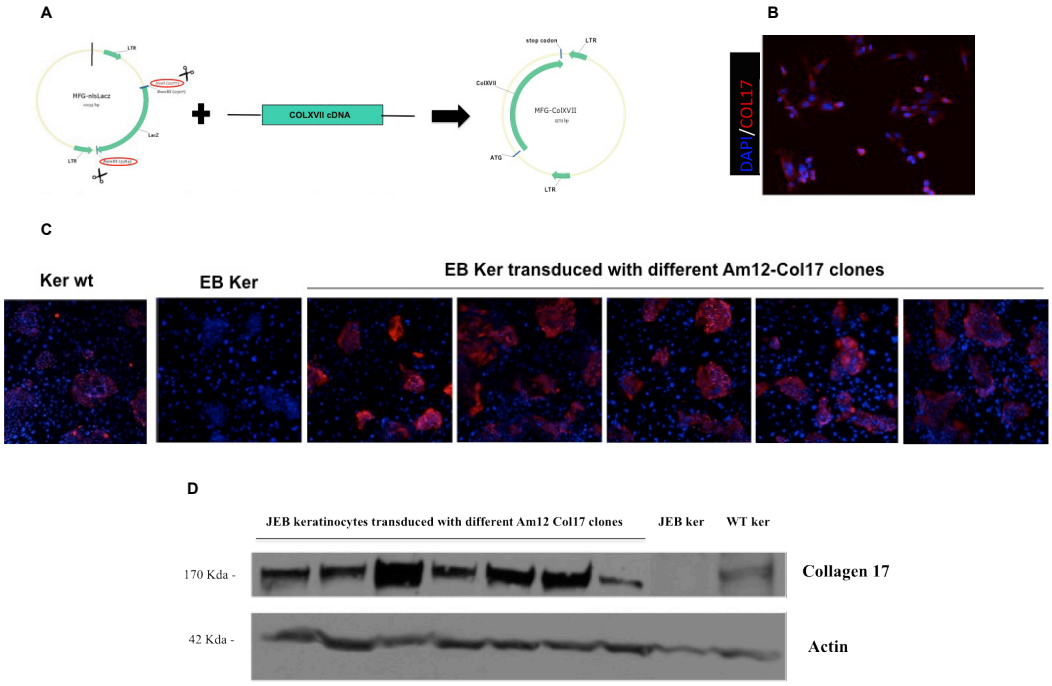


Figure 15. Am12-Col17 retroviral vector and keratinocytes transduction with different Am12 clones

A) Cloning strategy and schematic map of the Retroviral vector MFG –Col17

B) Am12-Col17 packaging cell line has been generated using viral supernatant obtained from 293 cell transiently transfected with the construct in A. Immunofluorescence analysis reveals Col17 expression in the Am12 cells and is representative of all the selcted clones tested on keratinocytes. C) Different positive clones of Am12-Col17 packaging cell line have been selected and tested on keratinocytes. expression of WT keratinocytes is shown in the left panel. Immunofluorescence analysis shows good expression of Col17 protein on EB keratinocytes transduced with different Am12-Col17 clones.

D) Western blot analysis of JEB-cultured keratinocytes before (lane 8) and after (lane 1-7) transduction with MFG-Col17 indicate complete reconstitution of Col17 synthesis in transduced cells. Col17 expression is compared to the normal synthesis in wild type keratinocytes. All the tested clones efficiently transduced keratinocytes leading to efficient Col17 expression, that was completely absent in JEB ker before transduction. Col17 expression levels are different from each clone because of the different number of viral integrations in keratinocytes. None of the selected clones give rise to recombinant form of Col17 protein.

Another critical aspect of gene therapy strategy concerns the integration of MLV retroviral vector in the human DNA. Because of the adverse events happened in the SCID-X1 gene therapy trial, it is of particular importance to evaluate the number of integration to minimize the risk of insertional mutagenesis.

To evaluate the integration pattern, we performed a Southern Blot analysis (data not shown) of the keratinocytes bulk cultures transduced with selected Am12 clones. According to the number of integration revealed by Southern Blot in the bulk population, we performed a real time to better quantify Col17 expression. The RT-PCR analysis identified Am12 clones that have low proviral integration on target cells and are good candidate for ex-vivo gene therapy approach (Figure 16). The relative fold shown is in relation to clone 1 that in Southern Blot revealed 7 integrated proviral copies.

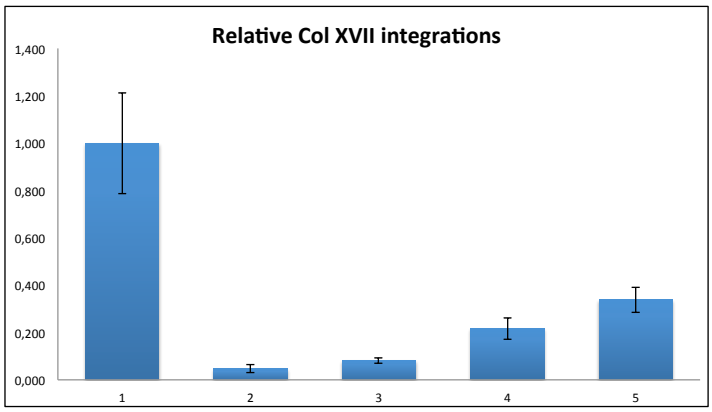


Figure 16. Relative Col17 integrations in keratinocytes culture

Real Time-PCR analysis of Col17 expression from bulk culture of keratinocytes transduced with different Am12 clones. Relative fold shown is in relation to clone 1 that in Southern blot analysis reveals 7 integrated proviral copies.

Among five AM12 clones we choose AM12-Col17 #124 (clone #4 in Real-Time analysis) since it combined good levels of Col17 expression as demonstrated by immunofluorescence and western blot analysis, together with the absence of eventual recombinant forms of Col17 protein. In order to full characterized #124 clone we performed a titration assay on 3T3 murine cells. Viral supernatant from packaging cell line was filtered and serially diluted to infect 3T3.

The resulting viral titer for #124 Am12 clone is $1.3*10^6$ that fits with the range of retroviral vectors that is between 10^4 and 10^6.

According to these results we proceed with the characterization of primary JEB keratinocytes transduced with Am12-Col17 #124 clone.

Primary keratinocytes transduction

Am12-Col17 efficiently transduce keratinocyte culture

Primary keratinocytes isolated from skin biopsy derived from two different JEB (Col17 negative) patients were used for transduction experiments with AM12-Col17 #124. Transduction was performed with the co-culture strategy (Figure 17A) and as shown in Figure 17 B-C a uniform cytoplasmic staining was observed in Col17-transduced keratinocytes, indicating a high efficiency of transduction of #124 clone. Moreover immunofluorescence performed on keratinocytes plated at clonal density demonstrated that clonogenic Col17-null keratinocytes were transduced with approximately 90% of transduction efficiency, which was maintained during serial cultivation (Figure 17D-E).

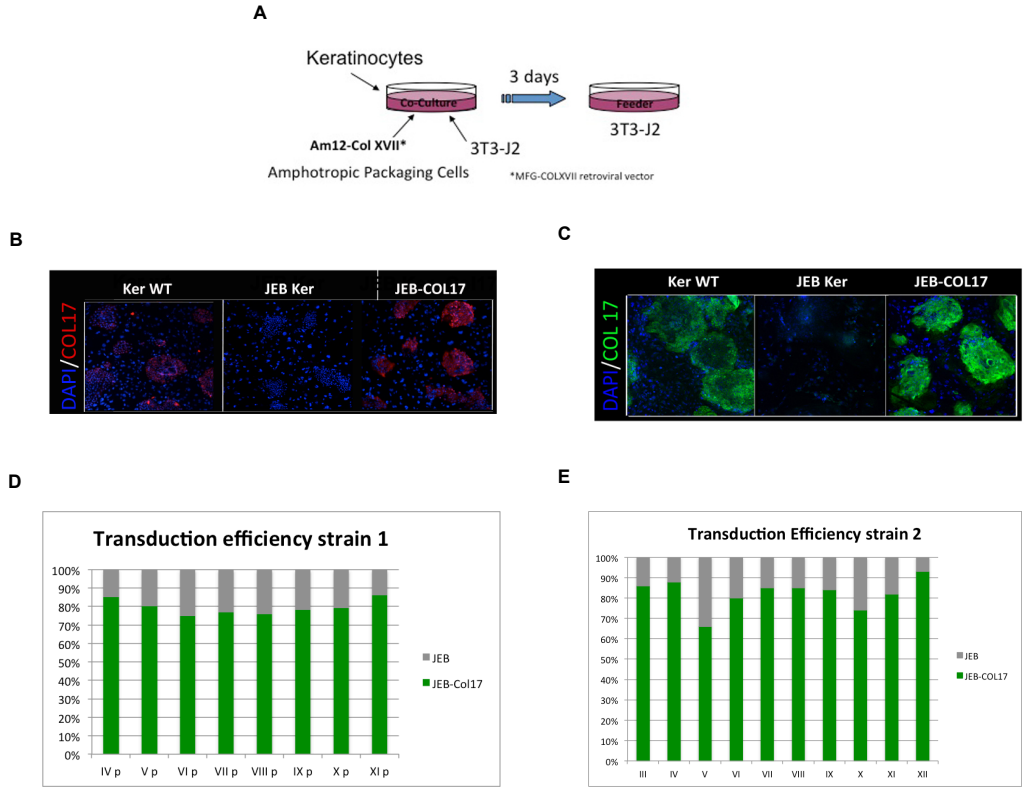


Figure 17. Keratinocytes transduction with selected Am12-Col17 clone

A) Scheme of co-culture infection strategy: Keratinocytes are plated on a co-culture of irradiated 3T3-J2 and Am12-Col17 for three days. After 3 days Keratinocytes were plated onto a normal feeder layer of 3T3-J2 fibroblasts untill reach sub-confluence and analysed for efficiency of transduction by immunofluorescence assay. B-C) Immunofluorescence analysis of Col17 expression from two JEB Col17-null keratinocytes before and after transduction as described in A. Col17 expression of WT keratinocytes is shown in the left panel. Anti-Col17 immunofluorescence shows good expression of Col17 protein on transduced keratinocytes with levels compared to wild type keratinocytes. D-E) Transduction Efficiency: Col17 positive colonies were counted on CFE dishes at each keratinocytes passage. High efficiency of transduction was maintained during culture passages in both transduced strains.

Transduced keratinocytes maintain clonogenicity in culture

We performed a life span of the transduced and untransduced keratinocytes in order to monitor eventual morphological or growth alteration We observed that both control and transduced cells displayed similar characteristics in term of growth and colony morphology. As shown in Figure 18A-B transduction with Am12 clone did not cause morphological changes or alteration of growth rate, so we can exclude cell toxicity effect of retroviral vector.

For each passage in culture we performed CFE assay to evaluate the percentage of clonogenic and abortive colonies both in control and transduced keratinocytes.

We demonstrated that transduction did not affect keratinocytes clonogenicity since in both strains the percentage of abortive and clonogenic of transduced colonies were comparable to their not transduced counterpart (Figure 18C-D). Moreover, Col17 correction induces a little increase in clonogenicity with a corresponding decrease of abortive colonies reflecting the improvement of the culture conditions.

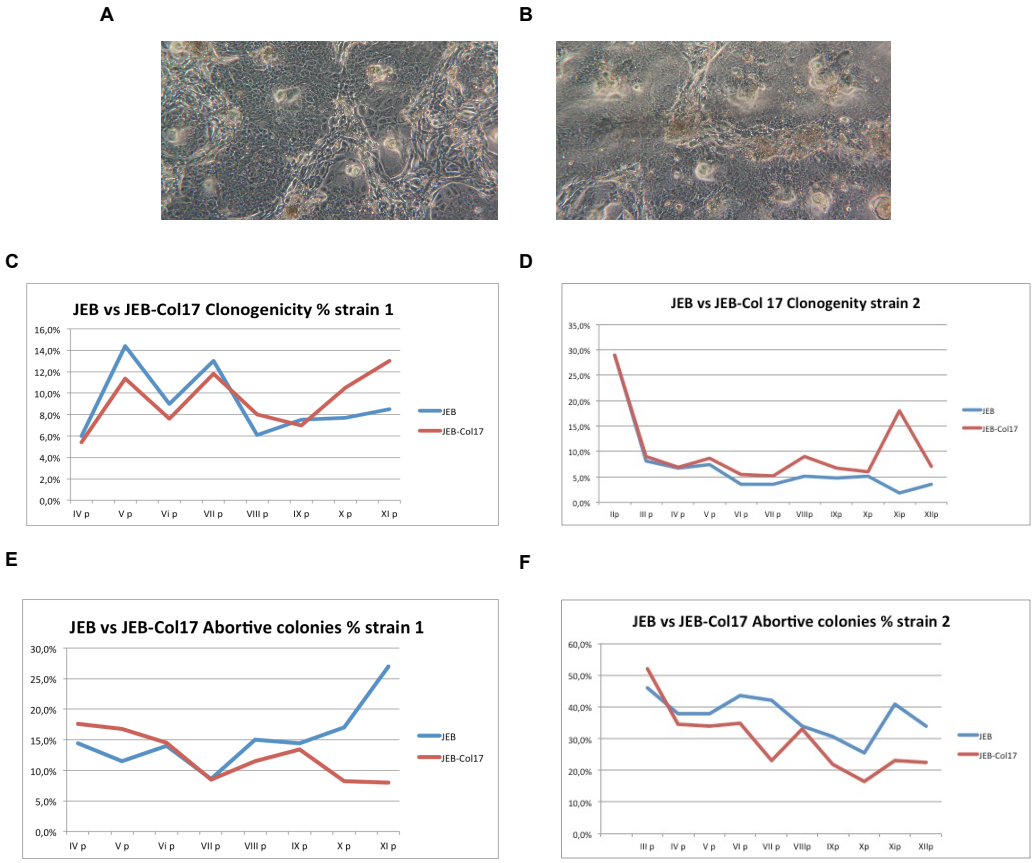


Figure 18. Clonogenicity of transduced cultures

A-B) Keratinocytes were transduced at the third passage of culture. We monitored transduced and not-transduced keratinocytes during serial cultivation. AM12 clone did not cause relevant morphological changes or alteration of growth rate of transduced keratinocytes (B), as compared to non-transduced cells (A). C-D) Clonogenicity: CFE assay shows that transduction using gamma-retroviral vector do not affect clonogenicity of JEB- keratinocyte compared to untransduced population. E-F) Abortive colonies counted on CFE assay resulted similar in transduced JEB vs Untransduced keratinocytes.

Am12-Col17 clone corrects stem cell in culture

Long-term restoration of JEB epidermis has been shown to require a defined number of transduced epidermal stem cells, detected as holoclones[1]. The transduction of holoclone forming cells is instrumental for the permanent regeneration of a fully functional, self-renewing transgenic epidermis.

To formally demonstrated that holoclones were properly transduced we performed a clonal analysis on JEB-Col17-transduced cells. Single cells were isolated from subconfluent primary transduced cultures and plated into multi well plates containing 3T3-J2 feeder layer. After 7 days, ¼ of each clone was transferred into a 100-cm2 dish and cultivated for 12 days. The dish was fixed 12 days later and

stained with Rhodamine B for the classification of clonal type [9,43,72] (Figure 19A). The transduction with Am12-Col17 #124 clone does not affect the stem cell population of JEB keratinocytes and three Holoclones have been isolated (Figure 19B).

Two out of three isolated holoclones resulted transduced with COL17 by western blot analysis (Figure 19D) and a immunofluorescence analysis (Figure 19C).

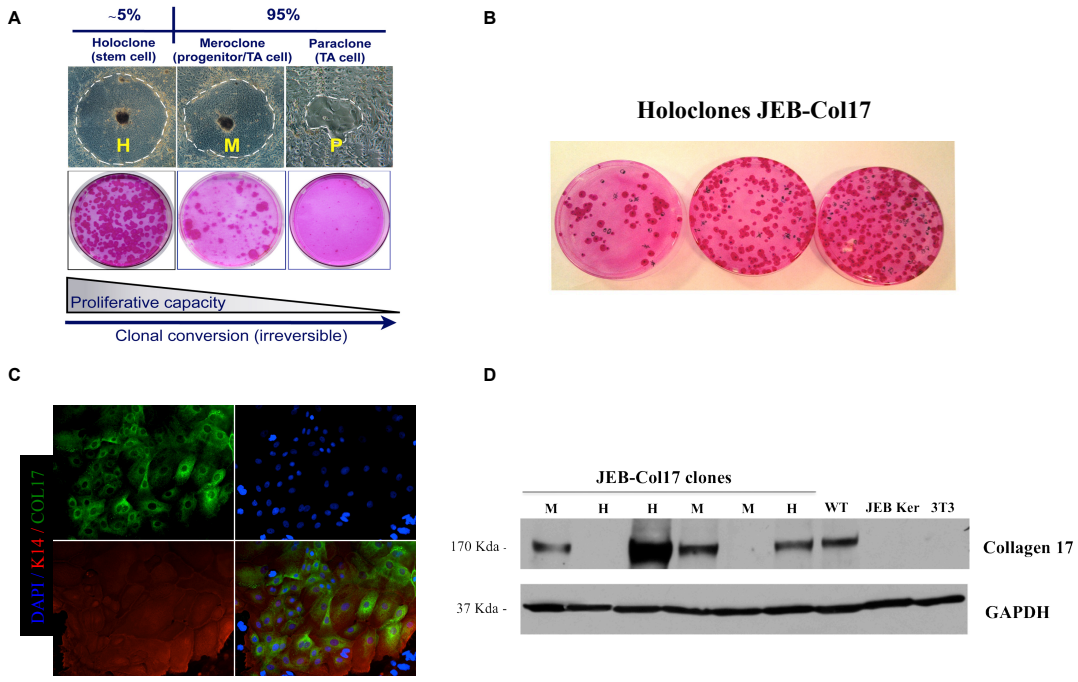


Figure 19. Epidermal stem cells transduced by Am12-Col17 clone

A) Schematic Rappresentation of clonal type classification. Clonal analysis allows to isolate three different type of clones from an heterogeneus primary keratinocytes culture: Holoclones, Meroclones, Paraclones. The Holoclone is generated by the epidermal stem cell. The Paraclone is generated by a transient amplyifying cell. The Meroclone is an intermediate type of cell and is a reservoir of transient amplyifing cell. B) CFE indicator dishes of three Holoclones isolated from JEB strain transduced with gamma-Col17-retroviral vector. C) Immunofluorescence staining on cells derived from isolated holoclone of JEB transduced culture. D Western Blot analysis performed on different type of clones shows that two out of three Holoclones (H) express Col17 at comparable or higher level than WT cultures.

Exogenous Col17 is deposited in an organotypic culture

In appropriate culture conditions, it is possible to mimics the structure of in vivo epidermis. In this regards, we generated an organotypic culture with de-epidermized derma from a skin biopsy plated with transduced and untransduced keratinocytes from a JEB patient.

The reconstituted epidermal sheet is embedded on OCT medium and subsequently cut in slides for immunofluorescence analysis. The correct localization of Col17 protein in transduced organotypic culture at dermo-epidermal junction demonstrates that exogenous Col17 is functional and released from corrected keratinocytes to the derma-epidermal junction in a way similar to a control one (Figure 20).

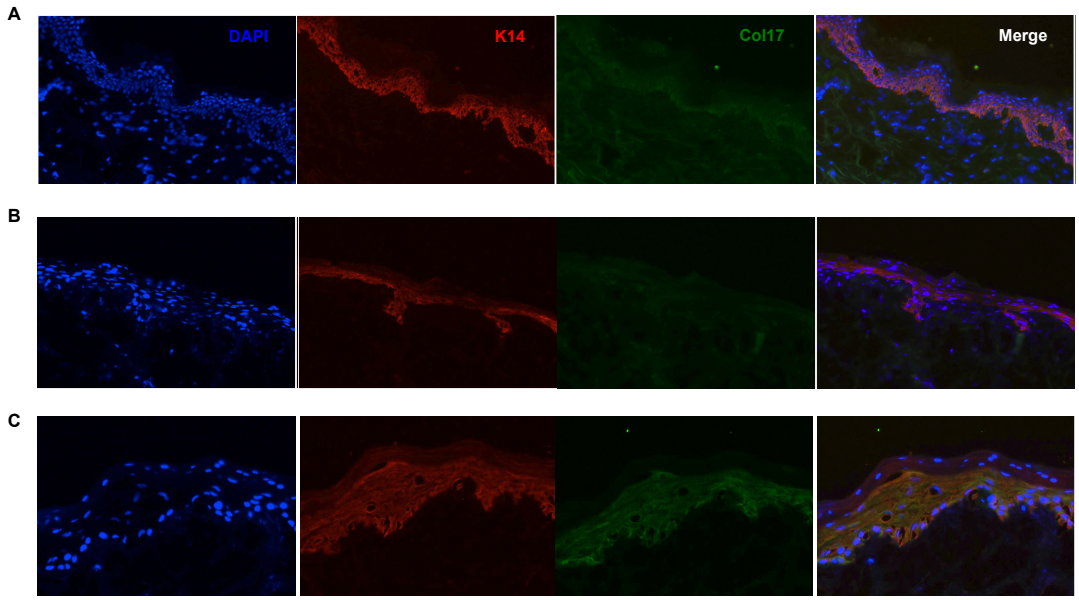


Figure 20. Organotypic cultures of transduced and not transduced keratinocytes.

Immunofluorescence analysis performed on skin biopsy (A) and on organotypic culture of JEB (B) and Col 17 transduced JEB (C). The reconstructed epidermis expresses keratin 14 (red). Transduced keratinocytes show a correct localization of Col17 (green) that is completely absent in JEB not transduced keratinocytes.

Col 17-JEB gene therapy: future steps

These data represent the pre-clinical assays for the establishment of a planned gene therapy clinical trial in Austria. Based on these results, a master cell bank of packaging clone Am12-Col17 #124 was made under full GMP/GLP standard by a qualified contractor (EUFETS GmbH). Moreover to evaluate the safety profile of MLV-RV transduction in terms of potential insertional mutagenesis we developed robust *in vitro* and *in vivo* studies to identify and estimate the putative genotoxicity events during the entire *in-vitro* and *ex-vivo* procedure for gene therapy of skin genetic disease. In particular, we are testing the ability of transduced cultures to grow in soft agar (soft agar assay), their dependence on the presence of growth factors in culture medium (growth factor dependent assay), and the eventual occurrence of an immortalized/transformed clone (serial passage assay).

Soft Agar assay is an anchorage independent growth assay and is considered the most stringent strategy for detecting malignant transformation of cells. With this assay we want to formally demonstrate that keratinocytes transduced with AM12 packaging cell line are not able to migrate and form colonies in agar. The growth factor dependence assay is performed to assessed whether transduced keratinocytes, are able to growth in the absence of standard growth factors and feeder layer acquiring a profile similar to transformed keratinocytes used as control (MCF7 cells). Serial cultivation assay consists in serial passage on the same type of support of the entire transduced keratinocyte populations. In this stressed growth condition it is easier to identify a putative cell with uncontrolled growth advance properties. This assay could confirm that none of the single keratinocyte of the transduced population is malignantly transformed.

A process validation is now on going for the GMP production of genetically corrected JEB epidermal cultures. The entire procedure is performed through the same steps established for the phase I clinical study made in 2006 and 2014 in Holostem [1,73]. The process for the generation of genetically corrected JEB epidermis consists of two phases: 1- generation of the drug substance, that cover the steps from skin biopsy to generation of transduced cells; 2-generation of drug products, that consists in the preparation of genetically corrected grafts suitable for transplantation.

The Col17 trial with the MLV retroviral vector will be submitted in Salzburg to the legal authorities. After regulatory approval appropriate patients will be selected and trasplantations will be planned.

SIN RETROVIRAL VECTOR

Vector map and viral titer

Although the Am12 clone efficiently transduces JEB keratinocytes, safety concern about the use of MLV derived vector lead to the development of a safer alternative using Ampho-pseudotyped SIN-γ-retroviral vector. For this purpose different (SIN) retroviral vectors expressing Col XVII gene have been produced by a qualified contractor (EUFETS GmbH) using an endogenous promoter: EF1α. Preliminary transduction experiments demonstrated that the supernatant generated from the transiently transfected HEK293 with pES.12-6 vector containing *COL17* cDNA had a low efficiency of transduction on keratinocytes. In order to ameliorate the virus production the *COL17* coding sequence was optimized in the splicing sites. With this modification, the viral particles produced by transient transfection of HEK293 are effective in infection process.

This supernatant was tested on JEB keratinocytes and in order to perform keratinocytes transduction at defined Molteplicity Of Infection (MOI) we performed a titration with an immunofluorescence analysis on infected HT1080 cell line. The resulting viral titer is $1.2 * 10^6$ infectious particle/ml.

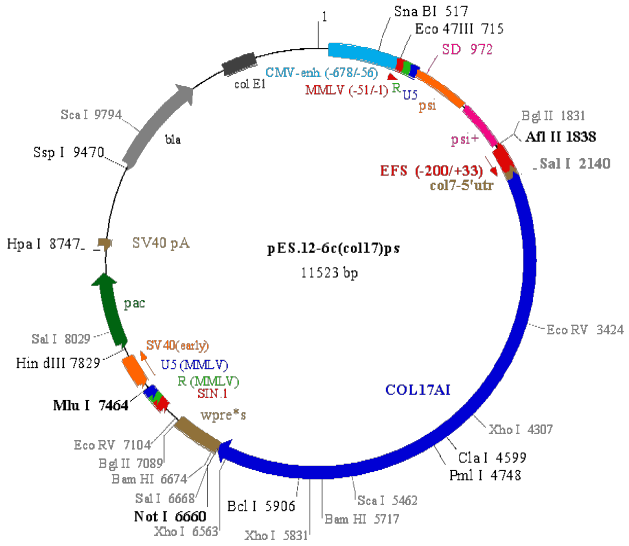


Figure 21. Schematic map of pES.12-6c(col17)ps vector (E1142)

vector elements:

CMV-enh.	Cytomegalus virus immediate early promoter enhancer region
MMLV (-51/-1):	MMLV-core promoter, contains optimized TATA-box
R, U5:	from MoMuLV (wt)
EFS:	Elongation factor-1a short promoter fragment
col7-5'utr:	human collagen 7 AI 5´-Utr (approx 40 bp)
pac:	puromycin resistance gene
pre*s:	**w**oodchuck hepatitis virus **p**osttranscriptional **r**egulatory **e**lement short version with deletion of all atg´s" and the X-protein ragment.
SIN.1:	deletions in MoMuLV-3´LTR: border (-448/-412 border; -74/- TATA-box mutated in core promoter)
SV40(early):	SV40 early promoter

JEB Keratinocytes transduction

Previous results on our laboratory demonstrated the difficulty to transduced keratinocytes with viral supernatant. For this reason, we had to establish an effective transduction protocol and we obtained acceptable results with the centrifugation of SIN-γ-*COL17* viral supernatant on Retronectin coated plates and subsequent plating of keratinocytes and irradieted 3T3-J2 cells (Figure 22A). Three days after transduction, keratinocytes were plated onto a new 3T3-J2 murine feeder layer.

Preliminary results with transient supernatant at different MOI (MOI20, MOI40) gave an efficiency of transduction of 70-80% measured by immunofluorescence (Figure 22B-C), in addition western blot analysis shows the correct expression of the protein in the absence of recombinant forms (Figure 22D). The amount of Col17 protein obtained with SIN-γ-retroviral vector transduction is lower than the one obtained with Am12-Col17 clone. This is probably due to the fact that SIN-γ-

retroviral strategy guarantees one integration per cell and it is much more comparable to a wild type expression.

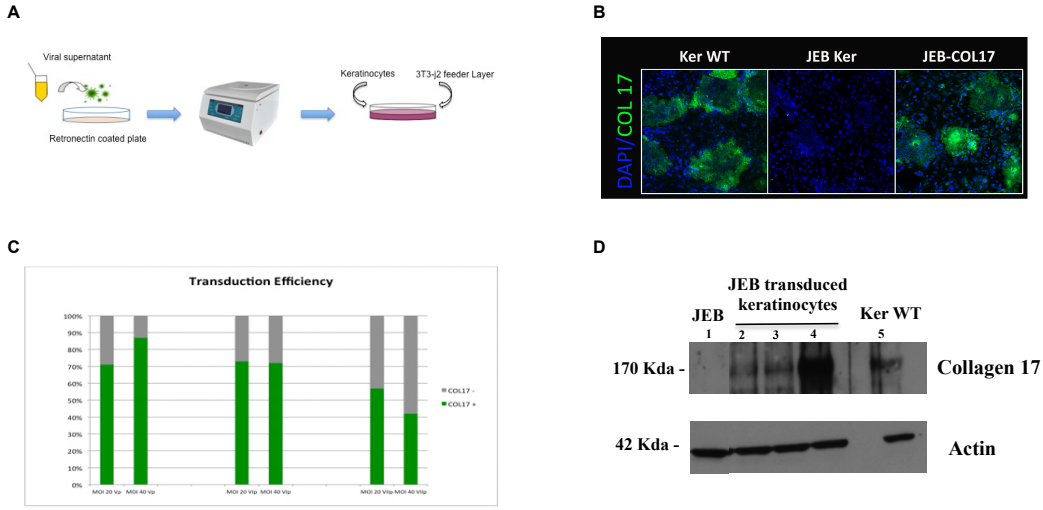


Figure 22. JEB keratinocytes transduction with SIN retroviral vector.

A) Scheme of Infection Strategy. Retronectine coated plates were centrifuged with viral supernatant for one hour. JEB keratinocytes and irradiated 3T3-J2 cells were plated simultaneously after supernatant centrifugation.

B) JEB keratinocytes were transduced as described in A. Immunofluorescence analysis of JEB transduced keratinocytes shows a good efficiency of transduction (about 70% as shown in panel C) with different levels of collagen 17 expression.

C) Transduction Efficiency: percentage of transduced cells were counted on mass colture. High percentage of transduction is preserved two passages after transdcution, the maintenance of high percentage of transduction during passages need to be investigated further.

D) Western blot analysis of JEB-cultured keratinocytes before (lane 1) and after (lane 2-3) transduction with indicate complete reconstitution of Col17 synthesis in transduced cells. In lane 2-3 are shown JEB keratinocytes transduced with pES.12-6Col17 at MOI 20 (lane 2) and MOI 40 (lane 3). E1142 vector efficiently transduced keratinocytes leading to efficient Col17 expression, that was completely absent in JEB ker before transduction (lane 1). Col17 expression levels are lower than in JEB keratinocytes transduced with AM12-Col17 clone (lane 4) because the Col17-SIN-RV has only one viral integration in keratinocytes. Col17 expression is compared to the normal synthesis in wild type keratinocytes (lane 5).

However, we observed a decrease of the Col17+ cells at third passage post transduction. The progressive decrease in Col17 expression is probably due to the low initial efficiency of transduction that is not sufficient to target the keratinocyte stem cell, which are able to maintain the long term expression of the transgene.

In order to address the loss of Col17 positivity during passages we need to performed further experiments with other strains and with a supernatant obtained from a stable SIN packaging cell line clones.
For this reason Eufets GmbH produced a stable SIN packaging line from which different stable clones have been isolated. We performed titration to each clone and selected the best ones to transduce keratinocytes.
The transduction was performed at MOI 20 on JEB keratinocytes but stable clones give rise to results similar to those obtained with transient packaging cell line. JEB transduced keratinocytes expressed Col17 protein in a percentage of 60% measured by immunofluorescence just after the transduction process, however the percentage decreased with passages.

Semian Foamy Virus (SFV) derived vector

Eufets GmbH also proposed to test an innovative retroviral vector with an envelope derived from Semian Foamy Virus (SFV) that is able to infect cells without cationic reagents (like polybrene and protamine sulfate) or adiuvants (like retronectin). This innovation would promote the transduction process and in the contest of gene therapy trial would be revolutionary. In order to address this issue we first use supernatant of SFV vector carring the gene encoding for GFP protein with a titer around $1.9 * 10^6$.
For keratinocytes transduction protocol we performed our experiments varying the number of cells, the time of incubation with viral supernatant, and the condition of infection (in adhesion or in suspension). Good transduction efficiency was reached at MOI 20 with 30.000 keratinocytes maintained in suspension with viral supernanat for 10 minutes. However, this condition assure a 43% of transduction, which is not sufficient to assure stem cell transduction.
In order to ameliorate the efficiency of transduction other tests are on going and once we individuate the best condition we will transduce JEB keratinocytes with SFV-Col17 viral vector.

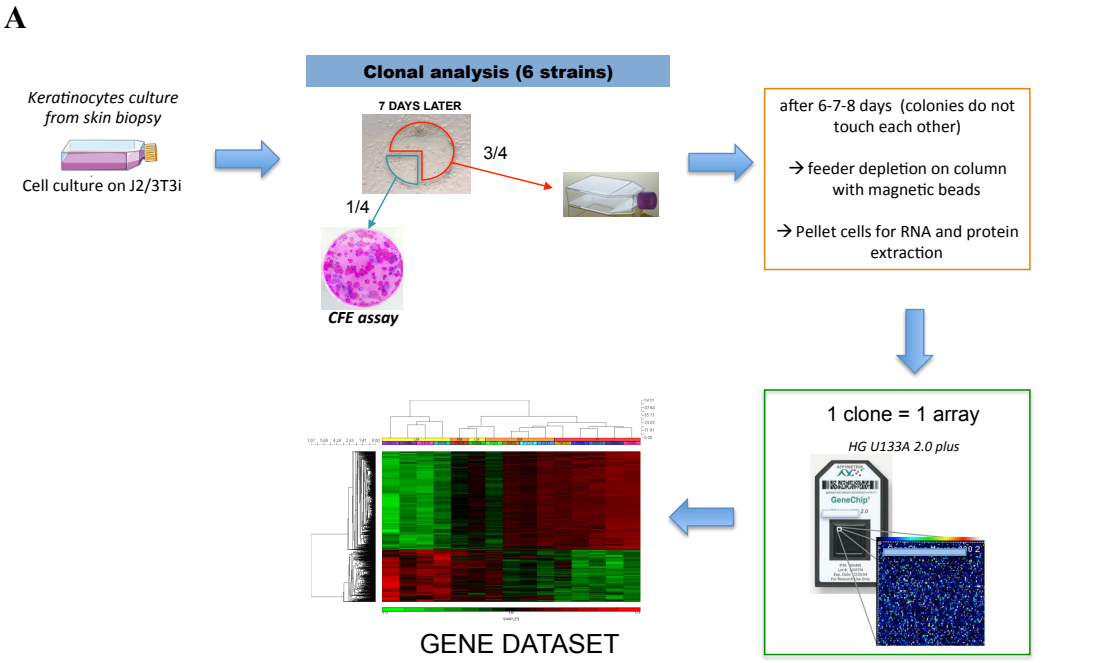
EPITHELIAL STEM CELL CHARACTERIZATION

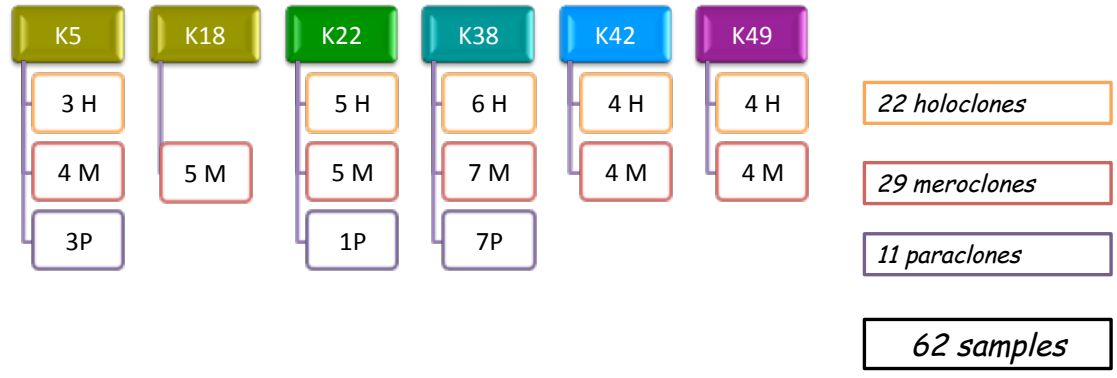
Transcriptional profile of Holoclone differs from that of Meroclones and Paraclones

The long-term restoration of JEB epidermis requires a defined number of transduced epidermal holoclones [1]. However, no epithelial stem cell markers have been identified that allow the prospective isolation and transduction of stem cell in culture. For this reason, the current gene therapy approach expect to reach 100% of transduction efficiency in order to transduce even the small percentage of keratinocyte stem cells present in the culture.

In order to identify KSC markers we decided to investigate the transcriptional profile of the different type of clones isolated from keratinocyte mass culture.

Clonal analysis of a heterogenic keratinocyte culture allows isolating and identifying three different clones in relation to the percentage of abortive colonies: Holoclone (H), Meroclone (M) and Paraclone (P) (Figure 23A). We performed clonal analysis from six strains and we collected RNA and protein from the primary culture derived from 22 H, 29 M and 11 P (Figure 23A1). We validated the identity of different clones also looking at the expression level of p63alpha that is highly expressed in H, partially expressed in M and absent in P (Figure 23B).





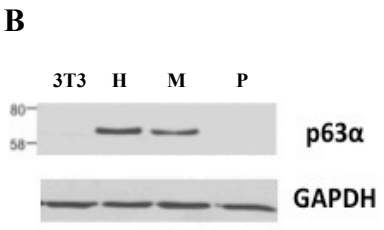


Figure 23. Different clones isolated from clonal analysis to perform microarray experiments.

A) Experimental plan

A1) The samples were collected from six different strains. We obtained 22 H, 29 M and 11 P

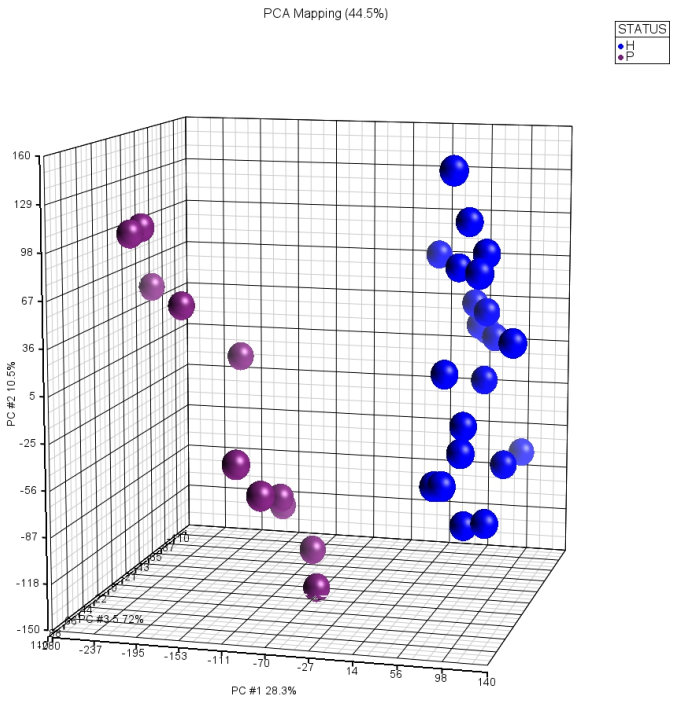
B) p63alpha is highly expressed in H, its expression decreases in M and is undetectable in P.

We used the Affymetrix Human Genome U133 Plus 2.0 array to analyse mRNA obtained from the progenies of different clones. Expression profiling by microarrays has been used so far as a very successful tool for the retrieval of important information about how the transcriptome is deployed in different cell types and how it varies during differentiation.

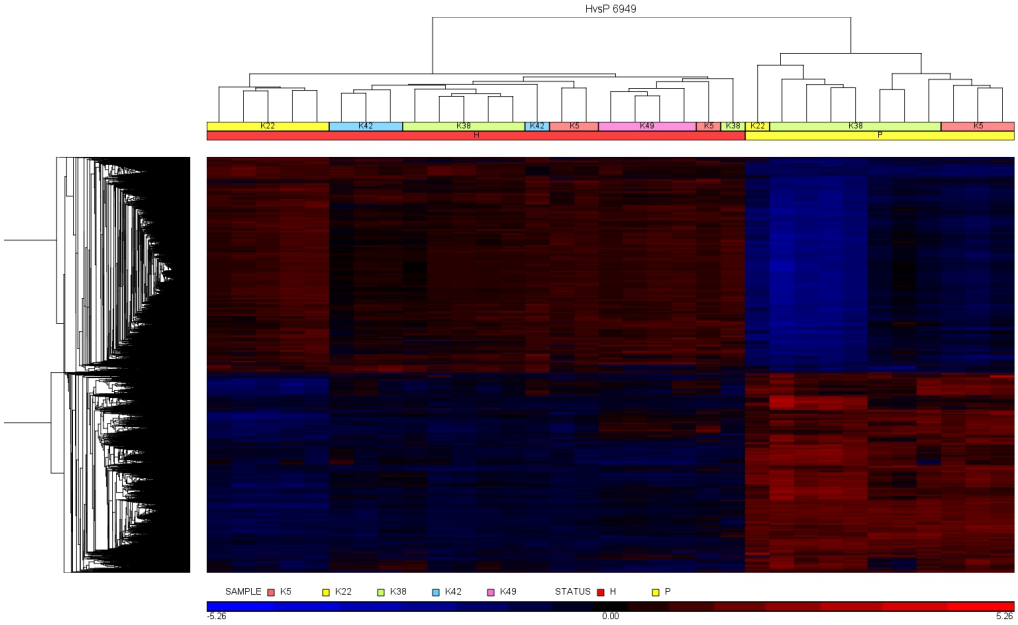
Samples were analysed for their biological status in the Principal Component Analysis (PCA). PCA is a statistical technique for determining the key variables in a multidimensional data set that explain the differences in the observations, and can be used to simplify the analysis and visualization of multidimensional data sets.

As shown in the Figure 24A the Principal Component Analysis show that the transcriptomic profile derived from Paraclone-derived cells (purple dots) is clearly different compared to the trascriptomic profile of Holoclone-derived cells (blue dots) (Figure 24A). From unsupervised analysis (Figure 24B) we can also observe that 6949 genes are differentially expressed between H and P with a p-value threshold set to p< 0,01 and fold-change thresholds (FC >1,5 < -1,5). Among them, 50% are upregulated (red genes) in H and downregulated (blue genes) in P and 50% are downregulated in H and upregulated in P.

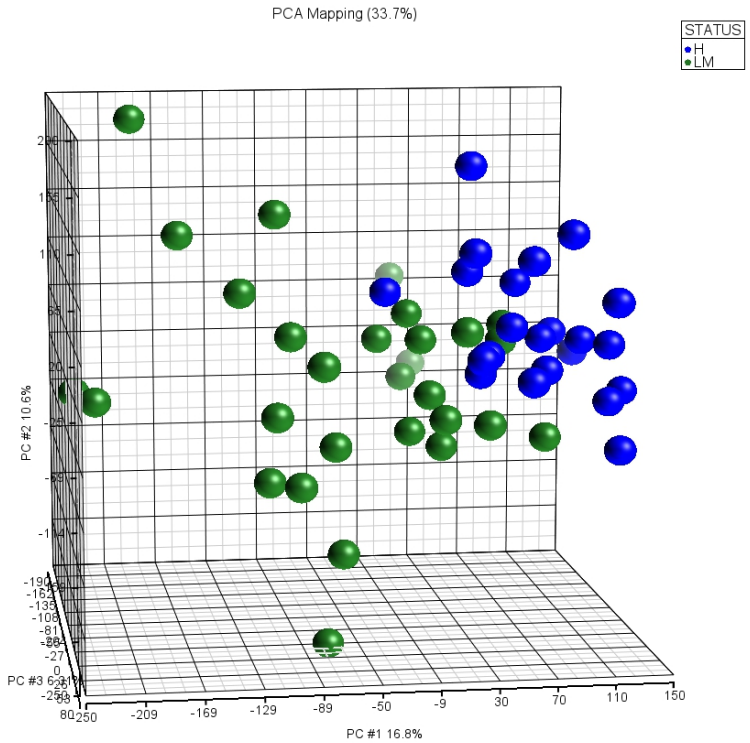
A



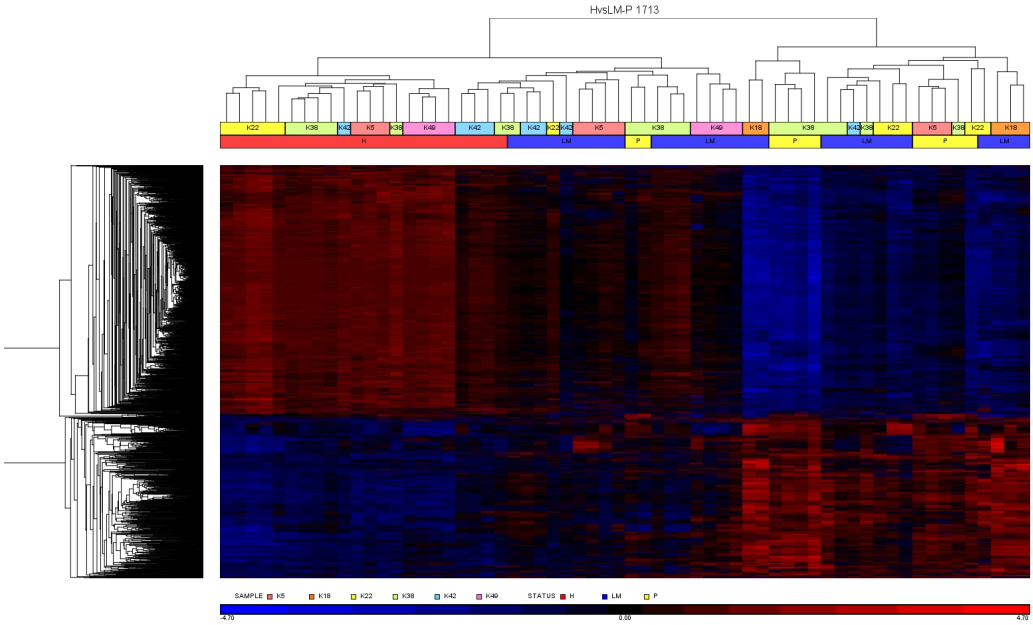
B


HvsP 6949
K22
K42
K38
K42
K5
K49
K5
K38
K22
K38
K5
H
P
SAMPLE K5 K22 K38 K42 K49
STATUS H P
-5.26
0.00
5.26

C


PCA Mapping (33.7%)
STATUS
H
LM
PC #2 10.6%
PC #3 6.3%
PC #1 16.8%
-250
-209
-169
-129
-89
-50
-9
30
70
110
150

D



E

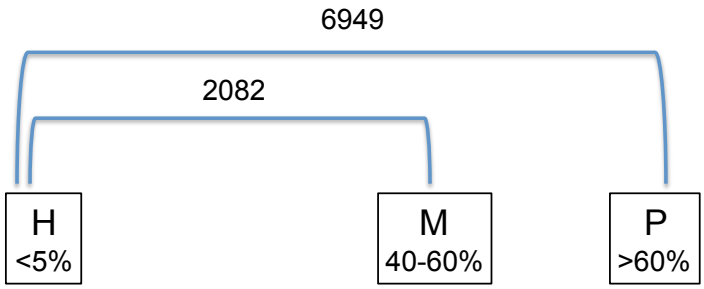


Figure 24. PCA and Unsupervised hierarchical clustering analysis.

A) Principal Component Analysis (PCA) shown the great distance from H (blue dots) and P (purple dots). B) Unsupervised hierarchical clustering analysis performed on arrays of H and P. Red patches represent up-regulated genes, while green patches represent down-regulated genes.

C) Principal Component Analysis derived from three of the strains used in microarray analysis. It shows that H (blue dots) are very different when compared to M (green dots). D) Unsupervised hierarchical clustering analysis performed on arrays of H and M. E) Graphic representation of differential expressed genes in H vs M and H vs P comparisons.

Paraclones are a class of clones that show a very small proliferation potential (only 10-20 doublings in culture) compared to Holoclone (that perform 180 doubling in culture). To verify that these differences are not ascribed only to a different proliferative potential but even to the presence, in Holoclones, of stem cells with self-renewing proprieties, we also compared Holoclone-derived cells with Meroclone-derived cells. Meroclones have a high proliferative potential but they do

not self-renew in vitro. Also the comparison between H and M show a quite different PCA (Figure 24C).

The heat map analysis (Fig. 24D) show that 2082 genes are differentially expressed in H compared to M. Moreover we notice that all 2082 genes are contained in the 6949 genes differentially expressed between H and P (Figure 24E).

Most interesting genes and functions differentially expressed in Keratinocyte Stem Cells

In order to identify the most relevant molecular interactions, functions and pathways differentially expressed in the transition between SC and TA, microarray data were analyzed using the network-based Ingenuity Pathways Analysis tool.

Ingenuity Pathway Analysis (IPA) is a system that transforms a list of genes into a set of relevant networks based on extensive records maintained in the Ingenuity Pathways Knowledge Base (IPKB). This knowledge base has been abstracted into a large network, called the Global Molecular Network, composed of thousands of genes and gene products that interact with each other.

The following class of "upstream regulators" were upregulated in holoclones compared to M and P: FOXM1; CSF2; EP400; Alpha Catenin; MYC; JAG2; MBD2; SATB1; FOXO1; NCOA3

The following cellular functions are differentially expressed in H compared to M and P:

- upregulated in H and downregulated in M e P: S phase, M phase, Interphase, Cytokinesis, DNA Repair, Checkpoint control, Excision repair, Homologous recombination.
- downregulated in H and upregulated in M e P: Necrosis, Apoptosis, Cell death, Cell movement, Migration, Chromosomal instability, DNA damage, Chromosomal aberration, Breakage of DNA.

FoxM1 : PUTATIVE STEM CELL MARKER?

Among these classes of upstream regulators we choose to focus our attention on FoxM1 transcription factor: it results upregulated of 2,9 fold in the comparison H vs M and 4,5 fold in the H vs P; moreover, the pathway containing FoxM1 target genes are upregulated in H vs M (Figure 25A).

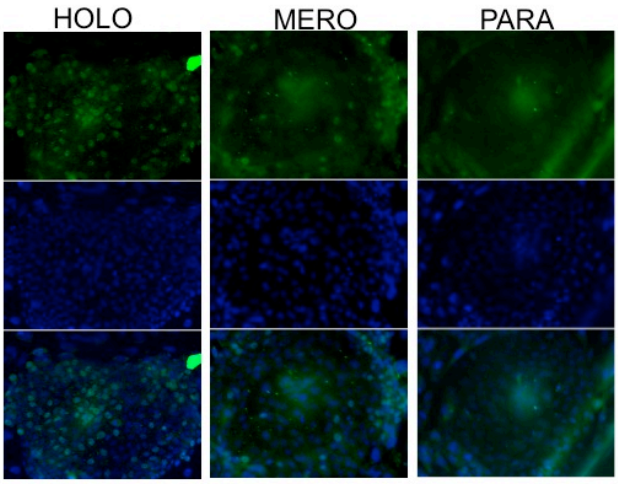
To confirm these bioinformatic data we analysed the protein level expression between the progenies of Holoclones, Meroclones and Parclones by immunofluorescence and western blot analysis. The Immunofluorescence analysis (Figure 25B) revealed in all the Holoclone-derived cells a nuclear expression of FoxM1, whereas in the Meroclone-derived cells only few cells resulted FoxM1 positive and in Paraclone-derived cells none of the cells stained positive. The western blot analysis confirmed this great difference of FoxM1 expression in the three clonal type, because it is expressed only by Holoclones-derived cells and is not detectable in Meroclones and Paraclones derived cells (Figure 25C). In addition, to investigate the localization of Foxm1 *in vivo*, we perfomed an immunofluorescence analysis on skin sections.

Nuclear FoxM1 is mainly expressed at the basal layer of epidermis, often with a clustered pattern. Moreover, FoxM1 co-localizes with p63+ keratinocytes indicating that, also in vivo, FoxM1 is expressed in the stem cells compartment. However, as shown in Figure 25D, not all p63 positive cells express FoxM1, suggesting that FoxM1 could be a more stringent marker of stemness.

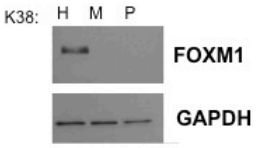
A

	p-value	H vs M	p-value	H vs P
FoxM1	2,39E-12	2,9089226	9,8E-14	4,50643

B



C



D

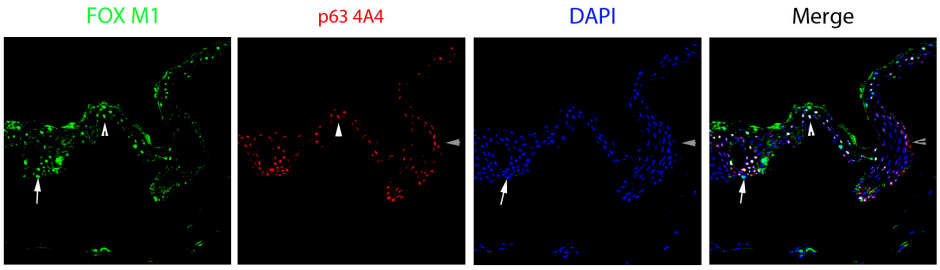


Figure 25. FoxM1 in vivo and in vitro expression on keratinocytes.

A) Foxm1 fold changes in HvsEM, HvsLM and HvsP comparisons. Transcriptional differences are significative in HvsLM and HvsP. B) Immunofluorescence on different clones progenies: Holoclone (H), Meroclone (M) and Paraclone (P). FoxM1 (green) is clearly express by holoclone derived cells and sometimes from Meroclone culture, whereas is completely absent in Paraclone one C) Western Blot analysis of protein extracts from H, M and P derived cells of keratinocyte strain name K38. FoxM1 is expressed only by Holoclones and its expression is normalized on GAPDH expression. D) Immunofluorescence performed on skin section, FoxM1 (green) is located principally at basal layer of epidermis, and p63 (red) has a distribution coherent with litterature. White arrows evidentiate Foxm1 positive and p63 negative cells.

DISCUSSION

Epidermolysis Bullosa represents a devastating rare genetic skin disorder for which there are only palliative cures, that provide temporary relief from the symptoms. Recent advancement in molecular biology and regenerative medicine have fostered new therapeutic approaches for EB. Among these, cell and gene therapy strategies using different viral systems have been developed [1,47,74,75], in order to be applied in clinic. However only one successful clinical trial, using MLV based retroviral vector, for the treatment of a patient affected by Junctional Epidermolysis Bullosa, gave the concrete possibility to definitively treat this genetic disease with an *ex-vivo* genetic approach.

Based on the results obtained with Lamβ3 corrected skin engraftment performed on two different patient [1,40] (Bauer et al. Submitted), we developed an ex vivo gene therapy strategy with an MLV-derived retroviral vector carrying the *COL17* cDNA, to treat JEB patients with mutations on *COL17* gene. The nature of Col17 protein is similar to Lamβ3 since both are structural proteins localized on the basal lamina and their synthesis and assembly is regulated by basal keratinocytes; for this reason it is feasible to develop a genetic therapeutical approach for *COL17* correction, similar to that elaborated for Lamβ3.

Once an appropriate number of cells from an EB patient biopsy has been isolated and cultured in vitro, the transduction of epidermal stem cells in culture is an essential condition in order to obtain a long term skin restoration [1,72].

The isolation of one Col17 Am12 clone able to transduced 90% of keratinocyte population, assure to genetically correct keratinocyte stem cells that normally constitute 5% of cultured keratinocyte[12]. Indeed, we were able to isolate transduced holoclone with a clonal analysis performed on transduced Col17 JEB keratinocytes, and we formally demonstrate that retroviral transduction did not affect stem cell population in culture.

Moreover, we demonstrated that retroviral transduction did not affect the clonogenicity, since transduced cultures showed a life span similar to the control one and in addition the morphology of keratinocytes was not impaired with the transduction protocol.

Regarding the safety in using the Am12 clone 124 we demonstrated a low integration profile in the bulk population of transduced keratinocyte. However other safety assays are necessary in order to exclude the risk of transforming events induced by insertional mutagenesis. In particular, we are testing the ability of transduced cultures to grow in soft agar (soft agar assay), their dependence on the presence of growth factors in culture medium (growth factor dependent assay), and the eventual occurrence of an immortalized/transformed clone (serial passage assay). If we will not observe any risks of insertional mutagenesis in our culture system these preclinical data will be submitted in Salzburg to legal authorities for the authorisation of a phase I/II clinical trial.

However the use of MLV-derived retroviral vectors, where the exogenous gene is under the control of LTR promoter, has raised safety concerns regarding the risk of insertional mutagenesis. Indeed, in a different system, as the hematopoietic cells, the insertion of LTRs in human chromosome could result in endogenous gene dysregulation, as demonstrated in preclinical studies [76] and clinical trials [77].

For this reason, novel retrovirus-based vectors have been developed. They ensure long-term and safe correction of genetic deficiencies in keratinocytes, decreasing the risks of insertional mutagenesis. Therefore, we tested a Self-Inactivating (SIN) γ-retroviral system based on a scalable vector production technology that fulfills the requirements for future clinical applications thus greatly enhancing patient safety and facilitating regulatory processes. The advantages for the use of γ-retroviral SIN vectors are: 1) the robust, scalable production technology of gamma-retroviral vectors (in contrast to lentiviral vectors); 2) the documented safety record of redesigned γ-retroviral SIN vectors; 3) cultured keratinocytes undergo extensive proliferation and are thus easily transducible with this vector system, at a low multiplicity of infection.

The aim of this part of the project was the development of self-inactivated (SIN), γ-retroviral vector carrying *COL17* cDNA in codon-optimized form, under the control of an endogenous promoter. We elaborated a new transduction protocol using viral supernatant centrifuged on Retronectin® coated plates to allow an efficient transduction of keratinocytes. Preliminary results do not show toxicity of the SIN vector on transduced keratinocytes, however we do not obtain a transduction efficiency comparable with the Am12 clone and for this reason it is

necessary to optimize the transduction protocol. Moreover, it is necessary to demonstrate that SIN-retroviral system is able to transduced the keratinocyte stem cells fraction without affecting holoclones and to investigate the safety profile of this new approach.

The possibility to selectively isolate and transduce keratinocyte stem cell fraction, could represent a revolutionary step towards the gene therapy approaches in clinic. For this reason we tried to define a keratinocyte stem cell profile that can be used in clinic in order to allow the identification and -eventually- the selection of a pure population of stem cells for gene correction.

According to the notion that clonal analysis is the only method to isolate a pure population of holoclones [9], we compared the transcriptome of stem cells (H) and transient amplifying cells (M and P). Through bioinformatical analysis of trascriptomic profile, we demonstrate that it is possible to discriminate Holoclones from TA cells. Indeed the transcriptomic profile of Meroclone derived cells is quite different from the transcriptomic profile of Holoclone derived cells. Moreover, these differences are confirmed and even amplified when Holoclone-derived cells are compared to Paraclone-derived cells. These results suggest that stem cells properties gradually decrease toward transient amplifying cells while differentiated genes gradually increase to define a transient amplifying cells. The bioinformatic analysis allows to identify the pathways that are differentially regulated in stem cells compared to transient amplifying cells. Functions like cell cycle progression or DNA repair result upregulated in holoclones, whereas cell motility or apoptosis result downregulated, reflecting some characteristics identified in quiescent stem cell of epidermis and hematopoietic system [78-81].

The transcriptomic profile outlined for holoclones has similarities with stem cell profile observed in other system [82-84], and strongly supports the definition that holoclones is considered the clone derived by an epithelial stem cell.

Among all the genes upregulated in Holoclone we choose to investigate the role of *FOXM1*, a transcription factor involved in cell cycle progression and stem cell maintenance in different systems [52,54,61,67,70]. Indeed, in our analysis both FoxM1 itself and its target genes are upregulated in stem cells compared to TA cells, suggesting a role for FoxM1 in regulating keratinocytes stemness.

Western blot analysis reveals an high level of FoxM1 protein expression in holoclones compared to meroclones and paraclones confirming the bioinformatic

data and suggesting a possible effect of this protein in holoclone-paraclone transition, moreover immunofluorescence analysis shows that holoclone derived cells have a higher nuclear expression of FoxM1, in contrast with paraclones where the expression is almost absent. These observations confirm the microarray analysis and provide evidences of the possible relevant role of FoxM1 in Holoclones respect to paraclone.

In-vivo its expression is restricted to some cell clusters in the basal layer of the epidermis. Moreover we notice that FoxM1+ cells are even p63+, even if many cells p63+ are FoxM1 negative, suggesting a restricted role of FoxM1 in a subpopolation of p63 positive cells, until now the most reliable marker of keratinocytes stem cell [12].

Based on these positive preliminary data, ongoing gain and loss of function studies would be useful in order to define the role of FoxM1 in stem cells and keratinocytes homeostasis. Using shRNA delivered by lentiviral transduction, we are stably deleting FoxM1 protein from keratinocytes in order to observe the effects on the life span of the culture. The suggested role of FoxM1 in stemness maintenance would be demonstrated with a shorter life span of shRNA transduced keratinocyte compared to the untransduced one. Moreover, absence of Holoclones demonstrated with a clonal analysis performed at second and third passage after gene silencing, would demonstrate the requirement of FoxM1 in Holoclones maintenance. On the other hand, the overexpression of *FOXM1*, obtained with lentiviral transduction, would sustained the life span of the FOXM1 transduced keratinocytes culture and favor the maintenance of Holoclone forming cells during passages.

Definitively if FoxM1 would satisfy all these characteristics, it could be a promising epithelial stem cell marker, which could be used to improve the ex-vivo gene therapy approach. The selection of holoclones enriched population could allow to efficiently transduce stem cell population also with viral systems that do not have an high efficiency of transduction but represent an interesting alternative to retroviral vectors for their capacity to integrate in a specific site (Sleeping Beauty Transposon system, Zinc Finger Nuclease, CRISPR-Cas 9) [85-87]. Moreover, the possibility to enrich the initial population in stem cells is essential in those cases in which the cultured graft risk to be lost because of a poor content of stem cells. The continuous evolution of Epithelial stem cell research open new

opportunities in the treatment of severe skin disorders, the development of new gene therapy approaches together with the discovery of new stem cell markers would result in life-saving approaches especially for children affected by different forms of EB.

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