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Combined gene and stem cell therapy approach to correct ocular defects in p63-related diseases

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ABSTRACT

Background

p63 gene is essential for the regeneration of adult epithelia and the maintenance of the proliferative status of basal keratinocytes. Mutations in p63 gene can cause at least five different syndromes (EEC, ADULT, AEC, LMS, and RHS) as well as two non-syndromic disorders (SHFM and NSCL). The three main phenotypic characteristics of p63-associated disorders are: (i) ectodermal dysplasia; (ii) split hand/foot malformation and (iii) orofacial clefting. The progressive keratopathy associated with limbal stem cell failure results in a vascularized pannus, leading to corneal clouding and visual impairment. In patients with bilateral limbal stem cells deficiency, transplantation of cultured autologous oral mucosal stem cells (OMESCs) sheets could represents an effective alternative to reconstruct the ocular surface. However, being genetic disorders, stem cells will have to be genetically modified.

Aim of the study

Allele specific silencing of mutated transcript mediated by siRNAs has yet been seen as potential therapeutic strategy in p63-mutated patients, in order to counterbalance the loss of stem cells (Barbaro et al., 2016). But the limits of siRNAs approach are that it is a transient and mutant-specific correction. So the ultimate outcome of my doctoral work has been to develop permanent gene therapy approaches to correct the genetic defect and to design a therapeutic strategy using the transplantation of this genetically modified stem cells.

Methods

A self-inactivating (SIN) lentiviral vector, with transgene expression (ΔNp63α) driven by cellular promoter with short-range enhancer activity (K14), was produced in order to restore the WT phenotype in mutated cells. It was evaluated if the behavior of transduced mutated cells was comparable with the WT. Luciferase reporter assay was performed to test the capability of p63, in its WT or mutated forms, to activate K14 promoter.

Results

We characterized OMESCs from patients with EEC syndrome and demonstrate a proof-of-principle to correct the underlying genetic defect by allele-specific silencing. It was designed a lentiviral vector similar to that used in junctional epidermolysis bullosa (JEB), modified with WT-ΔNp63α fused EGFP instead of laminin-5, and different cell lines were transduced. The signal of tagged EGFP is restricted in epithelial p63-expressing stem cells, demonstrating the possible use of this kind of vector in EEC syndrome therapy approaches based on genetic correction of oral mucosal keratinocytes.

This approach, by acting on the amount of WT-p63 instead that mutated, has the great advantage of been mutation-independent, potentially useful not only in EEC syndrome but in all disease correlated with p63 mutations. In fact it was tested, by using luciferase assay, the activation of K14 promoter/enhancer by WT- or mutated-ΔNp63α. Results demonstrate that mutated-ΔNp63α are unable to activate the promoter, ability restored by adding increasing amount of WT-ΔNp63α.

So it was tested the ability of this lentiviral vector to act in primary OMESCs. WT and R304Q transduced keratinocytes show an amount of ΔNp63α approximately 2-times higher than negative control, correlates with higher life-span (WT-OMESCs = 10-11 passages; transduced-WT-OMESCs = 12; R304Q-OMESCs = 5-6; transduced R304Q-OMESCs = 7) and CFE assay results.

Discussion and conclusion

Reported important results demonstrate that combined gene and stem cell therapy approaches for reconstructing the ocular surface represents an effective alternative for corneal blindness in mutated-p63 patients. It was demonstrated an improvement in the phenotype of R304Q cells transduced with our vector.

Our hypothesis is that, using a lentiviral vector carrying ΔNp63α, whose expression is driven by K14 promoter/enhancer that is regulated by p63 itself, the increment of p63 would be not too high but presents an expression WT- stem cells like, recovering normal phenotype.

Abbreviations used in the thesis

ADULT: acro-dermato-ungual-lacrimal-tooth; AEC: ankyloblepharon-ectodermal defects-cleft lip/palate; DBD: DNA binding domain; EEC: ectrodactyly, ectodermal dysplasia, clefting; HSs: DNase I hypersensitivity regions; JEB: Junctional epidermolysis bullosa; K14: keratin-14; LMS: limb mammary syndrome; LESC: limbal epithelial stem cell; LSCD: limbal stem cell deficiency; NSCL: non-syndromic cleft lip; RHS: Rapp-Hodgkin syndrome; OMESC: oral mucosal epithelial stem cell; mucosal; qPCR: quantitative PCR; SAM: sterile α motif; siRNA: short interfering RNA; SHFM: isolated split hand/foot malformation;

INTRODUCTION

p53 superfamily

p63 is the most recently discovered but most ancient member of the p53 superfamily (A. Yang et al., 1998). Human p53 is translated from a single mRNA with a single open reading frame. Its homologs p63 and p73 both share the hallmark features that identify p53 across all species – an acidic, N-terminal transactivation (TA) domain, a central highly conserved core DNA-binding domain (DBD), and a C-terminal oligomerization domain (OD) (Ko and Prives, 1996; Levine, 1997) (Figure 1). At sequence level, p63 and p73 are more similar to each other than each is to p53, suggesting the possibility the ancestral gene is a gene resembling p63/p73, while p53 is phylogenetically younger (Kaghad et al., 1997; A. Yang et al., 1998).

Unlike p53 gene, which encodes essentially one major transcript, each of p63 and p73 genes contain two separate promoters that direct expression of two fundamentally different classes of transcripts:

- TA p63/p73 containing the N-terminal transactivation domain;
- ΔNp63/p73 lacking the acidic TA domain, which is derived from an alternative promoter and initiation codon in intron 3 (A. Yang et al., 1998).

Because the ΔN isoforms retain the ability to bind to DNA, it was originally proposed that they act primarily as dominant-negative inhibitors of the transcriptionally active p53 family members, preventing apoptosis and cell cycle arrest (Bénard et al., 2003; Murray-Zmijewski et al., 2006). However, the presence of alternative transactivation domains indicates that the ΔN proteins directly regulate gene expression (Helton et al., 2006; Kouwenhoven et al., 2010; Viganò et al., 2006).

Both TA- and ΔN- transcripts can be alternatively spliced at the carboxy-terminus, leading to α, β, and γ isoforms. Thus both p63 and p73 genes encode at least 6 isoforms,

depending on specific alternative promoter usage and alternative splicing (Augustin et al., 1998; A. Yang et al., 1998).

While p53 behaves as a canonical tumor suppressor gene, both p63 and p73 play a major role in ectodermal differentiation and neurogenesis, respectively (Strano et al., 2001).

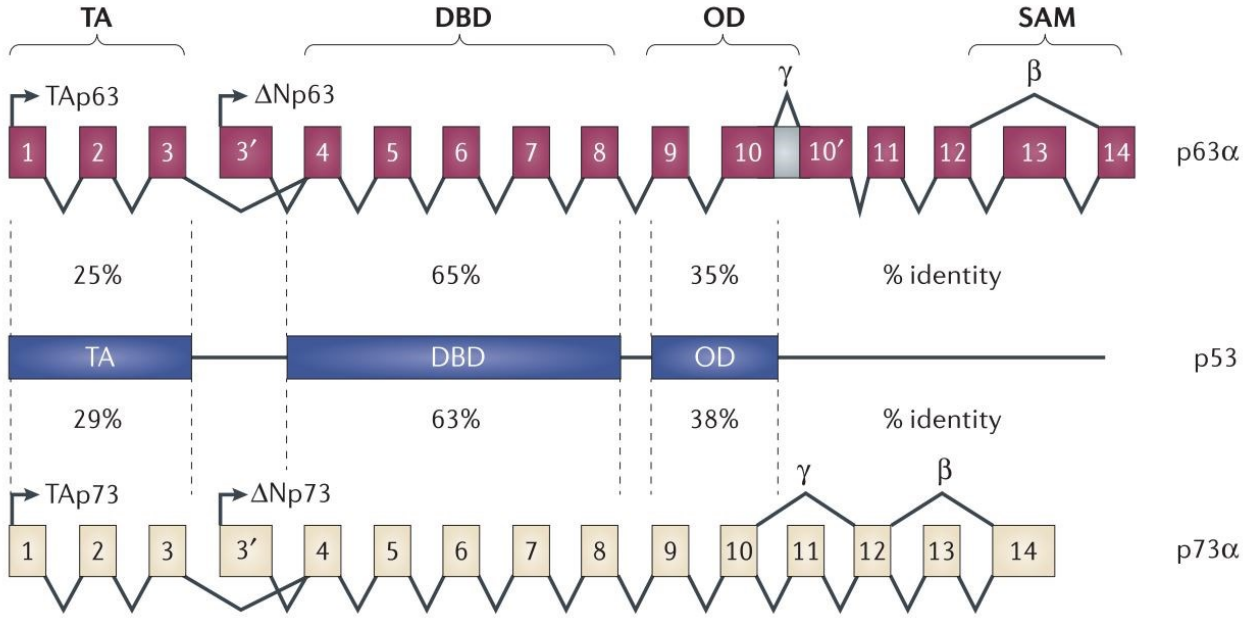

Figure 1
p53 superfamily members. It was reported percentages of homology among p53 and p63/p73 domains.

p63

<u>p63 structure</u>

p63 gene is located on long arm of chromosome 3 (3q27-29) (Figure 2A). It comprises several types of domains, depending on the isoform (Moll and Slade, 2004) (Figure 2B):

- TA: transactivation domain. For a long time the ΔNp63 isoform that does not contain the TA domain was thought to be a dominant repressor. It has been shown however that ΔNp63 also exhibits transactivation activity and contains:

a. $TA^{\Delta N}$: an alternative activation domain at N-terminus (Bénard et al., 2003; Murray-Zmijewski et al., 2006) and
b. TA2: another activation domain positioned between aminoacids 410 and 512, and encoded by exon 11 and 12 (Ghioni et al., 2002). This further activation domain is found in α and β isoforms, both of TA- and ΔNp63;

- DBD: DNA binding domain, present in all isoforms and crucial for the binding to the DNA;
- OD domain: oligomerization domain found in each isoform, it's essential for the di-/tetramerization of p63;
- SAM: sterile α motif, located at the C-terminus, known to be important for the protein-protein interaction, and it's present only in the longest α isoforms;
- TI: transactivation inhibitory domain, encoded by exon 13 and 14 and found only in α isoforms, it's able to block transactivation by masking few residues of the N-terminal TA domain intra-molecularly (van Bokhoven and Brunner, 2002).

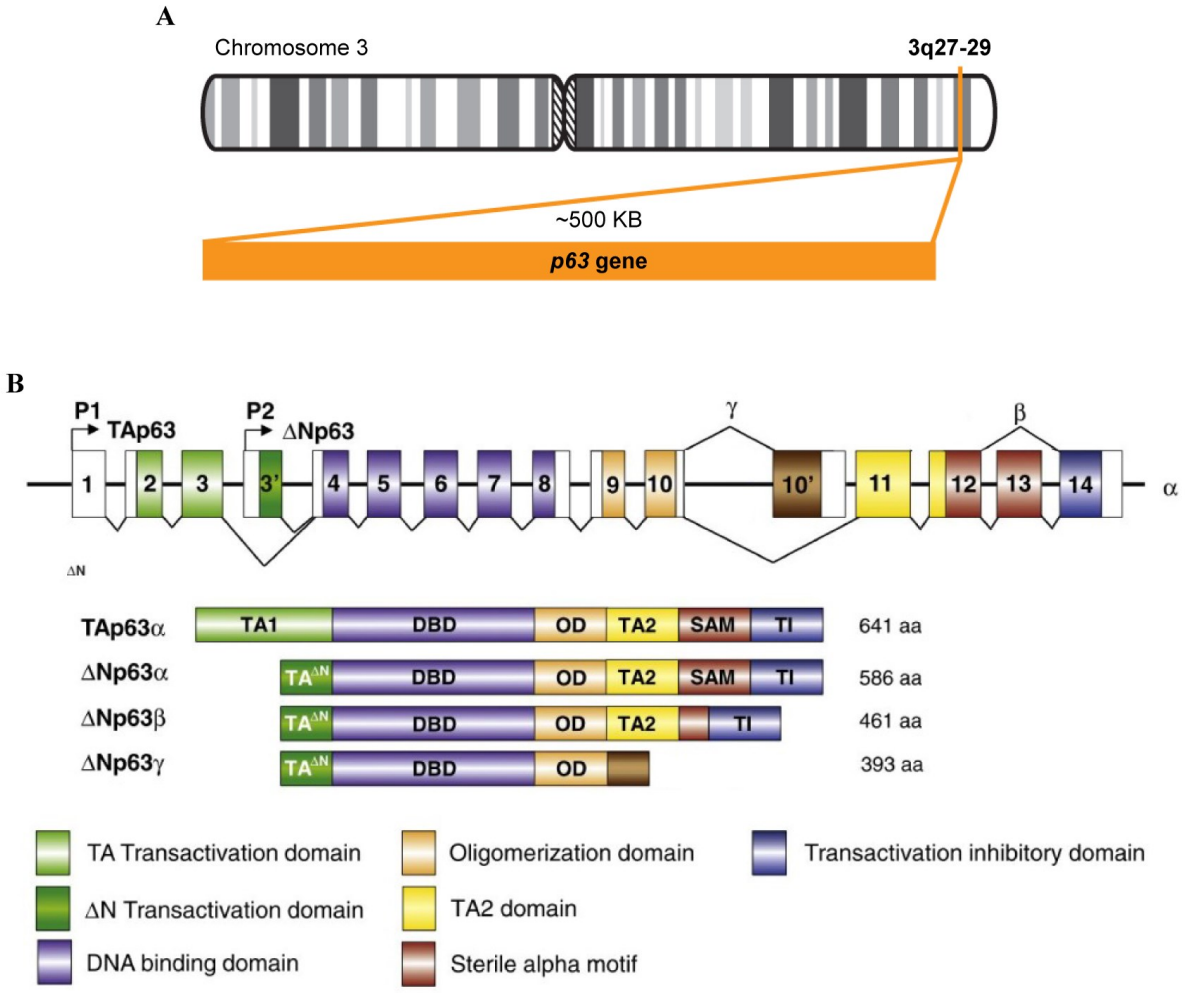

Figure 2
(**A**) p63 gene located at 3q27-29. (**B**) Exon schema and corresponding domains of the p63 gene. Alternative promoter use produces TA (transactivation) and ΔN (N-terminally truncated) isoforms, and alternative splicing produces C-terminal variants (α, β and γ). Alternatively spliced form of exon 3 and 10 are designated as 3' and 10' respectively.

All p63 proteins encode the DBD, which is approximately 65% identical at the aminoacid level to the DNA-binding domain of p53 (and 85% to the DBD of p73), and the OD with about 35% identity to that of p53 (60% to that of p73) (A. Yang et al., 2002). The TA domain of p63 and p53 share 25% of aminoacid sequence, while TA domain of p63 and p73 have 40% of similarity (Figure 1).

Some p53 target genes including Bax, MDM2 and p21 can be transactivated by TAp63 isoforms (Jost et al., 1997; A. Yang et al., 2002).

TAp63α and ΔNp63α contain also the SAM and TID domains (Mangiulli et al., 2009).

p63 binds to DNA as either a homo- or heterotetramer, with isoform composition of the tetramer possibly determining transactivation activity. p63 may also form mixed dimers or tetramers with p73 at relatively higher affinity than with p53, suggesting functional cross talk to regulate transcriptional activity (Davison et al., 1999; Natan and Joerger, 2012). p63 appears to form a dimer of dimers, with monomers consisting of a β-strand followed by two helices (H1 and H2) that adopt a Z-shaped double-hairpin conformation with little intramolecular contact between structural elements. Monomers dimerize via intermolecular antiparallel β-sheet interactions and antiparallel packing of the H1 helices, with important hydrophobic contacts made by key leucine, valine, tyrosine, methionine, and isoleucine residues. Tetramers are formed by hydrophobic H1-H1 interactions and H2-mediated contact where the H2 helices from the primary dimer clasps the adjacent dimer, packing the tetramer in an orthogonal fashion via H1 helices arrangement (Natan and Joerger, 2012). Analysis of p63 DBD shows higher similarity to that of p73 than p53, and appears to bind a 10 bp DNA sequence containing a "CATG" motif with A/T-rich flanking regions (C. Chen et al., 2011).

p63 expression

p63 is expressed in the surface ectoderm (in embryonic stem cells) during embryogenesis and in adult tissues it is restricted to the epithelial stem cells. Immunohistochemistry of p63 often shows strong nuclear-localized staining in basal epithelial cells. Additionally, TA and ΔN isoforms appear to be differentially expressed in particular tissue types. TAp63 variants are prevalent in the heart, testis, kidney, thymus, brain, and cerebellum. ΔNp63 transcripts are detected heavily in epithelial cells, kidney, spleen, cornea and thymus, but not in the heart, liver, testis, or brain (Dötsch et al., 2010; A. Yang et al., 1998).

p63 function

Unlike p53, which has distinct and measurable effects on a cellular level, p63 appears to have observable effects primarily at tissue level. p63 acts as a motif-specific transcriptional activator or repressor (A. Yang et al., 1998). It plays a critical role in the maintenance of progenitor-cell populations that encourage epithelial development and morphogenesis (Romano et al., 2009), and it is the gene with the most striking effects on the development of stratified epithelia (Koster et al., 2004; Mills et al., 1999; A. Yang et al., 1998, 1999). Ablation of the p63 gene in mice results in the absence of these epithelia (Mills et al., 1999; A. Yang et al., 1999). In particular, p63−/− mice show major defects in limb and craniofacial development, as well as a striking absence of stratified epithelia.

This could be explained both by the inability of p63−/− ectoderm to develop into epithelial lineages (Mills et al., 1999) and by the lack of stem cell features necessary to sustain epithelial morphogenesis and renewal (A. Yang et al., 1999). In humans, mutations of the p63 gene cause disorders of the epithelia and of nonepithelial structures whose development depends on the epithelial functions (Rinne et al., 2007; van Bokhoven and McKeon, 2002).

ΔNp63, once thought to serve as a dominant negative regulator due to its lack of a full TA domain, has recently been implicated in transcriptionally activating and repressing target genes such as keratin 5 and keratin 14 to dictate early epithelial development and determine keratinocyte cell fate and lineage choices (Romano et al., 2009; A. Yang et al., 1998). ΔNp63 sustains the keratinocyte proliferative potential (Parsa et al., 1999) that is characteristic of stem cells. Yet, the expression of p63 by the majority of basal cells and by suprabasal cells, as assessed by the 4A4 antibody recognizing all p63 isoforms, has been considered too broad to be stem cell specific (Kaur et al., 2004; Sun and Lavker, 2004).

p63 and cancer

p53 is the most commonly mutated gene in human cancer, supporting its role as a crucial tumor suppressor; in contrast, the p63 gene is very rarely mutated in human tumors or cancer cell lines. p53 tumor suppressor gene properties is due to its DNA binding and transactivation of target genes which specify cell cycle and apoptosis. TAp63 protein, when over-expressed in human cells, also binds to p53 target gene, and induces cell cycle arrest, differentiation and apoptosis in a p53-like manner. (Jacobs et al., 2005; Mills, 2006).

ΔNp63 isoforms, unlike TAp63 isoforms which acts as tumor suppressors, can act as oncogenes (Lee et al., 2006). In fact, ΔNp63α is overexpressed in several epithelial cancers, often as a result of gene amplification (Choi et al., 2002; Hibi et al., 2000; Park et al., 2000). It is hypothesized that ΔNp63α promotes the survival and maintenance of proliferative capacity of both epithelial stem cells and cancer cells.

Otherwise, it was demonstrated that TGFβ-dependent cell migration, invasion and metastasis are opposed by p63 (Adorno et al., 2009). A subset of mutant forms of p53 directly interact (at level of core domain) with p63, thus the p63 transcriptional functions are antagonized (Gaiddon et al., 2001). In epithelial tumors, in presence of high level of p63 the epithelial-mesenchymal transition (EMT) doesn't occur, while loss of p63 specifically promoting the emergence of metastatic cancers in stratified epithelia (Flores et al., 2005).

p63 expression in the cornea

Keratinocyte stem cells govern the renewal of squamous epithelia by generating transient amplifying cells (TA cells) that terminally differentiate after a limited number of cell divisions (Fuchs and Raghavan, 2002; Gambardella and Barrandon, 2003; Potten, 2004; Watt, 2001). Corneal stem cells (keratinocytes) are segregated in the basal layer of the limbus, which is the zone encircling the cornea and separating it from the bulbar conjunctiva. The limbus contains radially-oriented fibrovascular ridges

known as the palisades of Vogt. TA cells that migrate from the limbus form the corneal epithelium (Sun and Lavker, 2004) (Figure 3).

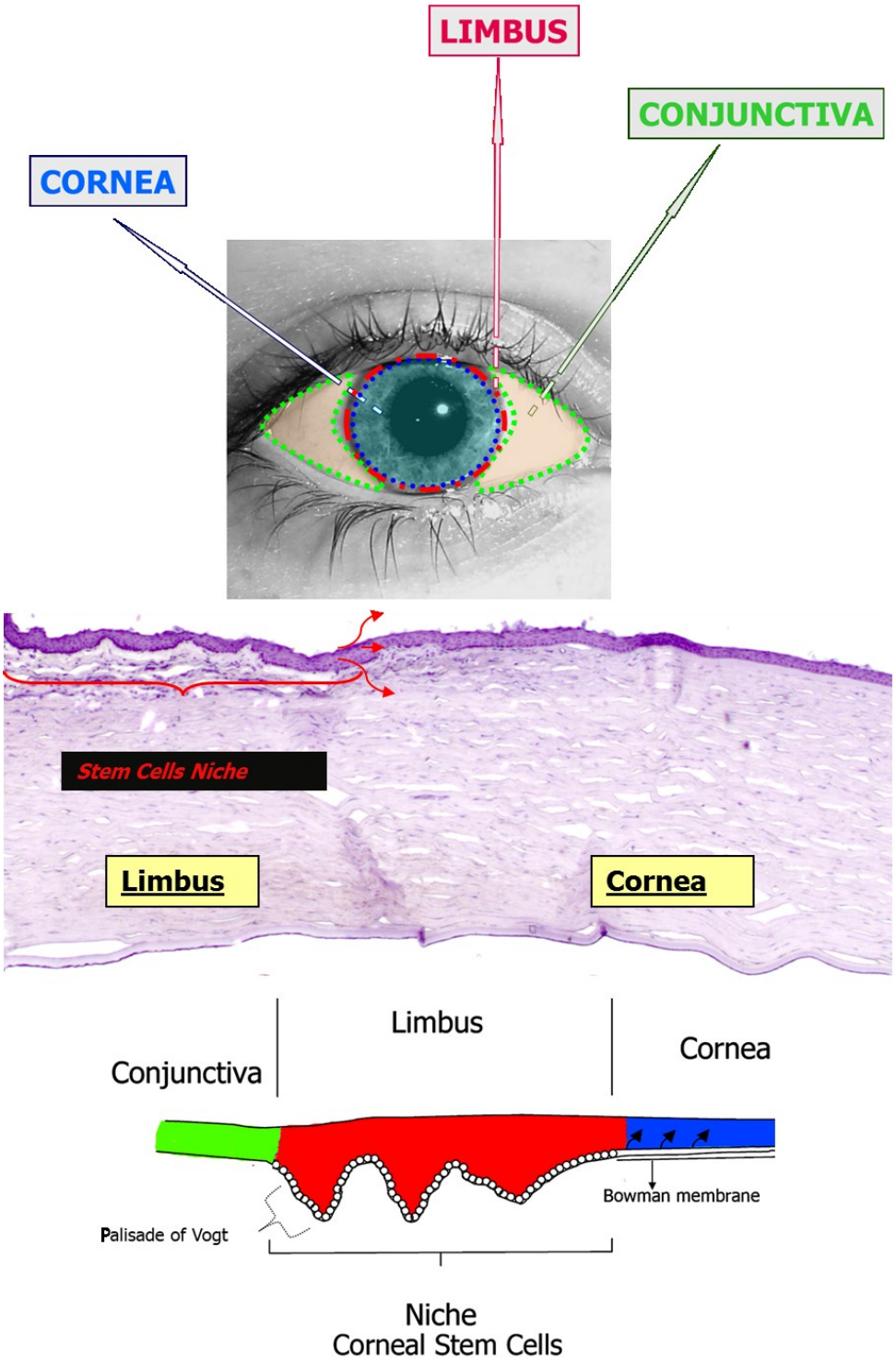

Figure 3
Corneal stem cells (keratinocyte) are segregated in the basal layer of the limbus, which is the zone encircling the cornea and separating it from the bulbar conjunctiva. The limbus contains radially-oriented fibrovascular ridges known as the palisades of Vogt. TA cells that migrate from the limbus form the corneal epithelium.

Clonal analysis of limbal epithelial cells show different types of cells (Figure 4) such as:

- holoclones: putative stem cells with a diameter of 6-10µm. These cells have a high proliferating capability with ≤ 5% aborted colonies and ≥ 100 cell doublings;
- meroclones: young transient amplifying cells with intermediate proliferating capacity having a diameter of 10-18 µm. These cells usually have 5-95% aborted colonies;
- paraclones: terminally differentiated cells with 15-20 cell doublings and very low proliferative capability. These cells are 18-36 µm long in diameter.

Human keratinocyte stem and TA cells, when isolated in culture, generate holoclones and meroclones, respectively (Barrandon and Green, 1987; Pellegrini et al., 1999; Rochat et al., 1994).

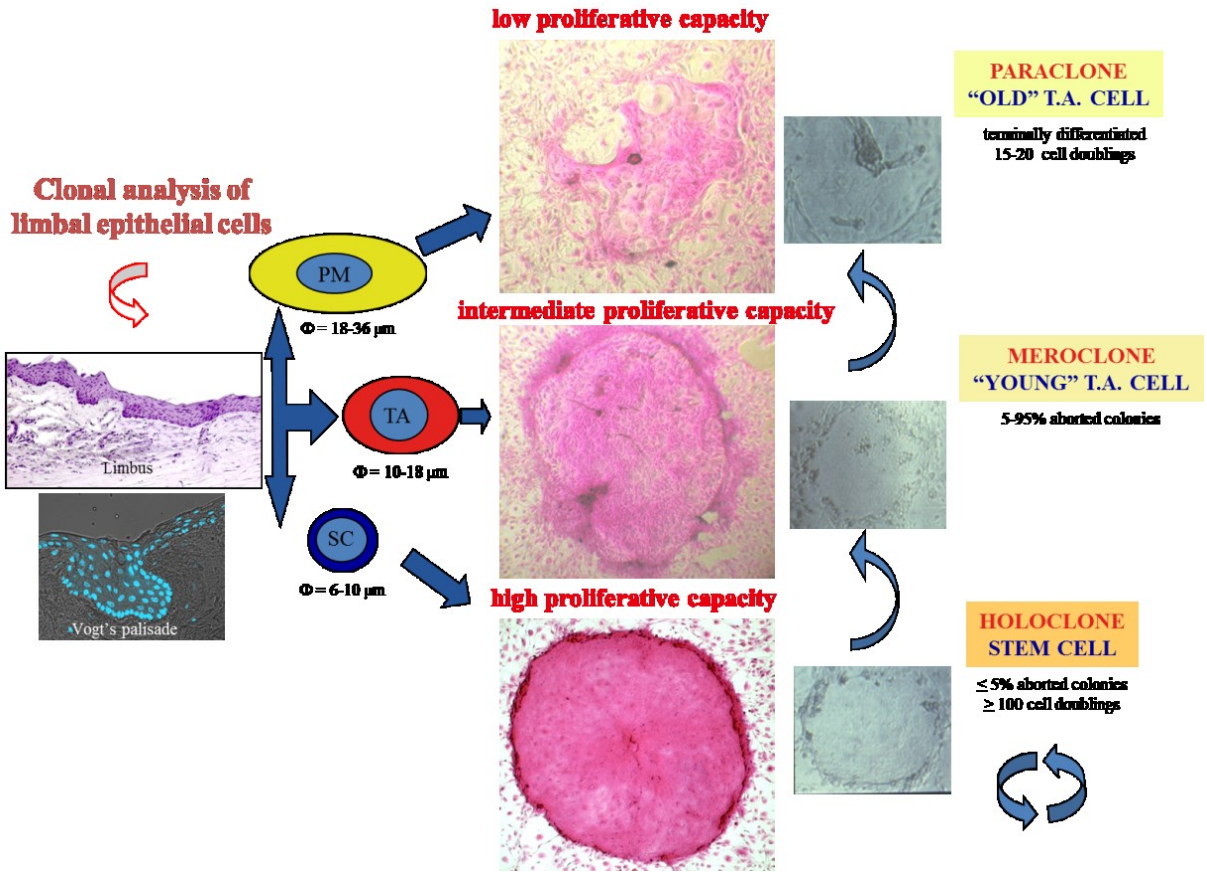

Figure 4
Clonal analysis of limbal stem cells (Barbaro et al., 2013).

That the limbus is the site of stem cell precursors of the corneal epithelium is clear for several reasons:

- the basal layer of the limbus lacks keratin 3 (a marker for corneal differentiation), whereas limbal suprabasal layers and all layers of the corneal epithelium express keratin 3 (Schermer et al., 1986);
- the limbus contains slow-cycling cells and holoclone-forming cells, but the corneal epithelium does not (Cotsarelis et al., 1989; Pellegrini et al., 1999);
- the corneal epithelial cells are not self-sustaining; they divide only a few times during their migration from the limbus to the central cornea (Lehrer et al., 1998);
- restoration of destroyed limbal/corneal epithelium requires limbal transplantation (Kenyon and Tseng, 1989) or grafts of autologous limbal cultures (Pellegrini et al., 1997; Rama et al., 2001; Tsai et al., 2000).

Depending on the conditions, limbal and corneal keratinocytes may contain all three ΔN isoforms. In the uninjured surface of the eye, ΔNp63α is present in the limbus but absent from the corneal epithelium. ΔNp63β and ΔNp63γ appear upon wounding and correlate with limbal cell migration and corneal regeneration and differentiation (Di Iorio et al., 2005) (Figure 5).

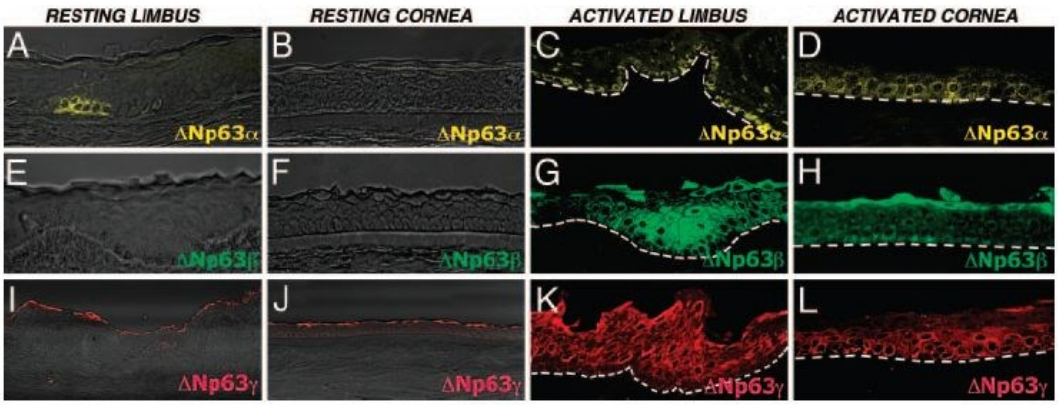

Figure 5
Cytological detection of ΔNp63α (**A**-**D**), β (**E**-**H**), and γ (**I**-**L**) transcripts in resting (unperturbed: **A**,**B**; **E**,**F**; **I**,**J**) and activated (wounded: **C**,**D**; **G**,**H**; **K**,**L**) limbal (**A**, **C**, **E**, **G**, **I** and **K**) and corneal (**B**, **D**, **F**, **H**, **J** and **L**) epithelia (in situ hybridization with antisense digoxigenin-labeled cRNAs specific for each of the ΔNp63 isoforms). Note the abundant expression of β and γ transcripts in activated but not in resting epithelia. The dotted line underlines the basal layer of activated limbal and corneal epithelia (Di Iorio et al., 2005).

The finding that p63 is specifically expressed by stem cells of human epidermis and limbal epithelium strongly suggests that the phenotype of p63−/− mice should be ascribed to a failure to maintain stem cells rather than to the inability of p63−/− ectoderms to form epithelial lineages during development. p63 is essential for regenerative proliferation in epithelial development, distinguishes human keratinocyte stem cells from their TA progeny, is expressed by the basal cells of the limbal epithelium (but not by TA cells covering the corneal surface), and is abundantly expressed by epidermal and limbal holoclones, but undetectable in paraclones (Di Iorio et al., 2005; Nylander et al., 2002; Pellegrini et al., 2001; Thurfjell et al., 2004). In human corneal epithelia, ΔNp63α is the major p63 isoform expressed and it is necessary for the maintenance of the proliferative potential of limbal stem cells and essential for regenerative proliferation in the ocular surface (Di Iorio et al., 2005). Limbal-corneal keratinocytes express not only ΔNp63α but also the ΔNp63β and ΔNp63γ isoforms. However, while expression of ΔNp63α is restricted to the limbal stem cell compartment, the expression of ΔNp63β and ΔNp63γ correlates with limbal cell migration, corneal wound healing and corneal differentiation (Di Iorio et al., 2006, 2005). ΔNp63α is expressed in a small amount of undifferentiated and small cells (stem cells). The percentage of these cells in primary cultures ranges between 3% and 8% and decreases progressively both during clonal conversion (the transition from holoclones to meroclones and paraclones) and serial propagation of stem cell in vitro (life-span).

p63-related disorders

Mutation in p63 gene can cause at least five different syndromes:

- EEC→ ectrodactyly, ectodermal dysplasia, clefting syndrome (OMIM 604292);
- AEC → ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (OMIM 106260);

- LMS→ limb mammary syndrome (OMIM 603543);
- ADULT→ acro-dermato-ungual-lacrimal-tooth syndrome (OMIM 103285);
- RHS→ Rapp-Hodgkin syndrome (OMIM 129400);

as well as two non-syndromic disorders:

- SHFM→ isolated split hand/foot malformation (OMIM 605289);
- NSCL→ non-syndromic cleft lip (Figure 6A).

The three main phenotypic characteristic of p63-associated disorder are:

- Ectodermal Dysplasia: the abnormal development or growth of tissues and structures from the outer embryonal layer (the ectoderm). This results in skin, hair, teeth, nails and several exocrine glands, such as sweat and sebaceous glands, abnormalities. In this condition the epidermis can be very itchy and hypopigmented, the amount of scalp and body hair is often diminished and hair can be wiry or curly. The number of teeth can be less than in health individual, that means that is a reduced number of teeth placodes. Nails are often dystrophic, thickened and discolored. The absence or reduced amounts sweat glands leads to a diminished perspiration, which can be life-threatening. Lacrimal duct defects and obstructions of the lacrimal duct impose a risk for conjunctivitis and corneal damage (Koul et al., 2014).
- Split hand/foot malformation: hands and feet are often malformed and have severe median cleft in the palm and/or in the sole. The clef usually occurs in conjunction with a lack of one or more central (2-3-4) digits, which is called ectrodactyly, it can be observe in hands and/or in feet (Figure 6B) (Kouwenhoven et al., 2015).
- Orofacial clefting: seen in the form of lip clefting or cleft palate, usually observed as part of a complex syndrome where other organs are also affected (Figure 6B) (Rinne et al., 2007).

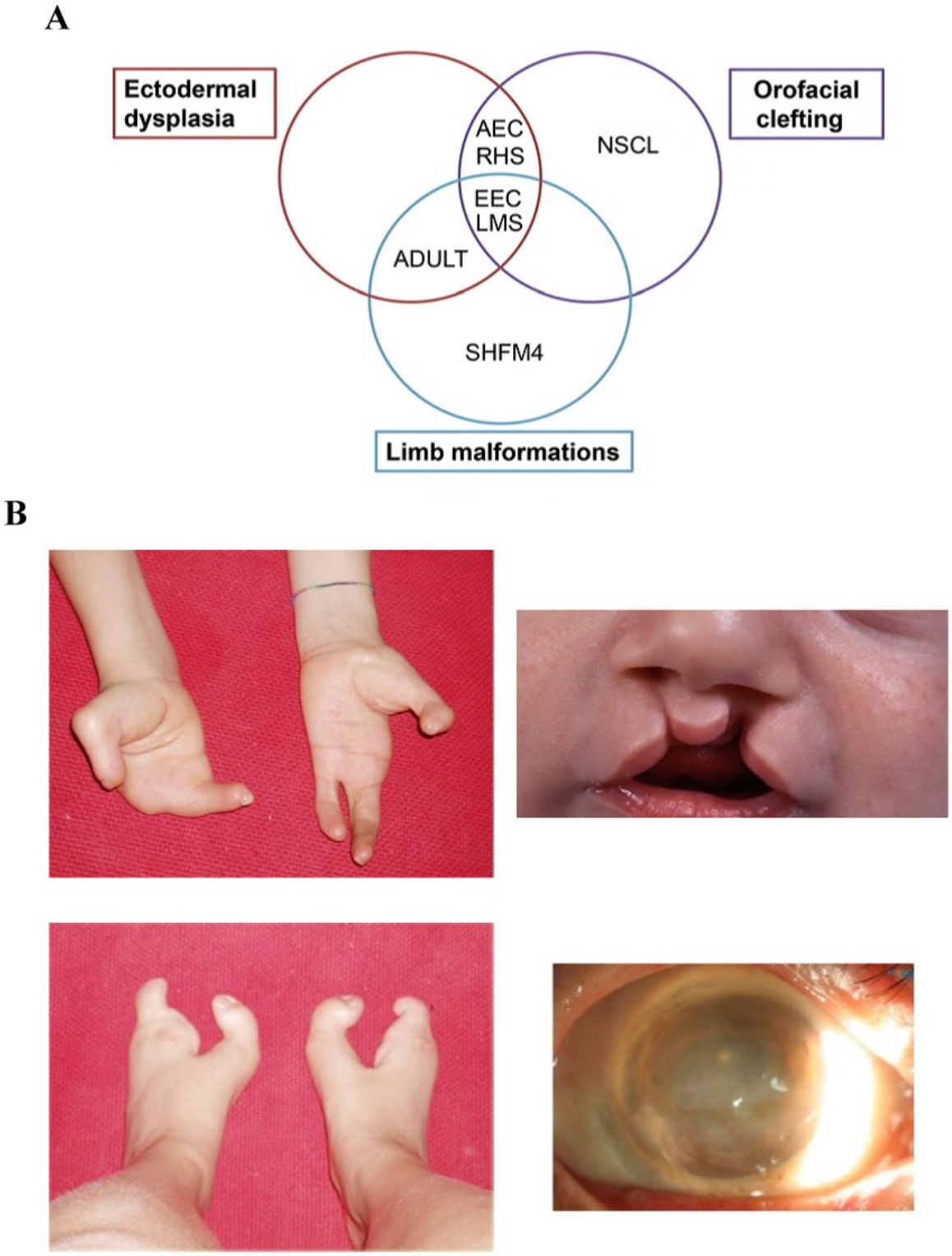

Figure 6
(**A**) Various combinations of ectodermal dysplasia, orofacial clefting and limb malformations are the hallmark of p63-associated syndromes. (**B**) EEC syndrome is the prototype of these syndromes and shows all three hallmarks and conjunctivalization.

EEC

The Ectrodactyly - Ectodermal dysplasia - Clefting (EEC) syndrome is a rare autosomal dominantly inherited disease with highly variable expression and reduced penetrance, characterized by multi-organ dysfunctions including ectrodactyly (split-hand-foot malformation), ectodermal dysplasia, facial clefting (mostly in the form of cleft lip and palate) and urogenital abnormalities (Koul et al., 2014; Nardi et al., 1992; Roelfsema and Cobben, 1996). It affects the skin, nails, hair, teeth, sweat glands and the ocular ectodermal derivatives (Figure 6B). Hypohidrosis is variable, the hair is sparse, fair and dry and eyebrows and lashes are often absent. The teeth are small and may be partially formed, and hypodontia and anodontia occur. The nails are thin, brittle and ridged, and tear duct abnormalities are common. Developmental abnormalities of the meibomian glands and lacrimal glands are present at birth.

While in childhood clefting and hand deformities are the main clinical features, during early adulthood ocular problems become the predominant clinical aspect of EEC syndrome (Käsmann and Ruprecht, 1997). Patients often show ocular surface alterations such as recurrent blepharitis and conjunctivitis, superficial microlesions of the cornea, spontaneous corneal perforation and ulceration, defective regeneration and poor reepithelialization following trauma or penetrating keratoplasty (PK).

EEC patients undergo to a progressive degeneration of the corneal epithelial tissue, generally reaching a peak in the third decade, leading to defective limbal function produced corneal ulceration and subsequent corneal neovascularization and/or scarring followed by corneal opacity and poor vision. Limbal stem cell deficiency (LSCD) of EEC patients is characterized by the absence of the limbal palisade, indicating extensive conjunctivalization and complete loss of stem cell from the limbus (Rinne et al., 2007). Progressive keratopathy with a dense vascularized corneal pannus leading to irreversible blindness (Mawhorter et al., 1985). The visual impairing was associated with advancing age but there is no apparent relationship between the limbal stem cell failure and severity of EEC (Di Iorio et al., 2012).

Alterations leading to these symptoms are:

- the atresia of the lacrimal duct system and the absence of meibomian glands causing tear film instability;
- the epithelial defects of the cornea.

While tear film instability and dry eye symptoms can be overcome with tear supplements, despite all the knowledge accumulated no curative treatments are currently available for the epithelial defects; current ones are only palliative.

The EEC syndrome is mainly caused by point mutations in the DNA binding domain of the p63 gene. Thirty-four different mutations have so far been reported, and at least 20 different amino acids are involved. Only 2 mutations are outside the DBD: one insertion (1572 ins A) and one point mutation (L563P) in the Sterile α-Motif (SAM) domain (Celli et al., 1999; Rinne et al., 2007). Five frequently mutated amino acids (R204, R227, R279, R280 and R304) were found in the EEC population, all located in the CpG islands (Hamada et al., 2002; Rinne et al., 2007). These 5 mutations account for almost 90% of all the EEC syndrome patients (Rinne et al., 2007; van Bokhoven and Brunner, 2002).

Even if he autosomal dominant inheritance suggest that these mutations have dominant negative effect, recent genotype-phenotype analyses for the five hot-spot mutations revealed significant differences between the corresponding phenotypes. For instance while cleft lip/palate is present in the R304 mutated population (80%), R227 patients seldom have cleft lip/palate. Syndactyly is completely absent in the R227 population, while 30−60% of the other hot-spot mutation population have syndactyly. Genito-urinary defects are frequently observed in R227 patients (40%), but less in other patients (Rinne et al., 2007). It thus seems that these hot-spot mutations might exert specific modifying effects as, for example, on promoters for p63 transcriptional target genes. All patients with R304Q, R279H, R279S, R311G, H208R, R280C and S272N show serious ocular problems and progressive loss of visual capacity (Di Iorio et al., 2012). Most of cases are sporadic, related to de novo mutations arising during early-stage development. Familial cases show an autosomic dominant inheritance with

variable penetrance. The incidence and prevalence of the EEC syndrome in the Italian and European populations are unknown. Males and females are equally affected.

LMS

Limb Mammary Syndrome (LMS) is an autosomal dominant disease caused by mutations in the N- and C- terminus of p63 gene: G76W, S90W, AA deletion in exon 14 (1743 del AA) and TT deletion in the exon 13 (1576 del TT) (last one will affect only the p63α protein isoforms, where it is predicted to cause a frameshift and a premature stop codon) and the stop mutation K632X (Rinne et al., 2007). The LMS phenotype resembles the EEC syndrome phenotype, but the ectodermal manifestations are milder. Clinically, the syndrome is characterized by severe hand and/or foot anomalies and hypoplasia/aplasia of the mammary gland and nipple. Clinical expression is extremely variable. Individuals with mild LMS have isolated athelia (Rinne et al., 2007). All three major categories of limb defects (i.e., deficiencies, duplications, and fusion/separation defects), as well as several combinations of these anomalies, were observed. Variation in the severity of the limb defects may be noticed, not only between individuals but also between the left and right hand/foot of one individual. Less frequent findings include lacrimal-duct atresia, nail dysplasia, hypohydrosis, hypodontia (absence of one or more teeth), ear-pits and cleft palate with or without bifid uvula. Skin and hair are spared (van Bokhoven and Brunner, 2002).

ADULT

Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT) syndrome is mainly caused by point mutation in exon 8 of p63, in which encoding for arginine is changed to glutamine or a glycine at position 298 (R298Q/G). Two other mutations are located in the N-terminus: N6H (mutation that affects only the ΔN-isoforms) and G134D (Amiel et al., 2001). Unlikely EEC, LMS does not affect DNA binding. Instead it's observed a gain of function effect for the ΔNp63γ isoform (Duijf et al., 2002). ADULT phenotype is very similar to LMS but the there is no orofacial clefting or presence of hair defects,

instead, these patients show neurodermitic signs – namely, exfoliative dermatitis of the digits – and excessive freckling (Rinne et al., 2007). Teeth, skin and nail defects are constantly present in ADULT syndrome (100, 93 and 100%, respectively).

AEC

Ankyloblepharon, Ectodermal defect, Cleft lip/palate (AEC) syndrome which is also known as "Hay-Wells syndrome", is caused by missense mutations located in the SAM domain (I510T, L514F/S/V, G518V, C522G/W, G530V, T533P, Q536L, I537T, S541F, I558T, R555P), splice-site mutation in front of exon 10 (3'ss intron 10) or deletions in TI domain (1742 del C, 1859 del A) (Rinne et al., 2007). The mutations in SAM domain affect only the α isoforms of p63 and are predicted to disrupt protein-protein interactions, by either destroying the compact globular structure of the SAM domain or substituting amino acids that are crucial for such interaction. Tentative evidence indicates that the effects of the SAM-domain mutations varies for different isotypes and at different DNA target sites. The hallmark of AEC syndrome is the occurrence of ankyloblepharon, a partial or complete fusion of the eyelids that is very rare in other p63-related syndromes (van Bokhoven and McKeon, 2002). It differs from the other conditions also because of the severity of its skin phenotype and the absence of limb malformations. The majority of patients have severe skin erosion at birth, but it would recover during the first year (van Bokhoven and Brunner, 2002). Ectrodactyly has never been reported, but 25% of patients has only mild syndactyly (Rinne et al., 2006).

RHS

Rapp-Hodgkin Syndrome (RHS) is caused either by point mutations in the SAM domain (I510T, S541Y, S541P) or deletions in the TI domain (1709 del A, 1721 del C, 1787 del G, 1859 del A). This condition is characterized by the association of anhidrotic ectodermal dysplasia with cleft lip/palate. Other common features include sparse, brittle, and dry hair with alopecia in adulthood together with hypohidrosis (a

reduced ability to sweat) and heat intolerance, dental anomalies (hypodontia - absent teeth -, cone-shaped incisors and enamel hypoplasia - thin or absent tooth enamel -) and dysplastic (misshapen or absent) nails. RHS and AEC are very similar both genotypically and phenotypically, the main differences are the absence of ankyloblepharon in RHS and the more severe skin phenotype in AEC (Rinne et al., 2007; van Bokhoven & Brunner, 2002).

Non-syndromic p63 conditions

SHFM

Patients with Split Hand/Foot Malformation (SHFM) showed only limb malformation (ectrodactyly and syndactyly) condition, thus without orofacial clefting or ectodermal dysplasia (Rinne et al., 2007; van Bokhoven & Brunner, 2002). The non-syndromic SHFM is caused by several mutations, delineating genetic heterogeneity: a point mutation in TA domain (R58C), a splice-site mutation in front of exon 4 (3'ss intron 4), four missense mutations in DNA binding domain (K193E, K194E, R280C, R280H), and two nonsense mutations in TI domain (Q634X, E639X) (Rinne et al., 2007). The two aforementioned nonsense mutations create truncations of eight and three amino acids, respectively, in the C-terminal end of the α isoforms that partially abolishes the action of the TI domain. In addition, the last five amino acids, KEEGE, may form an endoplasmic retention signal, suggesting that protein trafficking may also be impaired.

NSCL

The aminoacid change R313G is the only mutation causing the non-syndromic cleft lip/palate (NSCL) phenotype (Leoyklang et al., 2006). Since orofacial clefts are considered to have a multifactorial origin it is quite possible that these changes in the p63 gene impose a risk factor for facial clefting type (Rinne et al., 2007).

Mutation	Exon	Domain	Isoform		Syndrome
N6H	3’	$TA^{\Delta N}$	ΔN	α, β, γ	ADULT
R58C	3	TA	TA	α, β, γ	SHFM
G76W	4	-	ΔN, TA	α, β, γ	LMS
S90W	4	-	ΔN, TA	α, β, γ	LMS
3’ss intron 4	intron 4 / exon 5	-	ΔN, TA	α, β, γ	SHFM
G134D	4	-	ΔN, TA	α, β, γ	ADULT
L162P	5	DBD	ΔN, TA	α, β, γ	EEC
Y163C	5	DBD	ΔN, TA	α, β, γ	EEC
Y192C/D	5	DBD	ΔN, TA	α, β, γ	EEC
K193E	5	DBD	ΔN, TA	α, β, γ	SHFM
K194E	5	DBD	ΔN, TA	α, β, γ	SHFM
V202M	5	DBD	ΔN, TA	α, β, γ	EEC
R204L/Q/W	6	DBD	ΔN, TA	α, β, γ	EEC
H208Y	6	DBD	ΔN, TA	α, β, γ	EEC
R227Q	6	DBD	ΔN, TA	α, β, γ	EEC
C269Y	7	DBD	ΔN, TA	α, β, γ	EEC
S272N	7	DBD	ΔN, TA	α, β, γ	EEC
C273Y	7	DBD	ΔN, TA	α, β, γ	EEC
R279C/H/Q	7	DBD	ΔN, TA	α, β, γ	EEC
R280C/H/S	7	DBD	ΔN, TA	α, β, γ	EEC
R280C/H	7	DBD	ΔN, TA	α, β, γ	SHFM
R298Q/G	8	DBD	ΔN, TA	α, β, γ	ADULT
R304P/Q/W	8	DBD	ΔN, TA	α, β, γ	EEC
C306Y/R	8	DBD	ΔN, TA	α, β, γ	EEC
C308S/Y	8	DBD	ΔN, TA	α, β, γ	EEC
P309S	8	DBD	ΔN, TA	α, β, γ	EEC
R311G/K	8	DBD	ΔN, TA	α, β, γ	EEC
D312G/H/N	8	DBD	ΔN, TA	α, β, γ	EEC
R313G	8	DBD	ΔN, TA	α, β, γ	NSCL
A315E	8	DBD	ΔN, TA	α, β, γ	EEC
3’ss intron 10	intron 10 / exon 11	-	ΔN, TA	α, β, γ	AEC
I510T	13	SAM	ΔN, TA	α	AEC/RHS
L514F/S/V	13	SAM	ΔN, TA	α	AEC
G518V	13	SAM	ΔN, TA	α	AEC
C522G/W	13	SAM	ΔN, TA	α	AEC
1572 ins A	13	SAM	ΔN, TA	α	EEC
1576 del TT	13	SAM	ΔN, TA	α	LMS
G530V	13	SAM	ΔN, TA	α	AEC
T533P	13	SAM	ΔN, TA	α	AEC
Q536L	13	SAM	ΔN, TA	α	AEC
I537T	13	SAM	ΔN, TA	α	AEC
S541F	13	SAM	ΔN, TA	α	AEC
S541P/Y	13	SAM	ΔN, TA	α	RHS
R555P	14	SAM	ΔN, TA	α, β	AEC
I558T	14	SAM	ΔN, TA	α, β	AEC
L536P	14	SAM	ΔN, TA	α, β	EEC
1709 del A	14	TI	ΔN, TA	α, β	RHS
1721 del C	14	TI	ΔN, TA	α, β	RHS
1742 del C	14	TI	ΔN, TA	α, β	AEC
1743 del AA	14	TI	ΔN, TA	α, β	LMS
1787 del G	14	TI	ΔN, TA	α, β	RHS
1859 del A	14	TI	ΔN, TA	α, β	AEC/RHS
K632X	14	TI	ΔN, TA	α, β	LMS
Q634X	14	TI	ΔN, TA	α, β	SHFM
E639X	14	TI	ΔN, TA	α, β	SHFM

Table 1
p63 mutations causing disorders (Rinne et al., 2007).

Ocular surface reconstruction techniques

A clear cornea is essential for visual acuity and depends on stromal avascularity and epithelial integrity. Corneal renewal and repair are mediated by stem cells of the limbus, that comprise a minority of clonogenic cells in the limbus, while non-self-renewing transient amplifying progenitors with limited proliferative potential represent over 95% of cells with clonogenic activity (Pellegrini et al., 1999). In case of limbal stem cell deficiency (LSCD), that is when limbal stem cells are damaged or destroyed such as by ocular burns, flanking conjunctival cells migrate and replace corneal epithelium (Puangsricharern and Tseng, 1995). This process leads to neovascularization, chronic inflammation, and stromal scarring, resulting in corneal opacity and loss of vision. The only way to prevent conjunctival invasion is to restore the limbus by transplantation of either autologous limbal grafts (Kenyon and Tseng, 1989) or limbal cultures (Pellegrini et al., 1997). Transplantation of autologous LESC grafts has been shown to be successful for the treatment of acquired unilateral LSCD with percentages of success ranging between 60 and 80%. Long-term follow up of treated patients showed the stability of the regenerated corneal epithelium and improvement of vision (Pellegrini et al., 1997; Rama et al., 2001). Recently, a product based on autologous cultured corneal epithelial stem cells (Holoclar) has received a conditional marketing authorization by the European Medicines Agency (EMEA/H/C/002450), a further demonstration of the validity of such therapeutic strategy.

Also in p63-related disease the major cause of visual morbidity is LSCD, as a matter of fact it was reported that the diminished ability of limbal epithelial stem cells (LESCs) to regenerate fully stratified corneal epithelia is related to p63 mutations (Di Iorio et al., 2012). However, this type of treatment is not applicable and will not be successful in cases of bilateral LSCD, as in genetic syndrome (Daya and Ilari, 2001; Di Iorio et al., 2010).

Transplantation of allogeneic limbal epithelium is possible, but requires life-long systemic immunosuppression (the limbal tissue is highly vascularized, highly antigenic, and rich in Langerhans cells, which process and present antigen to T cells) and has a success rate that decreases gradually over time (graft survival rate of 40% at 1 year and 33% at 2 years) (Santos et al., 2005; Titiyal et al., 2014).

The source of limbal tissue can be a cadaveric eye (known as keratolimbal allograft [KLAL]) or a living donor who is usually related to the recipient (known as live-related conjunctival limbal allograft [Lr-CLAL]). Post-operative treatment usually involves long-term systemic immunosuppression, success rates seem to be better with Lr-CLAL than KLAL, and HLA-matching may lead to better survival rates of limbal transplants. Allogeneic limbal stem cell transplantation for bilateral LSCD is a relatively inexpensive, easily performed technique with reasonably good outcomes, but the most significant complication, be it KLAL or Lr-CLAL, is immunological rejection. Rejection episodes are recognized by onset of intense perilimbal congestion, epithelial breakdown, edema, and infiltration. Incidence of rejection is variable, and may be affected by recipient age, HLA matching, and use of immunosuppressive therapy. Focal recurrences of LSCD not threatening the visual axis may be observed until they are deemed to be progressive. (Vazirani et al., 2016).

The risks of immunological rejection and the need for an indefinite period of systemic immunosuppressive therapy after allogeneic transplantation have driven the search for autologous sources of cell-based therapy for bilateral LSCD. Over the past 15 years, a number of autologous cell types have been investigated for treating LSCD, including embryonic stem cells (Homma et al., 2004), conjunctival epithelial cells (Tanioka et al., 2006), epidermal stem cells (X. Yang et al., 2007), dental pulp stem cells (Monteiro et al., 2009), bone marrow- derived mesenchymal stem cells (Ma et al., 2006), hair follicle bulge- derived stem cells (Meyer-Blazejewska et al., 2011), and umbilical cord lining stem cells (Reza et al., 2011), but the most promising alternative would seem to be cultured autologous oral mucosal epithelial cell sheets (Nakamura et al., 2004; Nishida et al., 2004). Numerous studies have shown that oral mucosal epithelial stem

cells (OMESCs) are a feasible alternative to limbal transplants (Burillon et al., 2012; H.-C. J. Chen et al., 2009; Nishida et al., 2004), and an ideal substitute of LESCs in ocular surface reconstruction (Nakamura et al., 2003). After more than10 years of ex-vivo oral mucosal autograft involving 242 patients, its capacity to stabilize the ocular surface in bilateral LSCD has been thoroughly confirmed. Corneal transparency was restored and postoperative visual acuity improved remarkably. The clinical results seem comparable with those following transplantation of cultured LESC, although the rate of certain complications (corneal neovascularization and infections) is higher compared to cultured limbal stem cells. Postoperative management includes use of a soft contact lens to protect the transplanted epithelium, and topical steroid and antibiotic medication. No systemic immunosuppressive drugs are required (Utheim, 2015).

The final attempt to restore visual function when ocular surface reconstruction techniques fail in case of extreme dry eyes are keratoprosthesis, alternative to cell-based therapy for surgical management of bilateral LSCD. The donor cornea is sandwiched between a polymethyl methacrylate (PMMA) front plate with a stem and a back plate. The assembly is held in place with a titanium locking ring. The keratoprosthesis surgical technique is similar to that used in penetrating keratoplasty, apart from the fact that the crystalline lens is always removed. A large-diameter soft bandage contact lens is placed at the end of surgery, and is maintained indefinitely. The contact lens is changed every 3-4 months. The major complications encountered in keratoprosthesis implantation include retroprosthetic membrane formation, glaucoma, sterile corneal melts, vitritis, device extrusion, and endophthalmitis (Vazirani et al., 2016).

Gene therapy

Gene therapy is the introduction, within the cells responsible for a pathology, of a therapeutic gene whose expression is capable of curing the disease or relieve the

symptoms. Many human genetic diseases are clearly defined by a single gene defect, and the treatment of single gene disorders represents the prototype of gene therapy (Biffi et al., 2011; Burnett and Hooper, 2009; Petersen-Jones et al., 2012; D. Wang and Gao, 2014).

Gene transfer mediated by viral vectors is very efficient because exploits the natural ability of viruses to transfer heterologous gene material to specific target cells. Transduction with viral vectors leads to an abortive infection that introduces functional genetic information expressed by the genome of recombinant viral vectors into the target cell (Kay et al., 2001).

Among viral vectors, those derived by lentivirus show several advantage (Zufferey et al., 1998):

- capacity to mediate stable transgene integration, thus its long-term expression;
- absence of immune-mediated response (they do not transfer viral genes);
- relatively large cloning capacity;
- possibility of pseudotyping, extending vectors tropism;
- capacity to transduce nondividing cells.

Several vector safety modifications including self-inactivation (SIN), chromosomal insulators and tissue-specific promoters has been validated (Sinn et al., 2005). In particular, a strategy for transcriptional targeting of lentiviral vectors based on replacing the viral long terminal repeat (LTR) enhancer with cell lineage-specific, genomic control elements permits transgene expression that is higher, better restricted and show less position-effect variegation than that obtain by the same combination of enhancer-promoter elements placed in a conventional, internal position (Lotti et al., 2002).

Junctional epidermolysis bullosa (JEB), a severe and often fatal skin adhesion defect caused by mutation of the basement membrane component laminin-5 (LAM5), can be treated by lentiviral vectors in which transgene expression is driven by cellular promoter with short-range enhancer activity. Transcriptionally targeted lentiviral

vectors based on keratin 14 (K14) enhancer/promoter elements are capable of infecting epidermal stem cells, restricting their expression to the basal layer of transplanted epidermis, and producing enough LAM5 protein to correct the genetic defect in patient-derived cells *in vitro* and *in vivo* (Di Nunzio et al., 2008).

Gene therapy for corneal diseases

Gene therapy is imminent as a potential measure to treat inherited and acquired diseases of the eye. The corneal epithelium is a moist, thin, four cell layer stratified epithelium and unlike epidermis, it is non-cornified, i.e. it has no equivalent of the stratum corneum. In addition, the cornea is easily accessible, disease status is easily monitored and the surface to be treated is small. As the corneal tissue is rather easy to access compared to other tissues and organs and moreover due to its immune privilege, gene therapy becomes an ideal measure for treating cornea related diseases. The development of novel methods of introducing genetic material into cells (Hao et al., 2010), the identification of new therapeutic targets, and improved tissue selectivity may revolutionize our approach to current therapeutic challenges and treat currently incur- able illness and disease. The recent development of RNA interference (RNAi) technology is particularly attractive for developing gene therapy treatments to cure corneal genetic disorders. (Barbaro et al., 2016; Liao et al., 2011; Mohan et al., 2013). This process of sequence specific, post-transcriptional inhibition of gene expression has great potential to be developed as a novel therapeutic approach for a number of disorders where gene inhibition is predicted to be therapeutic (Bumcrot et al., 2006). A major challenge to development of therapeutics aimed at silencing mutant alleles is the requirement for a high-degree of sequence specificity. Remarkably, it has proven possible to develop potent, highly efficacious siRNA molecules that can discriminate a single nucleotide (Trochet et al., 2015)

So far, several experimental procedures have been performed for identification of innovative gene-based interventions as possible treatments of various corneal disorders: attempts to improve allograft survival using gene transfer technology

(Beutelspacher et al., 2006); controlling corneal scarring (Seitz et al., 1998); treatment of corneal alkali injury (Saika et al., 2005), corneal neovascularization (Lai et al., 2007; Yu et al., 2007) and corneal neuropathy and epitheliopathy caused by type-I and -II diabetes (Saghizadeh et al., 2010)

Keratin 14

Keratins constitute the largest subgroup of the family of intermediate filaments and represent the most abundant proteins in epithelial cells. (Fuchs and Weber, 1994). They exist as highly dynamic networks of cytoplasmic 10–12 nm filaments that are obligate heteropolymers involving type I and type II keratins. The primary function of keratins is to protect epithelial cells from mechanical and nonmechanical stresses that result in cell death. Other emerging functions include roles in cell signaling, the stress response and apoptosis, as well as unique roles that are keratin specific and tissue specific (several keratins are expressed in a differentiation-specific manner in epithelial tissues of various types, e.g. skin, hair follicles, cornea, conjunctiva, nails) (Coulombe and Omary, 2002; Ouellet et al., 1986). This differential expression of keratins has been useful in marking the boundaries of the different layers and in following/distinguishing proliferation and differentiation states in epithelial tissues (Fuchs and Green, 1980; Kinoshita et al., 2001; Martens et al., 2004; Ouellet et al., 1986).

Keratin 14 (K14) is a predominant cytoplasmic intermediate filament protein that is expressed at high levels in the proliferating basal cells of the mature adult epidermis. It is a faithful and abundant biochemical marker of stratified squamous epithelium, being its expression restricted to the basal layer of the epidermis. In addition, it is among the earliest epidermal markers, whose expression coincides with commitment of the single-layered surface ectoderm to an epidermal cell fate and stratification during mouse embryonic skin development (Byrne et al., 1994; Vassar et al., 1989).

Keratin 14 promoter

The K14 gene expression is restricted to the basal layer and is turned off when keratinocytes move to suprabasal compartments (Fuchs and Green, 1980). Previously *in vitro* and *in vivo* studies used the K14 putative promoter region (from -2500 to +30) to drive gene expression, demonstrating that it is sufficient to reproduce the basal layer–restricted expression of the K14 gene (Dellambra et al., 2000; Khavari, 2000; Sinha et al., 2000; X. Wang et al., 1997). Analysis on chromatin region of the K14 gene revealed DNase I hypersensitivity regions in the keratinocyte genome but not in the fibroblasts ones. Four specific sites for keratinocytes have been identified within a range of 3 kb to 5' of the starting site of K14 transcript (Figure 7) (Fuchs, 1995):

1. HSs-I – among of transcription start site (+1);
2. HSs-II – from -1450 to -1325 upstream of +1;
3. HSs-III – from -1750 to -1557 upstream of +1;
4. HSs-IV – at -2800 position upstream of +1.

Minimum promoter is located on -450/+30, containing TATA box and transcription start site. The 2.1-kb genomic fragment upstream of the K14 transcription start site, containing enhancers HSs-I, HSs-II and HSs-III, is sufficient to drive heterologous genes expression in the basal layer of transgenic mice skin (Vassar et al., 1989; X. Wang et al., 1997). Within the minimum promoter, the HSsI enhancer isn't sufficient to ensure epidermal-specific expression in human or murine keratinocytes, but play a role in regulating gene expression in relation to the cell differentiation stage (Sinha et al., 2000). Detailed mapping of this region identified a bipartite enhancer region of 700bp (from -2000 to -1300 containing HSs-II and HSs-III) that is sufficient to direct keratinocytes-specific transgene expression *in vitro* and *in vivo* when linked to a minimal promoter (Sinha et al., 2000; Sinha and Fuchs, 2001) and a 50-bp region immediately upstream of the TATA box (from -97 to -43) containing a putative negative regulatory (NR) element (Di Nunzio et al., 2008). Both HSs-II and HSs-III enhancer are unable to restrict gene expression. HSsII enhancer region present several binding sites for transcription factors including AP-1, AP-2, Ets and GATA which,

through both *in vitro* and *in vivo* studies, results essential keratinocyte-specific gene expression (Sinha et al., 2000).

Figure 7
Nucleotide sequence of keratin 14 promoter (-2100 → +30). In red are the TATA box and the transcription starting site; in black the relevant regulatory elements and in blue the transcriptional factors binding sites.

Keratin 14 expression in cornea

In developing human cornea, K14 was consistently expressed in the superficial epithelia across the entire cornea, both peripherally and centrally. With the appearance of the limbal dome K14 was present in all layers of the limbal epithelium, then regressed to the suprabasal and basal cells and was restricted to basal epithelia in the neonatal cornea, similar to its distribution in adult specimens (Di Girolamo, 2015; Eghtedari et al., 2016; Kurpakus et al., 1994).

Notably, studies performed on the developing mouse and rat cornea (Chung et al., 1992; Collinson et al., 2002) also support the notion that SCs are initially distributed across the ocular surface but eventually become confined to the limbus with time postconception. The importance of K14 for the cornea and its SCs population is confirmed by genetic ablation study (Lloyd et al., 1995).

Regulation of keratin 14 by p63

It was shown that the expression profile of ΔNp63 showed an identical pattern of keratin 14, suggesting that there is a clear correlation between the two. The fact that ΔNp63 and K14 are co-expressed in keratinocyte cell lines and that ΔNp63 activates the K14 enhancer suggests that ΔNp63 is an important transcriptional regulator of K14 (Romano et al., 2009). It was identified a DNA-binding complex that binds to a conserved sequence and is highly restricted to keratinocytes, that is important for the transcriptional regulation of the K14 gene. There are evidences that this complex is p63, specifically the ΔNp63a isoform (Romano et al., 2007).

This is further supported by the complete absence of K14 in p63 null keratinocytes and the fact that knockdown of p63 in keratinocytes leads to downregulation of K14 expression. (Koster and Roop, 2004). But the regulatory mechanisms that govern K14 gene expression must be more complex in vivo: in fact in a tissue- specific knockout mouse model, there is persistent K14 expression in the basal cells of the epidermis despite the ablation of p63 in adult skin (Keyes et al., 2005). One explanation for this phenomenon is the presence of other transcription factors may function in a compensatory manner allowing at least partial but detectable K14 expression. There has been a recent report suggesting that loss of expression of K14 in the absence of p63 is indirectly mediated by the transcription factor AP-2γ (Koster et al., 2006).

There are evidences that show the presence of a nuclear complex that is restricted to keratinocytes and binds to a specific conserved sequence present in the enhancer segment corresponding to HSs-II of the K14 gene (Romano et al., 2007).

AIM OF THE STUDY

Despite all the clinical and molecular accumulated knowledge, no definitive cures are currently available to treat the ocular alterations in p63-related diseases. Progressive limbal stem cell failure (due to p63 mutations) results in visual impairment. Therapies based on treatments with tear supplements are only palliative: they reduce the dry eye symptoms and tear film instability, but do not stop the progression of the disease. Furthermore common therapeutic strategies, such as penetrating keratoplasty (PK), are not a solution since limbal stem cell deficiency (LSCD) as soon as the donor epithelium is exhausted, conjunctivalisation occurs again. Similarly, autologous cultured limbal stem cell transplantation (cell therapy) cannot be a definitive cure for p63-mutated patients, since p63 mutations severely compromise the ability of autologous stem cells to regenerate the corneal epithelium.

Thus transplantation of genetically corrected autologous cultured oral mucosal stem cells could, therefore, represent an effective alternative for p63-mutated patients.

Allele specific silencing of mutated transcript has yet been seen as potential therapeutic strategy in EEC patients, in order to counterbalance the loss of stem cells (Barbaro et al., 2016). But the limits of siRNAs approach are that it is a transient and mutant-specific correction.

Our idea is that combined gene and stem cell therapy for reconstruction of the ocular surface could be an effective cure for corneal blindness in EEC patients. So the ultimate outcome of my doctoral work has been to develop permanent gene therapy approaches to correct the genetic defect and to design a therapeutic strategy using the transplantation of this genetically modified stem cells.

In particular, my project has focused on a “gene addition” approach. The aim is to recover functional and phenotypic features of p63-defective stem cells using a lentiviral vector in which WT-p63 expression is driven by epithelial stem cells-restricted regulatory elements. Lineage and temporal restrictions of this kind vector allow confining of exogenous WT-p63 expression in stem cells compartment and its

physiological downregulation during differentiation. Once established a “therapeutic window” able to restore WT-p63 physiological amount and recover functionality in mutated-OMESCs primary culture, the following steps would be to test effective efficacy and safety of this vector in *in-vivo* models.

METHODS

Human subjects

Patients with EEC syndrome were recruited from the United Kingdom Ectodermal Dysplasia Society (http://www.ectodermaldysplasia.org/), the Italian EEC society (http://www.sindrome-eec.it/) and referral from clinicians. All applicable institutional and governmental regulations concerning the ethical involvement of human volunteers were observed during this research. After obtaining consent, full medical and ocular histories were taken and a complete family history was recorded to document the pedigree. The clinical diagnostic criterion for EEC syndrome was that all EEC families or isolated cases had the main features of the syndrome (Buss et al., 1995; van Bokhoven et al., 2001):

- an ectodermal dysplasia affecting the skin, hair, nails, teeth, sweat glands, lacrimal ducts, or mammary glands;
- hand or foot abnormalities consistent with the split hand-split foot spectrum;
- cleft lip with or without cleft palate.

To quantify the phenotypic severity of EEC syndrome a previously published scoring system was used (Roelfsema and Cobben, 1996). Clinically, LSCD was diagnosed on the basis of well-established and reliable criteria:

- corneal conjunctivalization producing fibrovascular tissue (pannus);
- irregularity and permeability of corneal epithelium (revealed by fluorescein staining);
- anatomy of the limbal palisades of Vogt.

Cell cultures

Keratinocytes

Oral mucosal epithelial stem cells (keratinocytes) were harvested from oral mucosal biopsies (3×3 mm) of healthy subjects and EEC patients (with R279H or R304Q mutations). Biopsies were minced and trypsinized (Trypsin-EDTA 0.05%, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37 ºC for 2 h. Once isolated, cells were plated onto feeder layer (lethally irradiated 3T3-J2 mouse embryonic fibroblast cell line, $3\times10^4/cm^2$) and cultured in 5% CO_2 at 37°C, using a mixture 2:1 of Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific) and Ham's F12 medium (Thermo Fisher Scientific) containing 10% Fetal Calf Serum (FCS), penicillin-streptomycin (1%), glutamine (2%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.5 μg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), and epidermal growth factor (EGF, 10 ng/ml). Subconfluent primary cultures were trypsinized (10 minutes at 37°C), passaged at a density of 1.5×10^4 cells/cm^2 and serially propagated until exhaustion performing life-span experiments (Pellegrini et al., 1997).

HEK 293T

Human embryonic kidney cells stably transduced to express the Simian Virus 40 T antigen (HEK 293T) (Graham et al., 1977; Louis et al., 1997) were grown at 37°C / 5% CO_2 in DMEM containing 10% Fetal Bovine Serum (FBS). HEK 293T are typically passaged when 90% confluent at 1:8 to 1:15 ratio for general maintenance. A simple trypsin treatment (1-2 minutes at 37°C) is required to detach cells from the plate.

HaCaT

HaCaT cells, a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin (Boukamp et al., 1988), were grown at 37°C / 5% CO_2 in DMEM containing 10% FBS. Subconfluent cultures were pretreated with 0.2 mg/ml

EDTA in Phosphate Buffered Saline (PBS: NaCl 137mM; KCl 2.7 mM; Na_2HPO_4 10mM; KH_2PO_4 2mM; pH 7.4 in mQ water) (20 minutes at 37°C) in order to sequester calcium and weaken emidesmosomal adhesions, trypsinized (10 minutes at 37°C) and passaged at 1:10 to 1:20 ratio.

Colony forming efficiency assay

Colony forming efficiency (CFE) assays were performed at every passage. After trypsinization 1×10^3 keratinocytes were plated in 10-cm^2 dishes onto feeder layer and cultured at low density for 12 days. CFE were analyzed after crystal violet staining.

Site-directed mutagenesis PCR

Site-directed mutagenesis Polymerase Chain Reaction (PCR) was performed on pcDNA3.1ΔNp63αWT plasmid (Barbaro et al., 2016) to obtain different ΔNp63α mutations representative of p63-related syndromes. In order to introduce a single nucleotide change according to mutations that cause pathology, primers were designed with the following settings:

- both of the mutagenic primers contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid;
- desired mutation in the middle of the primer with ~10–15 bases of correct sequence on both sides;
- minimum size of 30 bp and maximum size of 50 bp;
- minimum GC content of 40%;
- termination in one or more C or G bases.

Primers used are shown in Table 2.

Primer name	Primer sequence (5' → 3')
EEC R304Q fw	CGCTGCTTTGAGGCCCAGATCTGTGCTTGCCC
EEC R304Q rev	GGGCAAGCACAGATCTGGGCCTCAAAGCAGCG
EEC R279H fw	GTGTTGGAGGGATGAACCACCGTCCAATTTTAATCAT
EEC R279H rev	ATGATTAAAATTGGACGGCGGTTCATCCCTCCAACAC
ADULT R298G Fw	GGGCAAGTCCTGGGCGGACGCTGCTTTGAGG
ADULT R298G Rev	CCTCAAAGCAGCGTCCGCCCAGGACTTGCCC
ADULT G134D Fw	CCAACACCGACTACCCAGACCCGCACAGTTTCGACG
ADULT G134D Rev	CGTCGAAACTGTGCGGGTCTGGGTAGTCGGTGTTGG
LMS G76W Fw	CAGTACACGAACCTGTGGCTCCTGAACAGCATGGACCAGC
LMS G76W Rev	GCTGGTCCATGCTGTTCAGGAGCCACAGGTTCGTGTACTG
LMS S90W Fw	GCAGATTCAGAACGGCTCCTGGTCCACCAGTCCCTATAACAC
LMS S90W Rev	GTGTTATAGGGACTGGTGGACCAGGAGCCGTTCTGAATCTGC
RHS S541Y fw	CCATCTATCAGATTGAGCATTACTACATGGATGATCTGGCAAGTCTG
RHS S541Y rev	CAGACTTGCCAGATCATCCATGTAGTAATGCTCAATCTGATAGATGG
RHS I510T fw	CCCACAGATTGCAGCACTGTCAGTTTCTTAGCGAGG
RHS I510T rev	CCTCGCTAAGAAACTGACAGTGCTGCAATCTGTGGG
AEC R555P fw	CCCTGAGCAATTTCCACATGCGATCTGGAAGG
AEC R555P rev	CCTTCCAGATCGCATGTGGAAATTGCTCAGGG
AEC C522W fw	GGTTGGGCTGTTCATCATGGCTGGACTATTTCACG
AEC C522W rev	CGTGAAATAGTCCAGCCATGATGAACAGCCCAACC
SHFM K193E Fw	CGCCATGCCTGTCTACGAAAAAGCTGAGCACG
SHFM K193E Rev	CGTGCTCAGCTTTTTCGTAGACAGGCATGGCG

Table 2
List of primers used in site-directed mutagenesis PCR.

5X HF Buffer, 7.5 mM $MgCl_2$; iProof™ High Fidelity DNA Polymerase (Bio-Rad, Hercules, California, USA), primers mix 10 µM, dNTPs mix 2.5 mM and 30ng of plasmid DNA were used (final reaction volume of 20µl). Reaction program used was:

Initialization	98°C	30 seconds	
Denaturation	98°C	50 seconds	18 cycles
Annealing	60°C	50 seconds	
Elongation	72°C	9 minutes	
Final elongation	72°C	7 minutes	
Final hold	4°C	∞	

Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the products were treated with Dpn I (New England Biolabs, Ipswich, Massachusetts, USA) in presence of 10X CutSmart® Buffer for 2 hours in a water bath at 37°C. The Dpn I endonuclease (target sequence: 5'-Gm^{6}ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA (DNA isolated from almost all E. coli strains is dam methylated and therefore susceptible to Dpn I digestion). The nicked vector DNA containing the desired mutations was then transformed into DH5α competent cells. Briefly, competent cells were slowly thawed in ice, then DNA plasmid added on competent cells, gently mix by flicking and incubated on ice for 30 minutes. After heat shock into a 42°C water bath for 45 seconds, tubes were put back on ice for 1 minutes and 200µl Luria-Bertani (LB) medium (bacto tryptone 10gr/L; bacto yeast extract 5gr/L; NaCl 10gr/L; pH 7.0 in deionized water) added. Transformed bacteria grew in 37°C shaking incubator for 1 hour and then were plated onto a 10 cm LB agar plate (15gr of Bacto Agar per liter of LB) containing ampicillin (100µg/ml). Only cells containing plasmid with ampicillin-resistance will be able to grow after 37°C overnight incubation. Colonies grown were further analyzed by Sanger sequencing (BigDye™ Terminator v3.1 Cycle Sequencing Kit; Thermo Fisher Scientific).

Luciferase Reporter Assay

For the luciferase assay, HEK 293T cells were plated at density of 5×10^4 cells per well in a 24-well plate and transfected 24 hours later. Lipofectamine 3000 (Thermo Fisher Scientific) was used as transfecting agent. A plasmid containing the luciferase gene under the control of the K14 promoter (20 ng) and expression vectors encoding for WT-, EEC R304Q-, EEC R279H-, RHS S541Y-, RHS I510T-, AEC R555P- and AEC C522W-ΔNp63α were used. When needed, the pcDNA3.1 empty vector (Thermo Fisher Scientific) was added to reach the total amount of DNA (100 ng) used in each

transfection. In all cases, 1 ng of NanoLuc® vector (pNL1.1.PGK; Promega, Madison, Wisconsin, USA) was cotransfected as a control of the transfection efficiency. Cells were transfected at 80%–90% confluence and incubated at 37°C / 5% CO_2 for 8 hours before medium change. Luciferase activities of cellular extracts were measured by using a Nano-Glo® Dual-Luciferase® Reporter (NanoDLR™) Assay System (Promega) and light emission was measured over 0.1 and 1 second using a VICTOR Multilabel Plate Reader (PerkinElmer, Waltham, Massachusetts, USA). All experiments were performed in triplicate.

Lentiviral vector

HS.NR.G (Di Nunzio et al., 2008) was built in the framework of the SIN pRRLsin-18.pptCMV-GFPwpre vector (Follenzi et al., 2002). Vector carried a deletion in the HIV-1 LTR (−418 to −40) that removes the U3 enhancer but not the minimal promoter. The U3 enhancer was replaced with a 600-bp fragment containing the HSs-III and HSs-II sites with their natural spacer plus the NR region of K14 enhancer/promoter (Lotti et al., 2002). In our mK14.ΔNp63α.EGFP (mini-K14) vector, the EGFP cDNA sequence of HS.NR.G was replaced with the 3.4-kb *Bam*HI/*Sal*I fragment encoding the full-length ΔNp63α cDNA fused to EGFP (Figure 8). Table 3 shows primers used.
Backbone (HS.NR.G) and insert (PCR product containing ΔNp63αEGFP sequence with restriction site of *Bam*HI and *Sal*I at 5' and 3', respectively) were cut with restriction enzymes *Bam*HI and *Sal*I (New England Biolabs), specific bands obtained were excised from agarose gel and purified with QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany). After dephosphorylation (Alkaline Phosphatase; Roche, Basel, Switzerland) of backbone, it was incubated together to insert with T4 DNA ligase (New England Biolabs) at 25°C overnight. Ligation reaction was used to transform Stbl3 competent cells. Colonies grown were analyzed by restriction enzymes and sequencing.

The vector was pseudotyped with vesicular stomatitis virus G protein (pVSV-G) by a third-generation lentiviral packaging system.

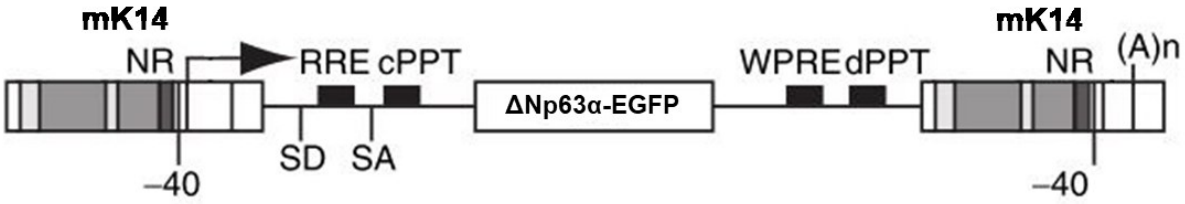

Figure 8
mK14.ΔNp63α.EGFP vector. The U3 enhancer (-418 to -40) was replaced by a 600-bp fragment containing the HSs-III and HSs-II sites (light grey boxes) with their natural spacer plus the negative region (NR, dark grey boxes). The HIV TATA box promoter was maintained. The arrow indicates active transcription start sites. Splice donor (SD) and acceptor (SA) sites, Rev-responsive element (RRE), central and distal polypurine tract (cPPT and dPPT), polyadenylation site [(A)n], and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) are indicated.

Lentiviral particles production

Viral stocks pseudotyped with the vesicular stomatitis virus G protein were prepared by transient cotransfection of HEK 293T cells using a four-plasmid system (the transfer vector; the pMDL encoding Gag and Pol; the pVSV-G encoding vesicular stomatitis virus G protein; and pRev encoding Rev) (Dull et al., 1998).
2.5×10^6 HEK 293T cells were plated in T150cm^2 flasks 3 days before the transfection with Lipofectamine 3000 (Thermo Fisher Scientific). DNA concentration of all plasmids were adjusted to 1µg/µl in TE buffer pH 8.0 (TrisHCl pH8.0 10mM; EDTA pH8.0 1mM). Following quantities of four plasmids was added in each flask: 20µg of mK14.ΔNp63α.EGFP; 10µg of pMDL; 10µg of pVSV-G; 10µg of pRev. After 8 hours incubation at 37°C / 5% CO_2 medium was slowly changed. 48 hours post-transfection supernatant containing lentiviral particles were harvested, centrifuged 10 minute at 950 rpm (revolutions per minute) at 4°C to pellet detached cells and debris and 0.45µm filtrated. Viral preparations were concentrated by ultracentrifugation (3 hours at 27000 rpm at 4°C) to increase titer. Viral stocks were stored at –80°C.

Viral titration by quantitative Real Time PCR

Viral titers were determined by transduction of HEK 293T cells with serial dilution of the vector stocks, and found to range between 10^8 and 10^9 transducing units/ml. Transduction efficiency was determined by measuring the number of copies of lentiviral vector stably integrated in HEK 293T target cells after transduction through quantitative Real Time PCR (qPCR). 5×10^4 HEK 293T cells were plated in 24-well plate 24 hours before the transduction, performed with serial dilution of the vector stocks in minimum amount of total medium per well (200µl). After overnight incubation fresh 1 ml of medium was added. At least 96 hours after transduction (to reduce the contamination from plasmids DNA transfected possibly co-purified with the vector) genomic DNA was extracted from each individual well using a DNeasy Blood & Tissure kit (QIAGEN) according to the manufacturer's recommendations.

Gag oligos were used for amplification of HIV-1 derived vector sequences and are specific for the 5' end of the gag gene. This sequence is present in all HIV-1 vectors, as it is part of the extended packaging signal. Alb oligos were used to normalize for the amount of genomic DNA and are specific for the human albumine gene (Table 3).

Quantification of integrated lentiviral sequences was performed by correlating the Gag-qPCR signal to a standard curve and normalized to Albumine results (https://jahia-prod.epfl.ch/files/content/sites/cpg/files/documents/Documents%20TCF/LV_titration_QPCRhuman.pdf). The reaction mix was composed by primer mix 10 µM, probes 10µM, TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific) and gDNA. The program used was:

50°C	2 minutes	
95°C	10 minutes	
95°C	15 seconds	18 cycles
60°C	1 minute	

All reaction was performed with 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA) and analyzed with 7900 SDS v2.4.1 software (Applied Biosystems).

Keratinocytes transduction

Transduction mixture containing 3×10^4 OMESCs, lentiviral particles and 8 μg/ml polybrene (Millipore, Burlington, Massachusetts, USA) were plated in 24-well plate onto feeder layer in minimum amount of total medium (200μl). After overnight incubation, fresh medium was added. Subconfluent primary cultures were trypsinized, passaged at a density of 1.5×10^4 cells/cm^2 and serially propagated until exhaustion.
5×10^4 HaCaT were plated in 24-well plate 24 hours before transduction with serial dilution of the vector stocks in the presence of 8 μg/ml polybrene. After overnight incubation, fresh medium was added.

Quantitative Real Time PCR (qPCR)

RNA was extracted from OMESCs using a RNeasy Mini Kit (QIAGEN). The retro-transcription protocol was performed following the manufacturer's instruction manual (M-MLV Reverse Transcriptase; Invitrogen, Carlsbad, California, USA). The reaction program used was: 25°C for 10 minutes, followed by 48°C for 60 minutes, 95°C for 5 minutes then the temperature drop to 4°C.
The reaction mix was composed by primer mix 10 μM, probes 10μM, TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific) and cDNA. The reaction program used was that describe above. The difference in relative ΔNp63α expression was performed using the $2^{-\Delta\Delta Ct}$ method. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as internal control gene. Primers and probes used are shown in Table 3.

Primer/Probe name	Primer/Probe sequence (5' → 3')	Type
Gag fw	GGAGCTAGAACGATTCGCAGTTA	qPCR
Gag rev	GGTGTAGCTGTCCCAGTATTTGTC	
Gag probe	[FAM]-ACAGCCTTCTGATGTTTCTAACAGGCCAGG-[BHQ1]	
Alb fw	GCTGTCATCTCTTGTGGGCTGT	
Alb rev	ACTCATGGGAGCTGCTGGTTC	
Alb probe	[TET]-CCTGTCATGCCCACACAAATCTCTCC-[TAMRA]	
ΔNp63α fw	GCATTGTCAGTTTCTTAGCGAG	
ΔNp63α rev	CCATGGAGTAATGCTCAATCTG	
ΔNp63α probe	[FAM]-GGACTATTTCACGACCCAGG-[BHQ1]	
GAPDH fw	CCACTCCTCCACCTTTGACG	
GAPDH rev	CATGAGGTCCACCACCCTGT	
GAPDH probe	[TET]-TTGCCCTCAACGACCACTTT-[TAMRA]	
ΔNp63α5'+BamHI	CGCGGATCCGCTAACATGTTGTACCTGGAAAACAATGC	lentiviral vector cloning
EGFP3'+SalI	CGCGTCGACGATATCTTACTTGTACAGCTCGTCCATGC	

Table 3
List of primers and probes used.

Western Blot

Cells were trypsinized and pelleted at 2000 rpm for 5 minutes. After washing in PBS, whole cell lysates were prepared using cold RIPA buffer (150mM NaCl; 1mM EDTA pH 8.0; 50mM TrisHCl pH 7.4; 1% Triton™ X-100; 1% Na-deoxycholate; 0.1% SDS in mQ water) containing protease inhibitor cocktail (cOmplete™ EDTA-free; Roche). After 20 minutes of ice incubation, cell lysates were centrifuged at 14000 rpm for 15 minutes and supernatant containing proteins were quantified with Micro BCA ™ Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations.

50 µg of proteins of each sample were denaturated at 99°C for 10 minutes, separated by SDS-PAGE (stacking gel 5% and resolving gel 8%) at 80V for 2.5 hours, and transferred to a nitrocellulose blotting membrane (Amersham ™ Protran ™ 0.2µm NC; GE Healthcare, Little Chalfont, UK) at 350 mA for 1 hour. The membrane was blocked in TBST (Tris Buffered Saline TWEEN® 20: 150mM NaCl; 50mM TrisHCl pH 7.4;

0.1% TWEEN® 20) containing 5% of Blotting-Grade Blocker (nonfat dry milk; Bio-Rad). Antibody used include rabbit anti-p63α (Cell Signaling Technology, Danvers, Massachusetts, USA), rabbit anti-GAPDH (Santa Cruz Biotechnology, Dallas, Texas, USA) and goat anti-rabbit horseradish peroxidase (HRP) conjugated (Abcam, Cambridge, UK). Chemiluminescence detection of HRP conjugated secondary antibodies signals was performed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare). Bands intensity were quantified with ImageJ analysis software and relative amount of p63 was normalized on GAPDH.

Confocal microscopy

Specimens were analyzed with an A1Rsi^{+} Laser Scanning Confocal Microscopy (Nikon Instruments, Melville, New York, USA). Image analysis was performed using the NIS-Elements Advanced Research (Nikon Instruments).

RESULTS

State of the art: siRNAs approach

Gene silencing could be an efficient strategy applicable to the majority of EEC patients. In fact only five p63 mutations account for almost 90% of EEC cases and the arginine (R) residues at position 304 (R304) and 279 (R279) are mutational hotspots, so the gene silencing approaches should be developed against a limited number of targets. As EEC syndrome results from heterozygous dominant-negative mutations in p63 gene, therapeutic strategies based on allele-specific gene silencing through RNAi could specifically inhibit the expression of disease-associated alleles without suppressing the expression of the corresponding wild-type alleles to phenotypically correct the stem cell population restoring function.

Oral Mucosal Epithelial Stem Cells (OMESCs) represent an alternative source of stem cells to Limbal Epithelial Stem Cells (LESCs) (Nishida et al., 2004) in EEC patients, characterized by complete stem-cell deficiency in both eyes (bilateral LSCD).

Compared to H-OMESCs from healthy controls, EEC-OMESCs showed (i) a more rapid decrease of colony forming efficiency (CFE) and clonogenic cell numbers (Figure 9A and B), (ii) a progressive increased percentage of aborted colonies during serial cultivation (Figure 9C), and (iii) an increased replicative senescence, as suggested by the lower number of cell passages in culture (Figure 9D) (Barbaro et al., 2016).

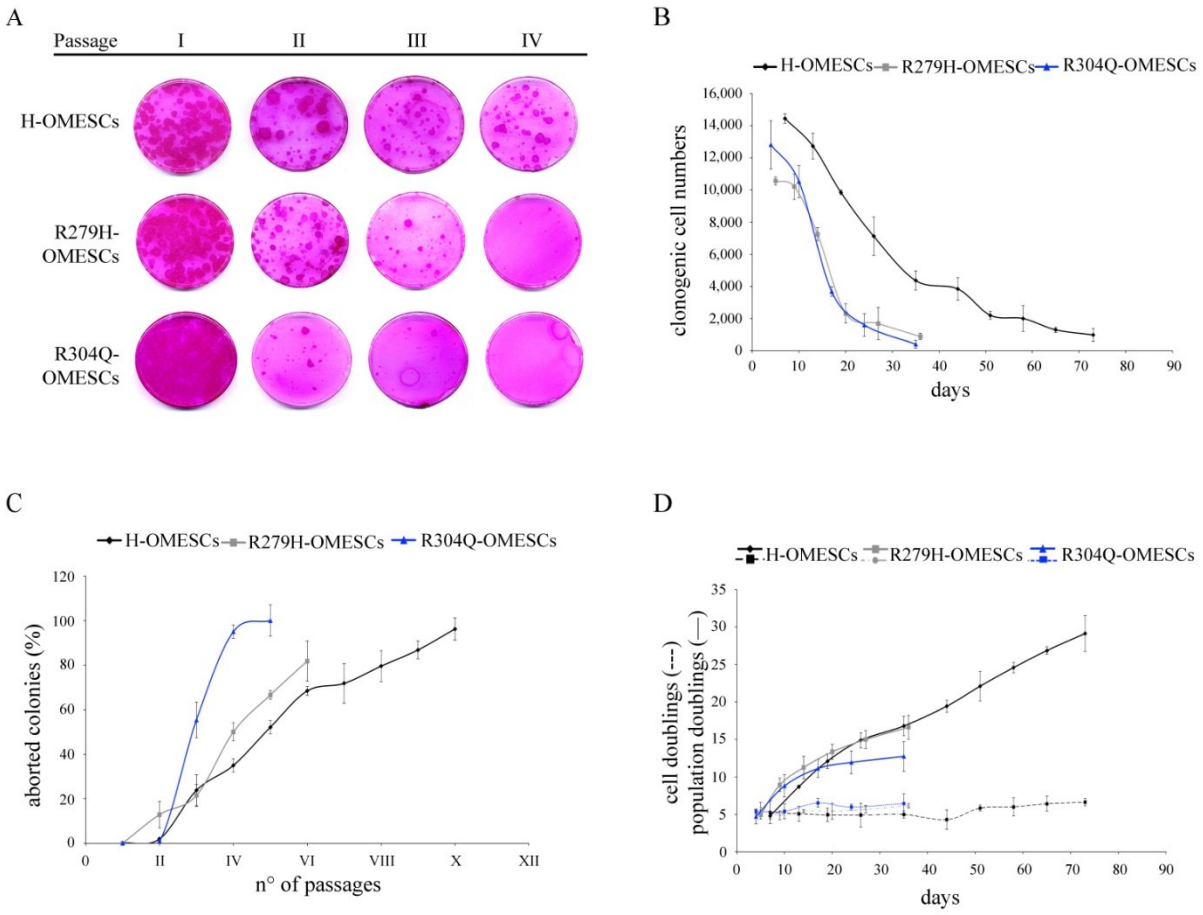

Figure 9
Life-span analysis of OMESCs from healthy (H-) or R279H- and R304Q-EEC donors. (**A**) Colony forming efficiency of healthy, R279H- and R304Q-OMESCs was evaluated at the cell passages indicated. Quantification of (**B**) clonogenic cells numbers, (**C**) percentage of aborted colonies, (**D**) cell doublings and cumulative population doublings of healthy (black line), R279H- (gray line), and R304Q- (blue line) OMESCs are shown. EEC-OMESCs were passaged 5–6 times compared to the 11 passages seen in H-OMESCs controls (Barbaro et al., 2016).

In order to evaluate the ability of p63-defective EEC-OMESCs to stratify and differentiate in a Human Keratoplasty Lenticule (HKL) model (Barbaro et al., 2009), primary H-OMESCs, R279H- and R304Q-OMESCs were seeded onto HKLs and grown for 21 days. Epithelia generated by EEC-OMESCs were hypoplastic with defects in epithelial thickness and cellularity with some areas devoid of cells and with flat irregular and terminally differentiated cells (Figure 10Figure 11A). Quantitatively, epithelial thickness was reduced when R279H-OMESCs (mean: 12.1 ± 7 µm; maximum: 21.3 µm) or R304Q-OMESCs (mean: 9.1 ± 3.0 µm; maximum: 14.5 µm) were compared to epithelia obtained from control H-OMESCs (mean: 56.4 ± 18.0 µm;

maximum: 82.3 µm). The number of cell layers in the epithelium was higher in HKLs seeded with H-OMESCs (mean: 3.9 ± 1.2 cell layers; maximum: 5.8 cell layers), compared to those with R279H-OMESCs (mean: 1.2 ± 1.3 cell layers; maximum: 2.2 cell layers) and with R304Q-OMESCs (mean: 1.1 ± 1.0 cell layers; maximum: 1.2 cell layers) (Barbaro et al., 2016).

Ultrastructural analyses with laser scanning confocal microscopy showed that epithelia from H-OMESCs were clearly organized into (i) basal column-shaped cells expressing p63, (ii) flat squamous superficial terminally differentiated cells, as indicated by the expression of keratin 3 and (iii) suprabasal cuboid wing cells, expressing 14-3-3σ, an early differentiation marker for stratified epithelia (Figure 10B). Tissues generated from R279H- and R304Q-OMESCs showed defects in both stratification and differentiation, thus resulting in severe tissue hypoplasia and lack of proper tissue polarity. We also observed reduced adhesion properties of the epithelium obtained by mutant cells.

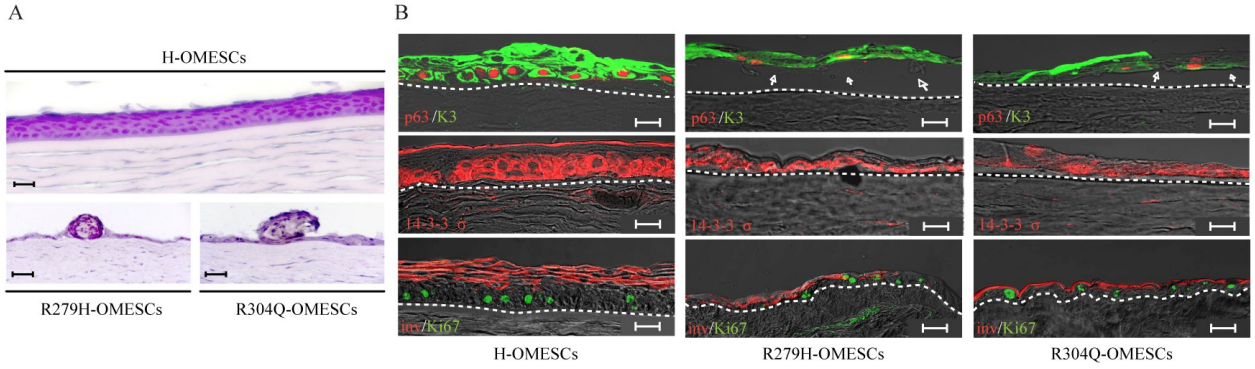

Figure 10
R279H and R304Q-p63 mutations lead to abnormal epithelial stratification and differentiation pathways. (**A**) Hematoxylin-eosin staining of epithelia generated on HKLs by healthy, R279H- and R304Q-p63 OMESCs. Scale bars: 50µm. (**B**) Immunofluorescence on epithelia derived from the above OMESCs using p63, cK3, 14-3-3σ, involucrin and Ki67 antibodies. Scale bars: 20 µm (Barbaro et al., 2016).

ΔNp63α expression in R279H-OMESCs was quantified using an absolute quantitative PCR (Ab-qPCR) at different passages in culture (I, III, V, VII, and XI) and compared to H-OMESCs and healthy limbal epithelial stem cells (H-LESCs). As shown in Figure 11, ΔNp63α decreased during serial cultivation, becoming very low after the 7th

passage. The rate of reduction was faster in R279H OMESCs, compared to H-OMESCs and H-LESCs. ΔNp63α expression was 3-fold higher in R279H-OMESCs compared to H-OMESCs, at the first passage. This observation correlates with the abnormal hyper-proliferation and invasion property of mutant R279H EEC primary cells, previously observed in the CFE and assay.

Importantly, the amount of ΔNp63α in primary H-LESCs was approximately 2-times lower than in H-OMESCs. This correlates well with their "cumulative population doubling ability" observed in vitro (H-LESC = 10 ± 2 passages; H-OMESC = 12 ± 2 passages) (Barbaro et al., 2007) and reflects the different "intrinsic life-span" and "clonogenic potential" of the two cell lineages. The longer self-renewal potential of OMESCs explains the successful use of oral mucosal as a source of stem cells, allowing epithelial regeneration to last for longer (Burillon et al., 2012; H.-C. J. Chen et al., 2009; Nishida et al., 2004). Our findings confirm that ΔNp63α is a robust stem cell marker: (i) its expression declines during the differentiation and senescence processes of primary epithelia stem cells in vitro and (ii) also provide further evidences of the premature ageing found in EEC-OMESCs.

To gain greater insight into the relationship between ΔNp63α expression and EEC pathogenesis, we evaluated the percentage of wild-type or mutated mRNA p63 expression through Allele-Specific (AS) quantitative PCR (AS-qPCR) (Barbaro et al., 2012). AS-qPCR demonstrated that the profile expression ratios of two transcripts were different in three passages analyzed (I, III, and V) with a decrease of the mutated p63 mRNA (Figure 11, green and red indicate mutant and WT- ΔNp63α expression levels, respectively).

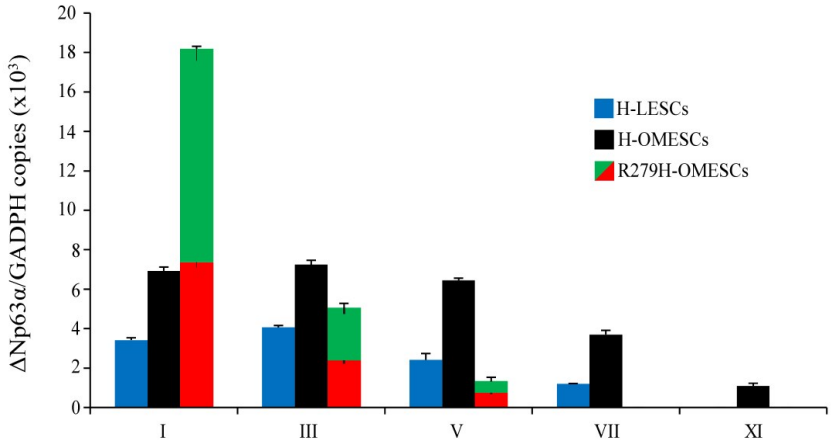

Figure 11
Ab-qPCR showing ΔNp63α copy number in total RNA from limbal epithelial stem cells (LESCs), H- and R279H OMESCs, and ASqPCR of R279H-p63 mutant during the passages. For the Ab-qPCR, results were normalized against GADPH. ASqPCR data indicate the percentage of R279H-p63 allele compared to WT-p63. Green and red indicate mutant and WT expression levels, respectively (Barbaro et al., 2016).

These results confirm that the presence of high R279H p63 mRNA affects the normal differentiation program and may induce the activation of an alternative and yet unknown regulation pathway, leading to premature stem cells loss.

To screen efficiently for siRNAs blocking the expression of mutant p63 with little or no effect on wild-type (WT) expression, we established two stable cellular lines: HEK293T cells, an epithelial cell line with no endogenous p63 expression, were transduced with 3rd generation lentiviral vector carrying WT- or R279H-ΔNp63α alleles fused to RFP or EGFP sequences, respectively.

Homozygous stable cell lines were used to separately transfect a collection of 19 locked nucleic acid (LNA) siRNAs (Elmén et al., 2005; Mook et al., 2007), designed to target the R279H-p63 mRNA region containing the single nucleotide change. Forty-eight hours post siRNAs transfection, allele specific down-regulation was assessed through ΔNp63α relative-qPCR. siRNAs (a, b and c) specifically down-regulated the R279H-ΔNp63α mRNA by approximately 90% (Figure 12A). Western-blot analyses demonstrated a significant and specific reduction of mutant ΔNp63α protein when siRNA-a was used (45 ± 4%), while protein down-regulation with siRNA-b and siRNA-c did not have consistent and reproducible effects (Figure 12B).

In order to evaluate the specificity of siRNA-a to (1) inhibit the R279H-ΔNp63α neo-synthesis, and (2) down-regulate its expression in time-dependent manner, we utilized transiently transfected HEK293T cell lines, as an in vitro cellular model of the EEC disease-related heterozygous state. The HEK293T cell line was co-transfected with a 1:1 ratio of both WT-ΔNp63α-RFP and mutant ΔNp63α-EGFP plasmids in the presence of the siRNA-a. Imaging analyses in the treated cell pool showed a specific and significant neo-synthesis inhibition of R279H-ΔNp63α-EGFP expression in approximately 80% of total cells at 48 h post transfection when compared to siNCT treated cells (Figure 12C).

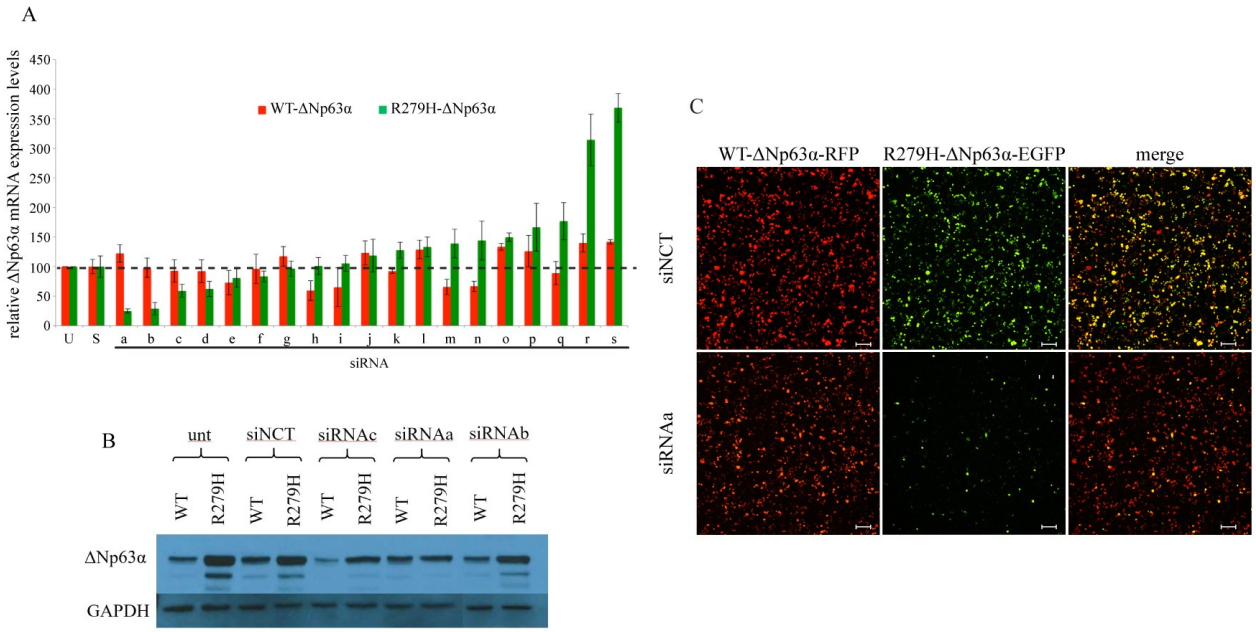

Figure 12
siRNA-a specifically inhibits R279H-DNp63a expression in a time-dependent manner in ΔNp63α expressing cell lines. (**A)** Relative-qPCR showing ΔNp63α mRNA downregulation in homozigous WT- and R279H-ΔNp63α HEK293T stable cell lines either untreated (U) or transfected with control (S) or ΔNp63α siRNAs. (**B**) Immunoblot detection of ΔNp63α in homozygous WT- and R279H-ΔNp63α HEK293T transfected with control (siNCT) or selected ΔNp63α siRNAs. (**C**) Scanning confocal microscope analyses of HEK293T cells transiently transfected with WT-ΔNp63α-RFP and R279H-ΔNp63α-EGFP plasmids, in combination with siNCT or siRNA-a, 48h post-transfection. Scale bars: 100μm (Barbaro et al., 2016).

To investigate whether siRNA-a could restore the functional phenotype in R279H-OMESCs, we repeated the migration and cell proliferation assays with OMESCs treated with either siNCT or siRNA-a. R279H-OMESCs treated with siRNA-a (but not

with siNCT) had a longer life-span and were able to be passaged in culture 7 times compared to 5-6 of untreated cells. Furthermore, siRNA-a treatment specifically mitigated the progressive loss of clonogenic cells in R279H-OMESCs, and a trend more similar to H-OMESCs has been achieved.

To test the ability of siRNA-a to restore the epithelial tissue phenotype, two simultaneous organotypic cultures of R279H-OMESCs were established using a device able to create two different and separated areas in a single HKL (Figure 13A). One area was treated with siRNA-a and the adjacent with a scrambled siRNA (siNCT); FITC-siRNAs were also added to track the siRNAs. At the end of the culture, histological sections of the tissue were taken to assess the ability of R279H OMESCs to correctly stratify following siRNA-a treatment (Figure 13B). Confocal microscopy demonstrated the presence of a well-organized and stratified epithelium of 4-5 layers limited to the area treated with siRNA-a, while the adjacent region showed the typical phenotype of p63-mutated cells. The resulting epithelium was characterized by basal expression of p63 and ΔNp63α while expression of keratin 3 and 14-3-3σ was mainly found in the upper cell layers. The basal cuboidal epithelial cells were anchored to the basement membrane and expressed laminin-β3 (Figure 13C and D).

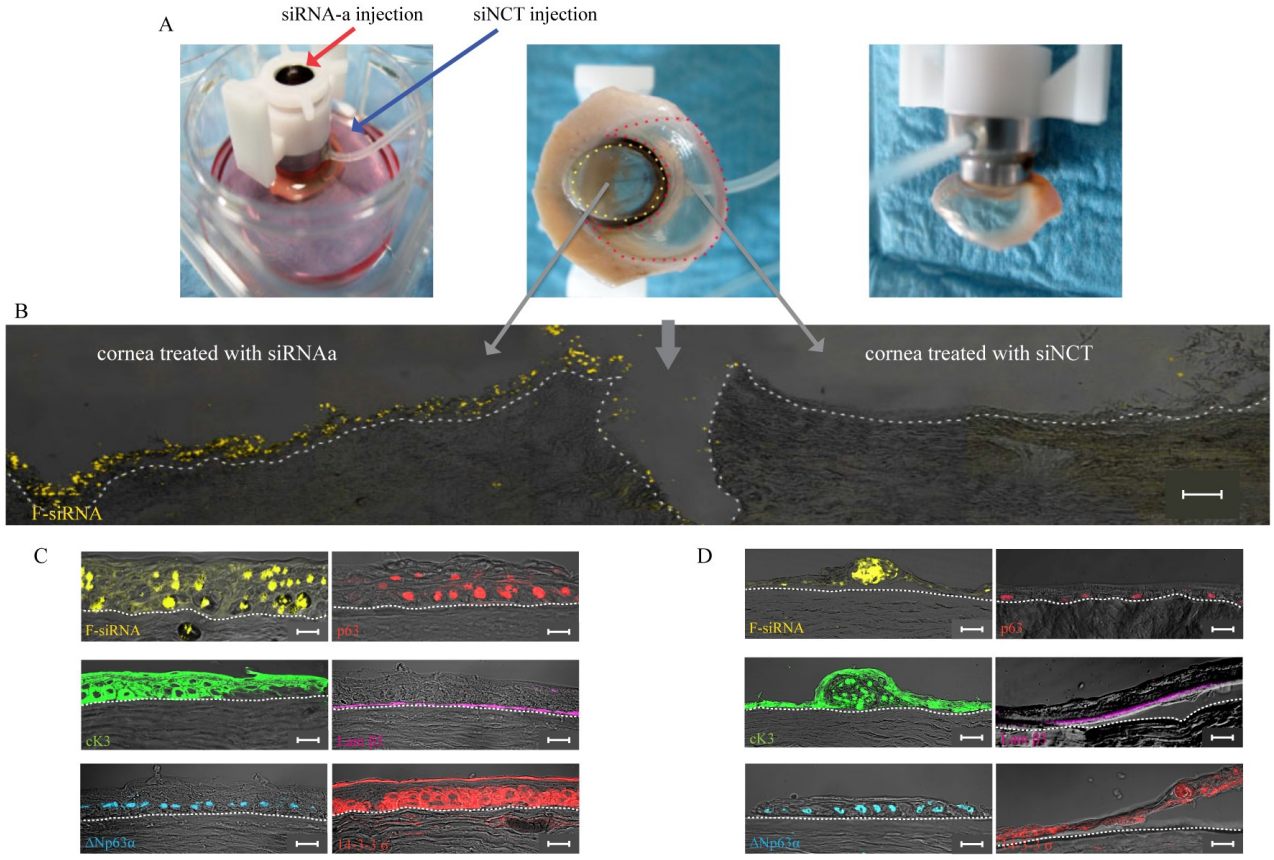

Figure 13
siRNA-a restores the epithelial phenotype, stratification and differentiation properties of R279H-OMESCs. (**A**) Photos of the device used to create two different areas in HKLs, treated with siRNA-a and control siRNA (siNCT) respectively, both in combination with a fluorescent placebo siRNA (F-siRNA). (**B**) Hystological images of organotypic cultures made with R279H-OMESCs, treated for at least 2 weeks with siRNA-a or siNCT, in combination with the fluorescent siRNA. Scale bars: 100µm. (**C** and **D**) Magnification of immunofluorescence images obtained with pan-p63, ΔNp63α, cK3, laminin-β3 and 14-3-3σ show the appropriate expression of these proteins. Scale bars: 20µm (Barbaro et al., 2016).

K14 promoter activation is dependent upon the DNA-binding activity as well the amount of p63

Expression of all major isoforms of p63 induces K14 expression and this induction is dependent upon the DNA-binding activity of p63 (Romano et al., 2007). We tested the induction of K14 promoter, mediated by WT- or different ΔNp63α mutated forms, by using luciferase assay. HEK 293T cells (not expressing K14) were co-transfected both with a plasmid in which luciferase expression is driven by the K14 promoter and with

one carrying WT- or different ΔNp63α cDNA mutated forms (EEC R304Q, EEC R297H, ADULT R298G, ADULT G134D, LMS S90W, LMS G76W, RHS 541Y, RHS I510T, AEC R555P, AEC C522W and SHFM K193E) (Figure 14A)

Interestingly, compared to WT-ΔNp63α-mediated activation, some p63 mutants (EEC-, RHS-, AEC-) are unable to activate K14 promoter, while others (ADULT-, LMS-, SHFM-) only show little differences in this capability. This behavior seems to be independent of specific site mutation. In fact, among DNA binding domain p63 mutants, EEC-mutations fail the activation of K14 promoter, while ADULT R298G and SHFM K193E induce it. On the other hand, mutations in different domains act as EEC ones (AEC- and RHS-) or contrarily (LMS-).

In order to evaluate whether the activity of ΔNp63α is as well amount-dependent, it was tested the induction of K14 promoter with increasing amount of ΔNp63α. Mutated cDNAs unable to activate K14 promoter were co-trasfected with different quantity of WT-ΔNp63α. Compared to WT-homozygous-like condition, cells that mimic mutated-hemyzygous conditions confirm the failure to induce luciferase expression, but this ability is restored by adding increasing amount of WT- ΔNp63α. Important, induction of K14 promoter prove to be dependent upon the amount of ΔNp63α, as demontrate by increasing of luciferase expression as a result of adding increasing amount of ΔNp63α (starting by heterozygous-like condition to further) (Figure 14B).

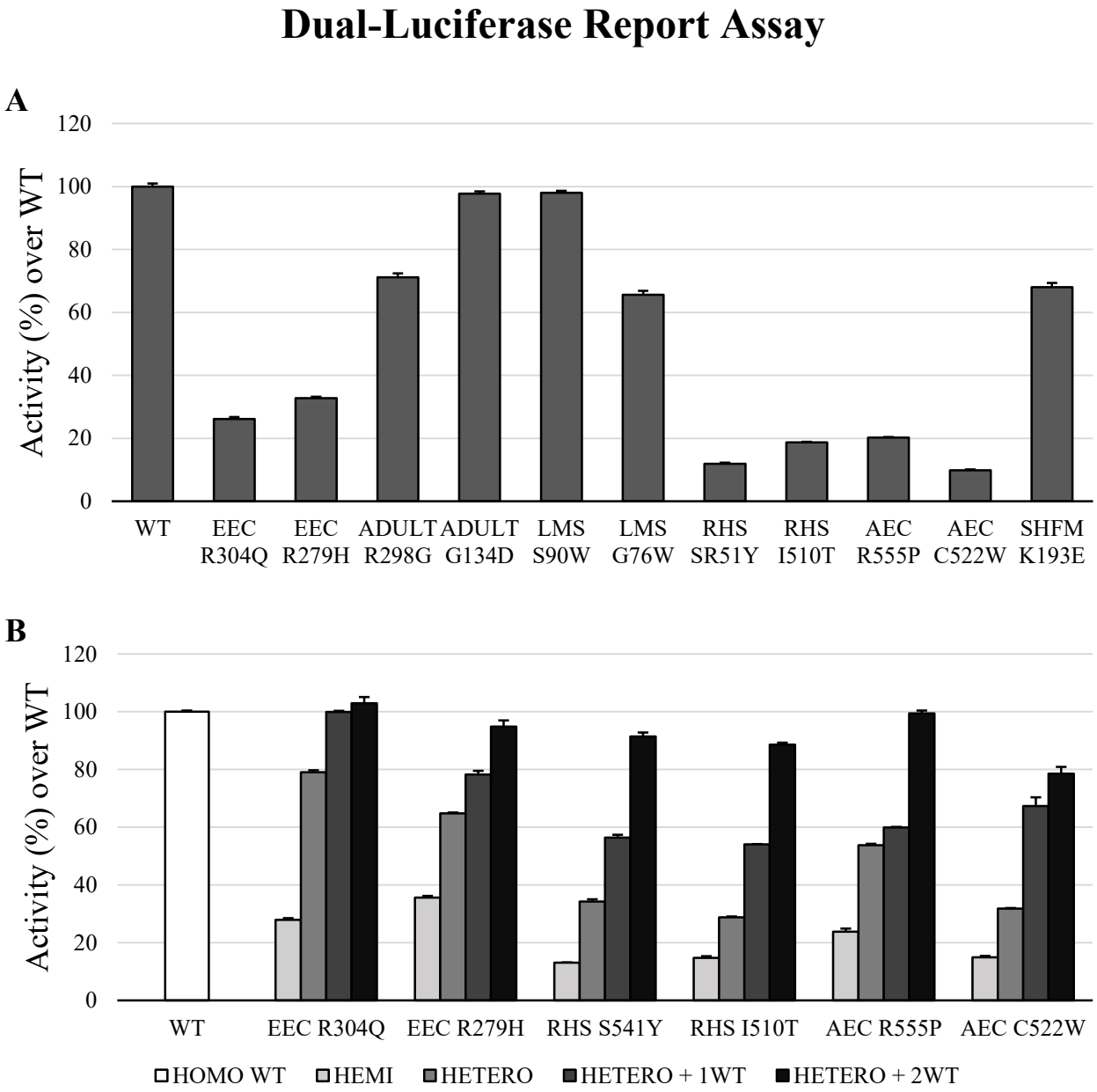

Figure 14
Transactivation potential of WT-ΔNp63α protein and some of its mutants, alone (**A**) or in different combination with each other (**B**) determined by Dual Luciferase Reporter Assay performing transient transfection into HEK 293T cells. Keratin-14 promoter, cloned in the luciferase reporter vector, was cotransfected along with the indicated ΔNp63α expressing plasmid. Transfection efficiency was normalized with a NanoLuc® vector. In graph **B**, homo refers to homozygous-like condition (40ng of WT-ΔNp63α), hemi is hemizygous-like (20ng of mutated plasmid); hetero is heterozygous-like (20ng mutated + 20ng WT); hetero+1WT is heterozygous condition with addition of a dose of WT-ΔNp63α (20ng mutated + 40ng WT) while hetero+2WT with addition of two doses of WT-ΔNp63α (20ng mutated + 60ng WT). Data in graph **A** were normalized on WT, in graph **B** on WT-homozygous model.

mK14.ΔNp63α.EGFP lentiviral vector restricted expression

Lentiviral vector used in mutated JEB keratinocytes to recover the wild-type phenotype (Di Nunzio et al., 2008) was modified, replacing laminin-5 with WT-ΔNp63α fused EGFP cDNA (mK14.ΔNp63α.EGFP). Transduction of different cell lines corroborated well-restricted expression of transgene. Indeed EGFP signal was detected only in cells expressing endogenous p63 such as HaCaT cells, while after transduction of HEK 293T cells tagged signal was absent (Figure 15A and B).

Western blot analysis also confirmed fluorescence data. In both untransduced and mK14.ΔNp63α.EGFP-transduced HEK 293T cells, ΔNp63α protein was absent. On the contrary, ΔNp63α protein levels in HaCaT cells were well detectable and easily distinguishable between endogenous and transduced (because of the presence of tagged EGFP fused to ΔNp63α) (Figure 15E).

Moreover, according to the restriction and high specificity of mK14.ΔNp63α.EGFP lentiviral vector, the transfection of WT-ΔNp63α in transduced-HEK 293T cells was able to activate the transgene expression, causing the appearance of EGFP signal (Figure 15D).

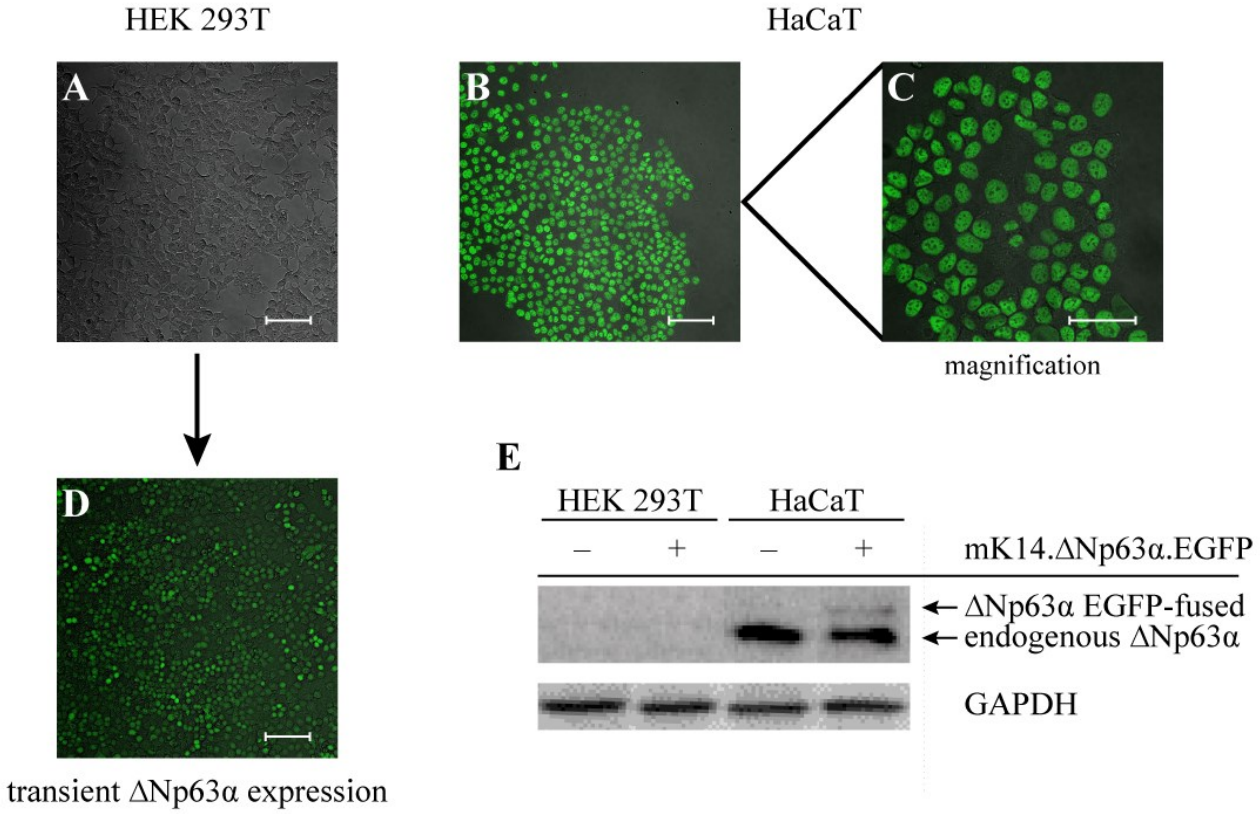

Figure 15
Transduction of HEK 293T and HaCaT cells with mK14.ΔNp63α.EGFP vector. Unlike transduced HaCaT cells (**B**; **C** shows magnification), transduced HEK 293T cells (**A**) don't express transgene. (**D**) The transfection of WT-ΔNp63α was able to activate the transgene expression. (**E**) Western Blot confirms the absence of ΔNp63α protein both in untransduced and transduced HEK 293T. In transduced HaCaT cells, ΔNp63α deriving by vector is easily distinguishable from endogenous because of the presence of tagged EGFP fused. Scale bar in **A**, **B** and **D**: 100µm. Scale bar in **C**: 50µm.

Primary OMESCs transduction

In order to investigate how ΔNp63α addition affected cell identity and growth, transductions of WT- and R304Q-OMESCs were carried out. qPCR performed on harvested cells after transductions showed an amount of ΔNp63α approximately 2-times higher than negative controls (Figure 16A).

Transduced R304Q-OMESCs had a longer life-span and were able to be passaged in culture seven times compared to 5-6 of untransduced cells (identical behavior showed by WT keratinocytes: 12 vs. 10-11 passages). Furthermore, mK14.ΔNp63α.EGFP transduction mitigated the progressive loss of clonogenic cells as also confirmed by the

results of the CFE assay (Figure 16B). In particular, at the last passage of R304Q negative control (6^{th}), transduced R304Q cells were more similar to WT showing increased colony forming efficiency (even more evident in transduced WT OMESCs). Nevertheless, the effect of this lentiviral vector is not everlasting, as a matter of fact both transduced keratinocytes stopped soon after their counterpart.

In addition, EGFP signal permitted to track transgene expression during lifespan. It was evident as ΔNp63α was highly expressed in holoclone-like cells. This expression was drastically lower in paraclone-like cells and further decreased during differentiation in culture passages (Figure 16B).

No significant alterations were observed in both keratinocytes and colonies morphology.

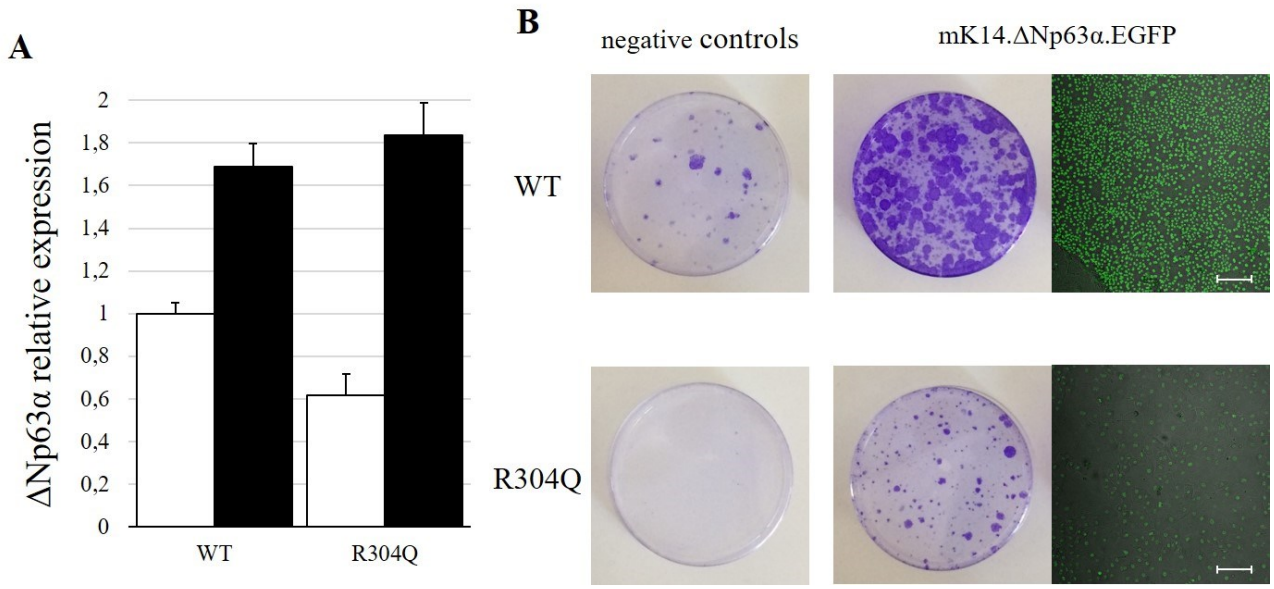

Figure 16
Primary OMESCs transduction. (**A**) qPCR reveals an increase of ΔNp63α expression in both WT and mutated transduced cells compared to negative controls. (**B**) Results of CFE assay at the 6^{th} passage (last passage of untransduced R304Q-OMESCs). mK14.ΔNp63α.EGFP transduction gives better clonogenic potential. Confocal images confirm the high transgene expression in stem cells and its decreasing during differentiation. Scale bar: 100 μm.

DISCUSSION

Various combinations of ectodermal dysplasia, orofacial clefting and limb malformations are the hallmark of p63-associated syndromes. The Ectrodactyly - Ectodermal dysplasia - Clefting syndrome (EEC), caused by mutations in the DNA-binding domain of p63 gene, is the prototype of these syndromes and shows all these three hallmarks. While in childhood clefting and hand deformities are the main clinical features, during adult age ocular problems become the predominant clinical aspect of the syndrome.

p63-related visual morbidity is due to a premature ageing and progressive deficiency of limbal stem cells, causing corneal blindness. As previously demonstrated (Burillon et al., 2012; Nishida et al., 2004), in patients with bilateral limbal stem cells deficiency, transplantation of cultured autologous oral mucosal epithelial stem cells (OMESCs) sheets, could represents an effective alternative to reconstruct the ocular surface.

Compared to healthy controls, EEC-OMESCs showed a more rapid decrease of colony forming efficiency with lower number of cell passages in culture. Organotypic culture generated by mutated cells showed defects in stratification, differentiation and adhesion, resulting in severe tissue hypoplasia and lack of proper tissue polarity.

We have already demonstrated (Barbaro et al., 2016), the potential use of knock-down strategies using siRNA allele-specific silencing. Compared to epithelial sheets generated by EEC cells with placebo treatment, those treated with siRNAs were more similar to healthy and well organized into characteristic epithelial layers, as shown by the expression of polarity and differentation markers.

Thus in young EEC patients, who still have stem cells in the limbus, eye drops containing siRNAs could counterbalance the loss of stem cells. Otherwise in adult EEC patients, who have a more severe limbal stem cells deficiency, gene silencing mediated by continuous supplying of siRNAs could improve the clinical outcomes of autologous oral mucosal keratnocytes graft (Figure 17).

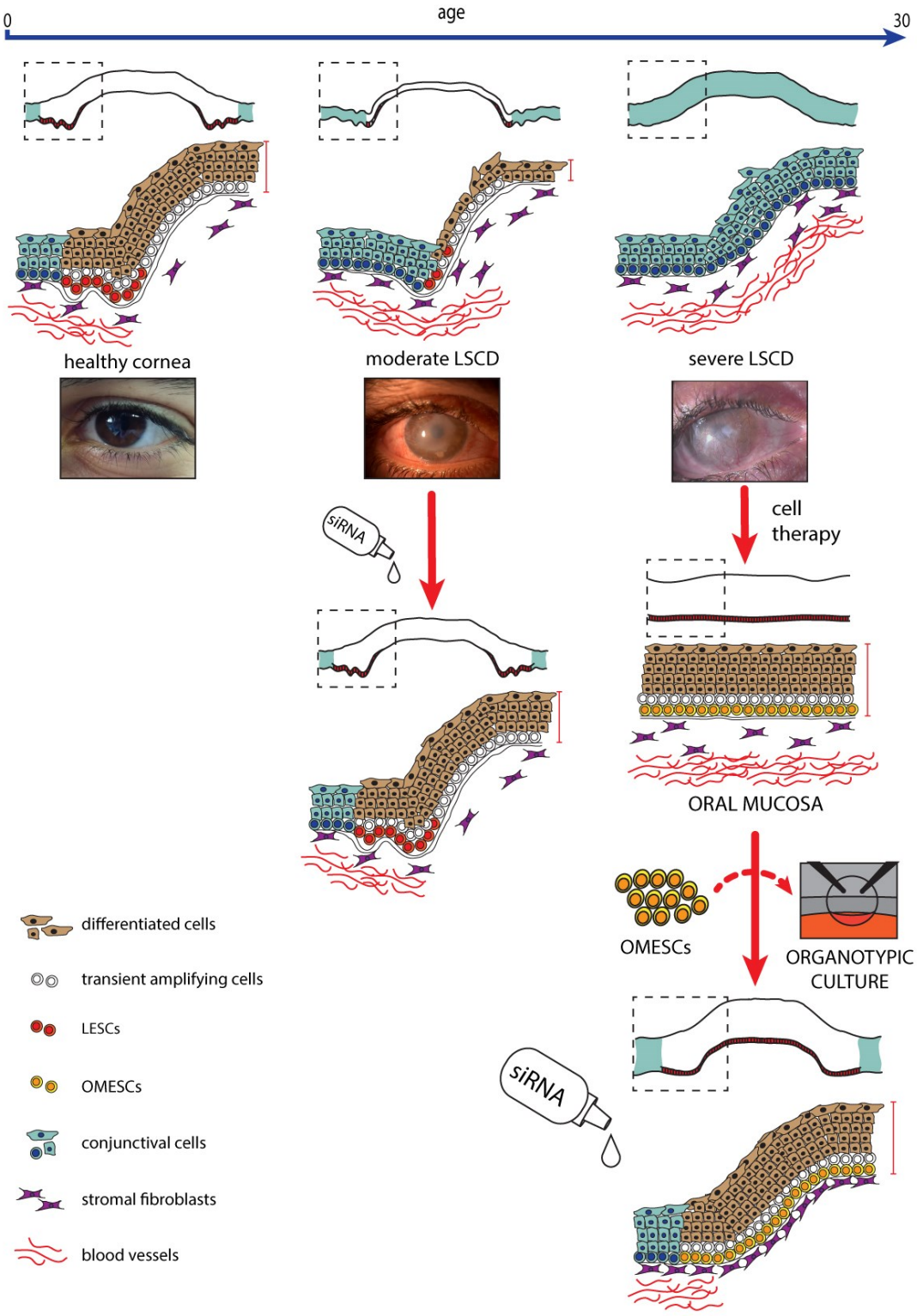

Figure 17
Model of siRNA-based therapeutic approach for LSCD treatment in EEC patients. p63-defective-LESCs have a reduced ability to repopulate the corneal epithelium, which leads to progressive visual impairment culminating in complete bilateral blindness, associated with conjunctivalization. Gene silencing approaches by means of allele-specific siRNAs is potentially a new way to revert the epithelial defects and, eventually, severe visual loss observed in young and adult EEC patients. In young EEC patients, who still have LESCs in the limbus, eye drops containing mutant-specific siRNAs could counterbalance the loss of stem cells. In adult EEC patients, who have a more severe LSCD and no limbus left, gene correction mediated by continuous supplying of specific siRNAs can improve the clinical outcomes of autologous OMESCs graft (Barbaro et al., 2016).

The idea behind our work is that combined gene and stem cell therapy for reconstruction of the ocular surface could be an effective cure for corneal blindness in mutated patients. But the limits of siRNAs approach are that it is a transient and mutant-specific correction. So the ultimate outcome of my doctoral work has been to develop permanent gene therapy approaches to correct the genetic defect and to design a therapeutic strategy using the transplantation of this genetically modified stem cells.

To achieve results I have evaluated several approaches of gene therapy. One of this is gene addition, hypothesys based on using a viral vector in which WT-p63 expression is driven by limbal stem cells-restricted regulatory elements, in order to restore two functional WT-p63 alleles. It was demonstrated that epidermòlysis bullosa, a severe and often fatal skin adhesion defect, can be treated by lentiviral vectors in which transgene expression, in this case laminin-5, mutated in this disease, is driven by cellular promoter with short-range enhancer activity (Di Nunzio et al., 2008). Using a basal cells–restricted regulatory element such as the keratin-14 gene enhancer, the authors demonstrated that transduced stem cells recover their functionality and wild-type phenotype.

Moving towards cornea biology, it was also demonstrated that in this tissue compartment K14 expression is restricted to epithelial progenitor cells, showing an identical pattern of ΔNp63α (Eghtedari et al., 2016). In addition, is well know that K14 enhancer is a direct transcriptional target of p63, so p63 itself could drive transgene expression (Romano et al., 2007). It's important to underline this induction is dependent upon the DNA-binding activity, and p63 mutated protein, abundant in EEC-patients, being unable to bind enhancer is also unable to activate it. Moreover this approach, by acting on the amount of WT-p63 instead that mutated, have the great advantage of been mutation-independent, potentially useful not only in EEC syndrome but in all diseases related with p63 mutations. In fact it was tested, by using luciferase assays, the activation of K14 promoter by WT- or different p63 mutants. Results demonstrate that mutated-p63 are unable to activate the promoter, but this ability is restored by adding increasing amount of WT-p63.

On this basis, it was designed a lentiviral vector in which LTR enhancer was replaced with K14-enhancer (mK14.ΔNp63α.EGFP), in order to obtain high and well basal-layer restricted expression of ΔNp63α, and transduced different cell lines. Transgene expression was restricted to epithelial stem cells, demonstrating the possible use of this kind of vector in therapy approaches of p63-mutated syndromes based on genetic correction of oral mucosal keratinocytes.

Moreover, according to the restriction and high specificity of our vector, the transfection of WT-p63 in trasduced cells without endogenous p63 is able to activate the expression of lentiviral transgene.

Thus mK14.ΔNp63α.EGFP lentiviral vector was used to transduced EEC-OMESCs and healthy controls. Both cells show increase in ΔNp63α RNA expression, and compared to negative controls, transduced cells showed a slower decrease of colony forming efficiency with longer life span.

CONCLUSIONS AND FUTURE PERSPECTIVES

These important preliminary results support the potential use of our lentiviral vector in p63-related disease therapy approaches based on genetic correction of oral mucosal keratinocytes. mK14.ΔNp63α.EGFP lentiviral vector shows lineage and temporal restrictions of transgene expression, allow confining of exogenous WT-p63 expression in stem cells compartment and its physiological downregulation during differentiation. Transduced primary mutated-cells show higher amount of ΔNp63α than negative controls, correlates with higher life-span and CFE assay results.

However, exceed the "dosage" of p63 could be extremely dangerous. In fact ΔNp63α is overexpressed in several epithelial cancers, often as a result of gene amplification (Choi et al., 2002; Hibi et al., 2000; Park et al., 2000). Also, using a lentiviral vector, multiple genome integration could cause genome instability. To overcome these limitations, before the transplantation of genetically modified stem cells (focusing primarily on demonstration of efficacy and safety in animal/human models), a therapeutic window of ΔNp63α should be established, in order to obtain the optimal ΔNp63α amount able to recover keratinocytes functional and phenotypic features.

The final goal will be to perform an autologous graft of genetically corrected OMESCs. Cells harvested from the patient should be transduced with our vector and used to reconstruct a healthy cornea to be transplanted (Figure 18).

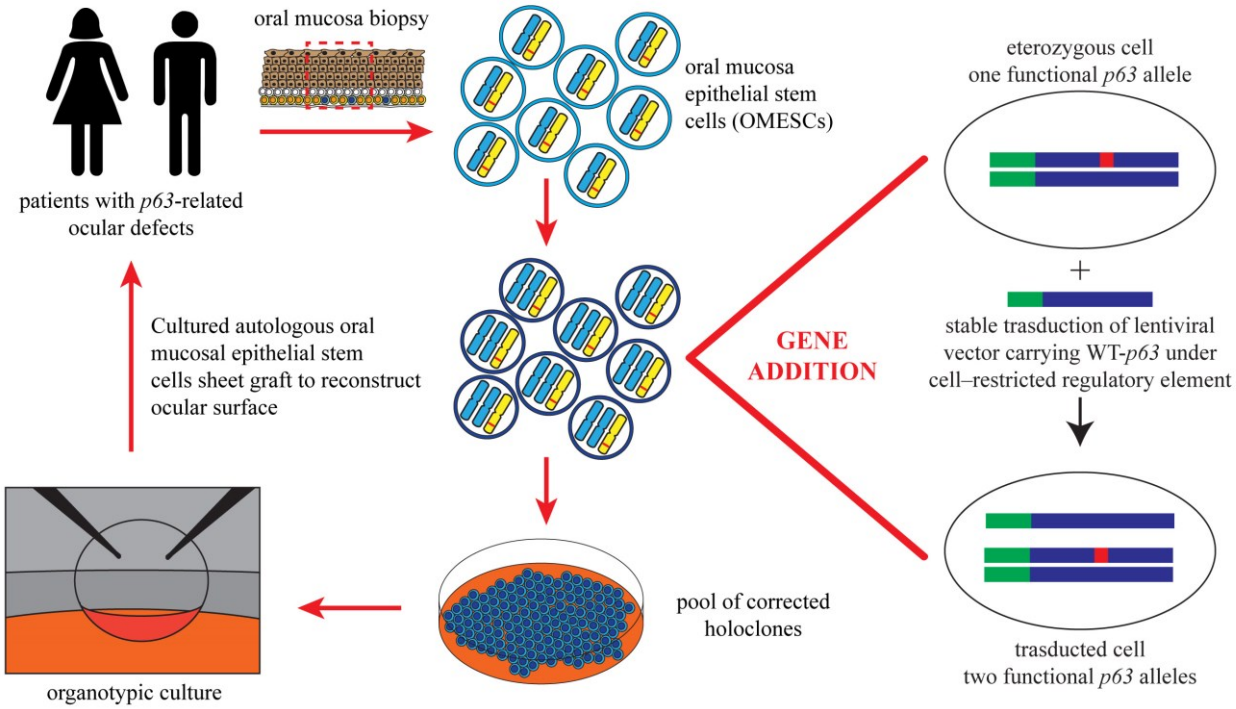

Figure 18
Model of combined gene and stem cell therapy approach to correct ocular defects in p63-related diseases. OMESCs harvested from the patients should be transduced with a viral vector in which WT-p63 expression is driven by cellular promoter with short-range enhancer activity (K14), in order to restore two functional WT-p63 alleles. This approach, by acting on the amount of WT-p63 instead that mutated, have the great advantage of been mutation-independent, potentially useful in all diseases related with p63 mutations. The final goal will be to perform an autologous graft of genetically corrected OMESCs.

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