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MOLECULAR AND REGENERATIVE MEDICINE

PhD School Cycle
XXV

THE NERVE GROWTH FACTOR (NGF) EFFECTS ON THE OCULAR SURFACE

Tutor:

Prof.ssa Graziella Pellegrini

PhD Student:

Athanasios Panaras

Head of PhD school:

Prof.ssa Rossella Tupler

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SUMMARY

The human corneal and conjunctival epithelia are constantly renewed during the life-time due to self-process depending on ocular epithelial stem cells (SCs).

Several ocular diseases (burns, autoimmune/trophic pathologies) may damage corneal and conjunctival SCs, leading respectively to Limbal Stem cells Deficiency (LSCD) and alteration in goblet cells numbers and mucin secretion. Sjögren's syndrome, a severe form of dry eye, and atopic keratoconjunctivitis are ocular surface diseases, which can be treated with NGF.

Nerve Growth Factor (NGF) has a pleitrophic action on ocular surface, exerting trophic support and modulation of corneal and conjunctiva healing. NGF eye drops promote corneal healing in patients with neurotrophic keratitis, often with LSCD, suggesting that NGF exerts a specific action on ocular surface SCs.

The overall aim of this project is to investigate the potential role of NGF and TrkANGFR/p75 NTR for the cell fate of conjunctival and corneal SCs.

In vitro studies were performed on both human conjunctival/corneal epithelial cell lines and primary cultures. TrkANGFR and p75NTR expression are studied in correlation with stemness (ΔNp63α, Bmi1, C/EBPδ), differentiation (14-3-3σ, K3/K12, K19/MUC1 and MUC5AC) and proliferation (Ki67/Edu) markers after NGF treatment. Conjunctival and limbal epithelial cells are exposed to NGF, specific TrkA/p75 inhibitors and siRNAs. The effects on cell fate are evaluated by cellular (colony forming efficiency assay, clonal analysis), biochemical (western blot, RT-PCR, flowcytometry, microarray) and immunohystochemical analysis.

We demonstrated that NGF induces an increase of goblet cells and stimulates the synthesis of the specific "gel-forming" mucin 5AC, essential for the stability of the tear film. In addition we obtained an organotipic culture of

conjunctival epithelial cells on fibrin-fibroblast scaffold, which expresses specific markers of epithelial differentiation.

In limbal epithelial cultures, NGF induces an increase of clonogenic cells and an increased proliferation. We showed that at clonal level NGF maintains the stem cell compartment slowing down the shift from an activated stem cell (early transient amplifying cell) to terminal differentiated cell.

These results may have relevance for basic research, clinical knowledge and pharmaceutical industry, since the project highlights the molecular mechanisms involved in the pathogenesis of ocular diseases, leading to potential therapeutic intervention.

INTRODUCTION

The epithelia of ocular surface

The ocular surface is the interface between the eye and the external environment.

The entire epithelial surface is anatomically consists of cornea, limbus and conjunctiva and is physically continuous with the eyelids and outbuildings such as primary and accessory lacrimal glands, meibomian glands and naso-lacrimal ducts (1).

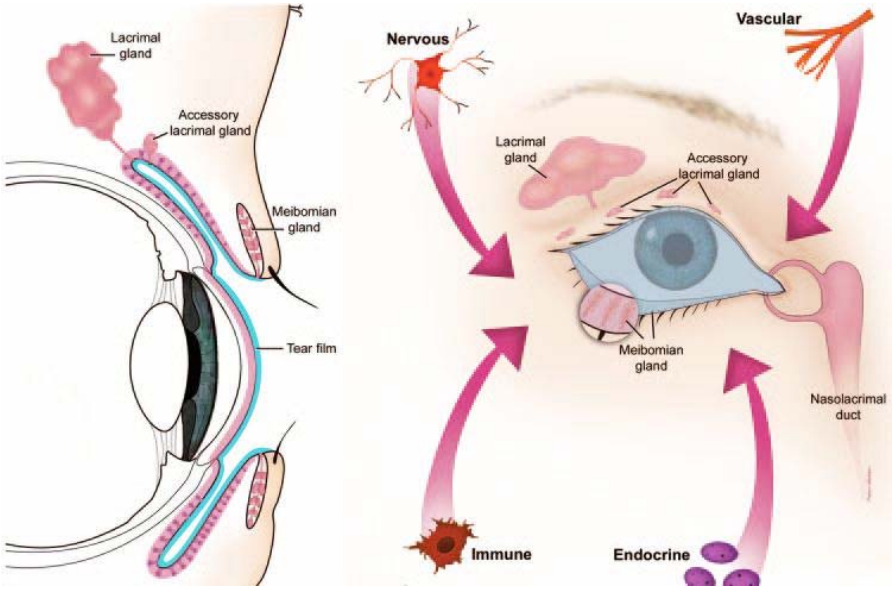


Fig1. System of ocular surface

The primary function of this system is to generate, protect and maintain a smooth refracting surface on the cornea through the production of the various components of the tear film:

- The corneal and conjunctival epithelia produce hydrophilic mucins which retain the tear film on the eye surface

- The primary and accessory lacrimal glands secrete water and protective proteins;
- The meibomian glands or tarsal glands ensure for the lipid layer of the tear film that prevents evaporation
- The nasolacrimal epithelial system absorbs components of tears and adjusts the loss of the latter contributing to maintain an appropriate level of the tear film.

Cornea

The cornea is a transparent avascular connective tissue (and as such immunologically privileged) which acts as a first barrier against the external environment of the eye. It is washed anterior from the tear film (optically important to smooth the micro-irregularities of the epithelial surface front) and posterior from aqueous humor. The human adult cornea measure 11-12mm horizontally and vertically 9-11mm, is about 0.5 mm thick at the center and its thickness gradually increases towards the periphery where it reaches the 0.7 mm. The cornea is replenished by diffusion of glucose from aqueous humor, while it receives oxygen by diffusion from the tear film that absorbs from the air (in reality a small amount of the oxygen demand of the cornea is satisfied for vapor diffusion from humor and limbal circulation).

The cornea consists of three different cell layers and two interfaces:

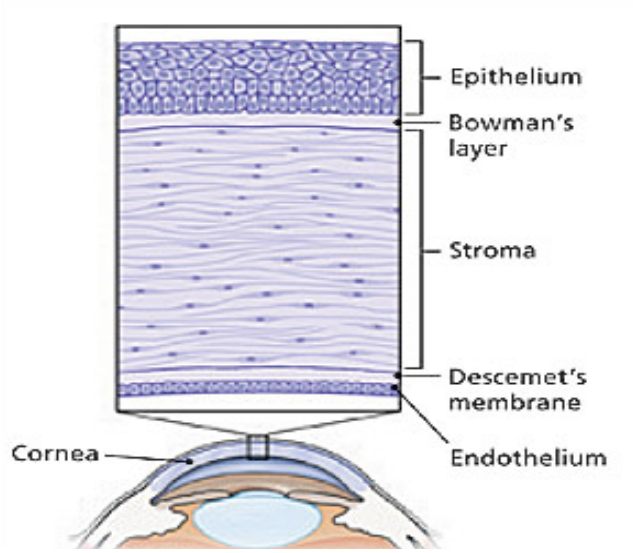


Fig 2. Structure of Cornea

A.

Corneal epithelium: is a stratified squamous and transparent epithelium devoid of goblet cells that rests on the underlying stroma avascular through a basement membrane called membrane Bowman.

The corneal epithelium is composed of three cell layers:

Superficial cells: forming 2-3 layers of terminally differentiated cells, are flat and polygonal and are linked to each other by means of occluding junctions that prevent toxins and microbes to penetrate deep into the cornea. The superficial cells possess numerous microvilli surface and secrete abundant glycoproteins that form a rich glycocalyx: these specializations of plasma membranes increase the surface area available for the absorption of oxygen and improve the hydrophilicity of the surface facilitating the distribution of the tear film [21].

Wing Cells: Underlying the superficial cells there are 2-3 cell layers "wing cells", so named because they possess the wing-like lateral extensions which extend from a more rounded cell body [12]. These cells are found at an intermediate stage of differentiation between the basal and superficial cells.

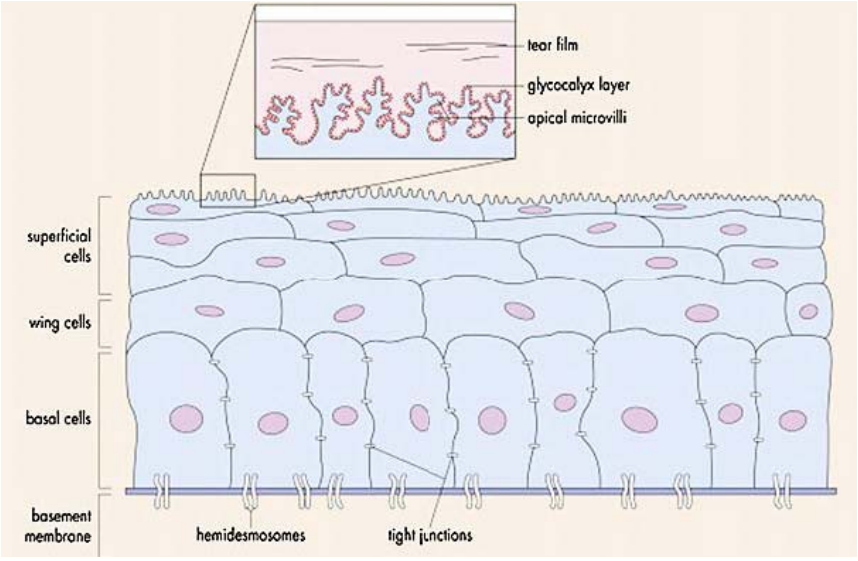


Fig 3. Cell Layers of the Corneal Epithelium

Basal cells: are the source of wing and surfacial cells and form a single layer of cuboidal cells that adhere to the basement membrane via hemidesmosomes. The latter are linked to the anchoring fibrils of collagen VII that cross the basement membrane and reach the corneal stroma, where they form plaques anchor together with collagen I, the principal component of the stroma.

Unlike to other mammals, in which the stem cells are uniformly distributed on the surface of the eye, corneal stem cells in humans are absent in the basal layer of the cornea central and peripheral and localized in the basal layer of the limbal zone.

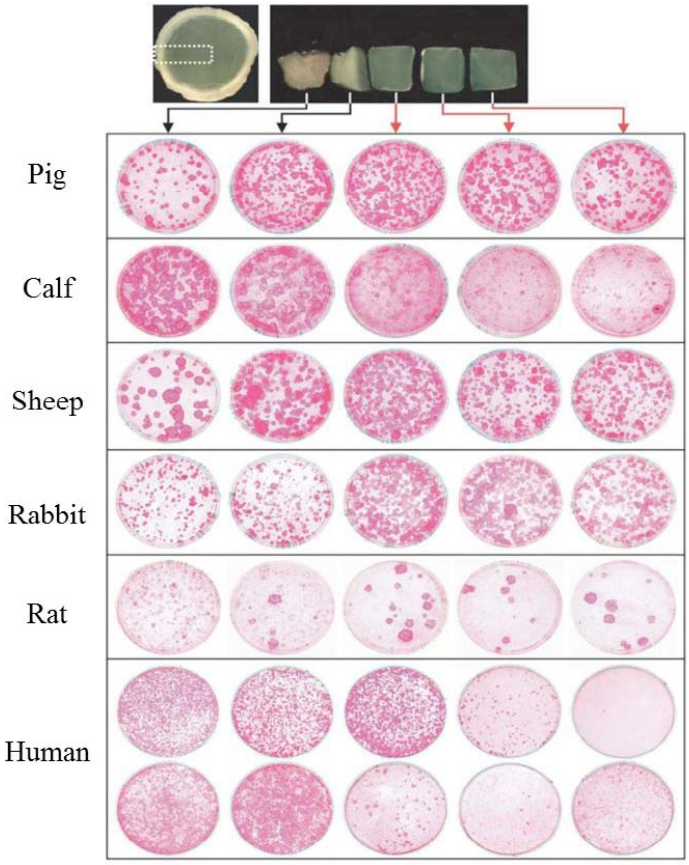


Fig 4. Different location of corneal stem cells in different mammals

Limbus

The narrow transition zone between the cornea and bulbar conjunctiva is called limbus.

In this region, the conjunctival epithelium becomes cuboidal stratified squamous epithelium typical of the cornea and blood vessels of the substantia propria ending in a rich vascular plexus important to provide nutrients and oxygen in corneal and limbal cells.

The limbal epithelium consists of several layers of epithelial cells organized in well-developed rete ridges, celled palisades of Vogt, devoid of goblet cells and populated by Langerhans cells and melanocytes (21) and in human is the niche of the corneal stem cells.

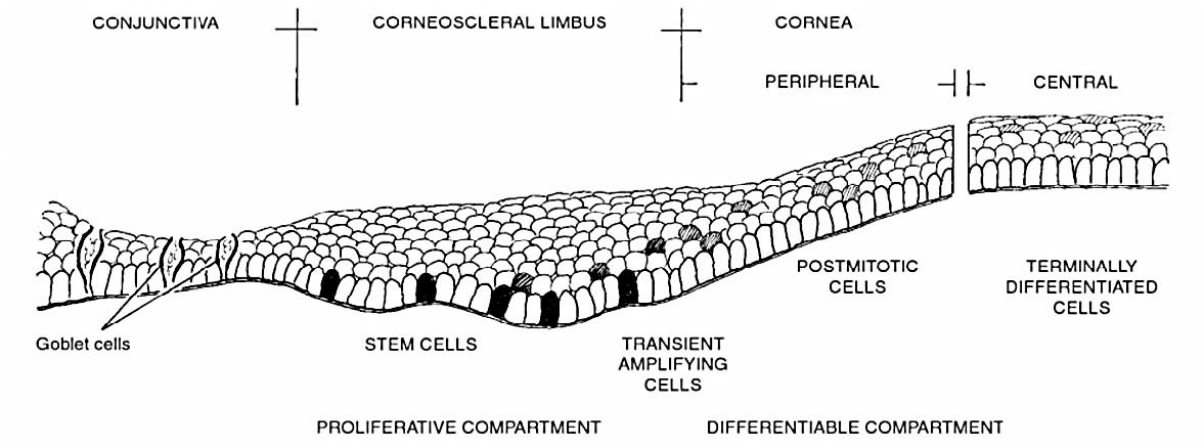


Fig 5. Representation of the limbal region, niche of the human corneal stem cells

During homeostasis, the limbal epithelial cells are thought to act as a barrier preventing conjunctival epithelial cells from encroaching upon the cornea, however following injury or pathological conditions (for example: cicatricial pemphigoid, Stevens Johnson syndrome, chemical burns, etc...) the conjunctiva can migrate to healing the wound on the cornea, this corneal conjunctivalization, causing chronic inflammation, painful corneal opacity and neovascularisation, with vision loss and significant changes in the local micro-environment.

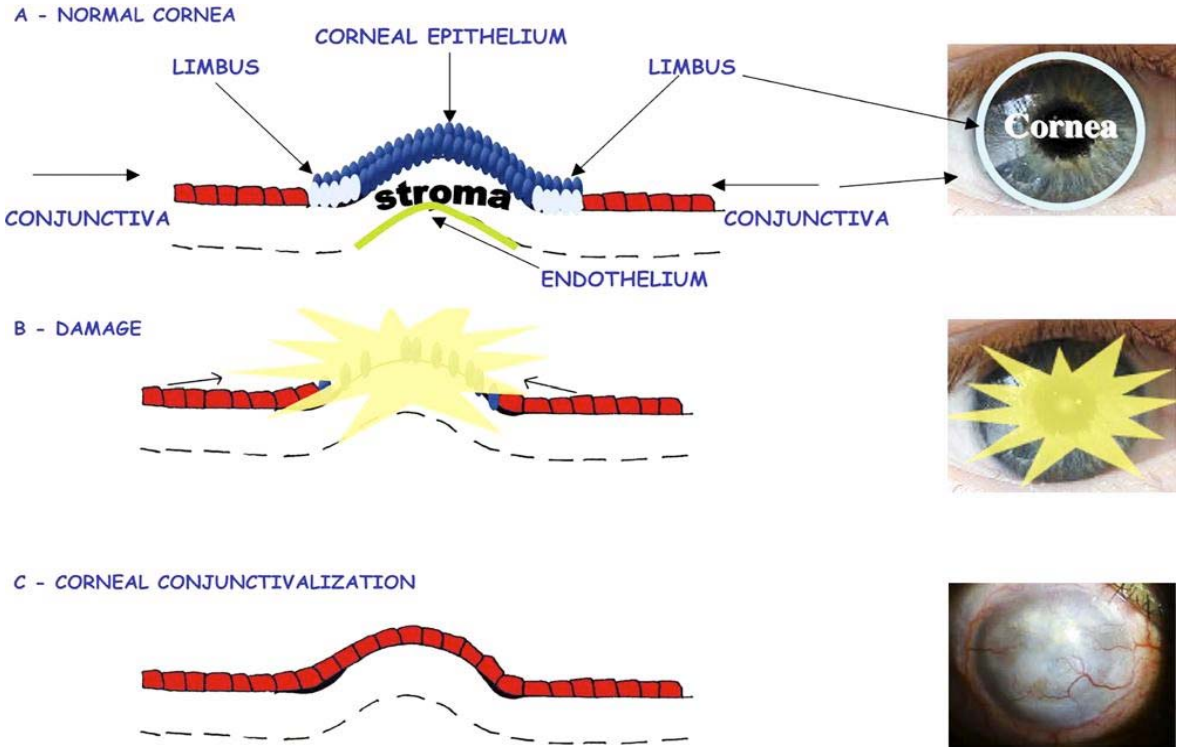


Fig 6. Corneal Conjunctivalizzation

This condition, analyzed by impression cytology, is characterized by the absence of specific markers of the corneal epithelium, a feature used to confirm the diagnosis of "Deficiency Limbal Stem Cells."



Fig 7. Special filter for impression cytology

B.

Membrane of Bowman: represents the basal membrane of the corneal epithelium whose components (mainly collagen type V and VII and laminin, but also proteoglycans, fibronectin and fibrin) are secreted by the epithelial cells themselves. The presence of the basal membrane between the epithelium and the stroma underlying basal allows the polarization of epithelial cells, in addition, this membrane provides a matrix on which cells can migrate and it is thought that this is important for the maintenance of a stratified and well-organized corneal epithelium (11).

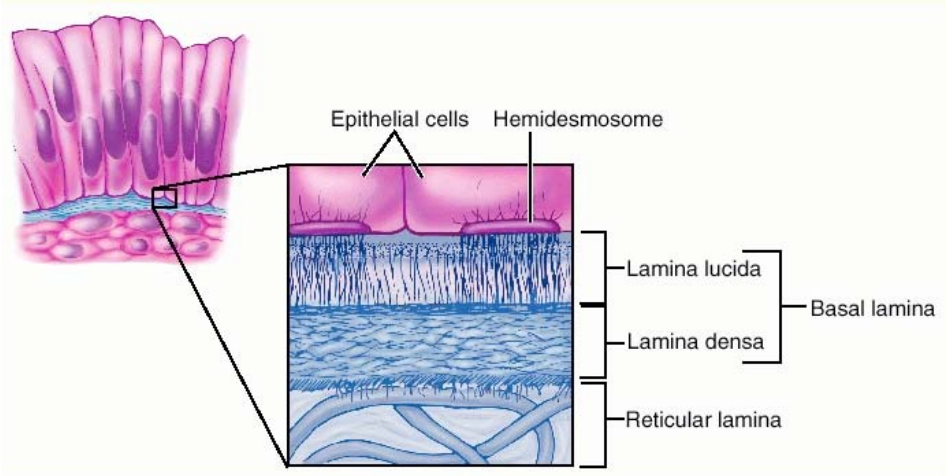


Fig 8. Basement membrane, comprising the basal lamina and reticular lamina.

C.

Corneal stroma or Substantia Propria:

About 90% of the corneal thickness is formed from the stroma, a layer of connective tissue composed of fibroblasts, extracellular matrix molecules and collagen fibers. It is responsible for the strength and shape of the cornea and differs from other collagen structures in the human body in its transparency. The transparency of the cornea is, in fact, mainly attributable to the arrangement of the fibers of collagen in the corneal stroma: these fibers are arranged in bundles called fibrils arranged parallel to each other to form layers or lamellae parallel to the ocular surface.

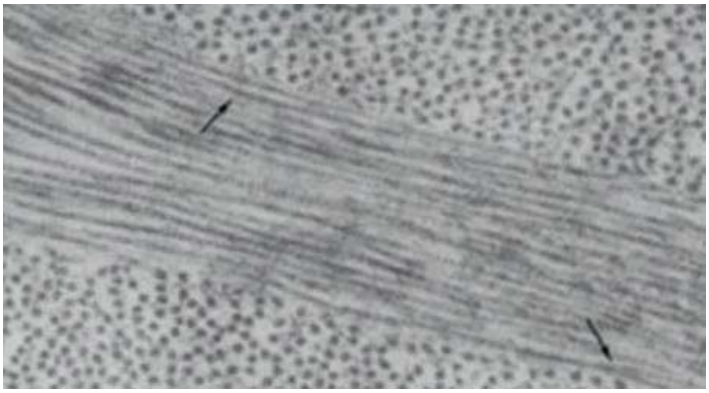


Fig 9. parallel arrangement of Collagen fibrils

Human corneal stroma The stroma of the cornea human contains about 200-250 distinct lamellae, each of which is rotated by an angle of 90 ° in relation to the adjacent collagen fibrils (13): the fibrils belonging to contiguous lamellae run, in fact, in perpendicular direction relative to each others.

Furthermore, the average diameter of each fiber and the average distance between the collagen fibers are relatively homogeneous and smaller than the wavelength of visible light (400-700nm): this anatomical arrangement is thought to be responsible for the fact that the diffusion of a light ray from each fiber collagen is neutralized by interference from other rays diffused, allowing light to pass through the cornea to the retina. If the diameter of collagen fibers or their distance becomes heterogeneous (as in the case of fibrous or edema), the incident rays are randomly spread and the cornea loses its transparency (11).

Stromal collagen fibrils are composed of collagen type I in heterodimeric complex with collagen type V: these complexes are surrounded by proteoglycans that help regulate the hydration and structural properties.

The keratocytes are the main cell type of the stroma and are involved in the maintenance of the extracellular matrix: in fact, they produce collagen,

glycosaminoglycans and metalloproteases crucial for the stromal homeostasis (13).

D.

Descemet's Membrane: constitutes the basal membrane of the corneal endothelium and is composed primarily of collagen IV and VII, laminin 411/511 and fibronectin.

E.

Endothelium or Posterior Epithelium: is formed by a single layer of polygonal cells (in a normal healthy cornea approximately 70-80% of endothelial cells are hexagonal) whose front surface rests on Descemet's membrane, while the posterior surface shape of microvilli projecting into the anterior chamber of 'eye, maximizing the area of the cell membrane exposed all'aqueous humor.

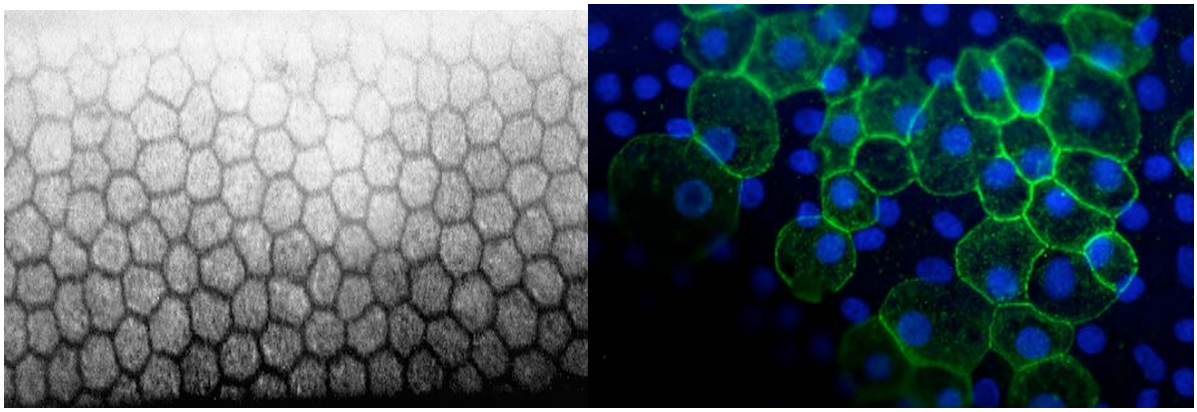


Fig 10. Endothelium

The main function of the endothelium is to regulate the water content of the corneal stroma by means of pumps Na + / K +-dependent ATP present in the basolateral membrane of endothelial cells: the stroma is maintained in a state of relative dehydration because water egresses from the corneal stroma following an osmotic gradient that goes from a relatively hypo-osmotic stroma toward a relatively hypertonic aqueous humor.

This passive bulk fluid movement is guaranteed by a process of active transport of ions necessary to generate the osmotic gradient.
the two most important ion transport systems are the membrane-bound Na+ and K+-ATPase sites and the intracellular carbonic anhydrase pathway. Activity in both these pathways produces a net flux of ions from the stroma to the aqueous humor.
The human corneal endothelium is not able to regenerate and has only limited repair capabilities: if the endothelial cells are destroyed by disease or trauma, those cells remain rapidly replace the injured region by enlargement ensuring the continuity of the endothelial barrier.
The transparency of the cornea also depends on a precise degree of hydration required to maintain the corneal stroma in the correct spatial orientation: the transport mechanisms present in the endothelium are, therefore, essential to adjust the corneal hydration and its transparency.
Without the correct pump function carried out by the endothelium, stroma would develop an edema and becoming swollen with water and assuming an opaque appearance.

The cornea performs several important functions:

- The corneal epithelium forms an effective mechanical barrier that protects the inner eye from the external environment: this barrier is the result dell'interdigitazione of cell membranes and the formation of occluding junctions between adjacent cells.

- Together with conjunctiva and lacrimal film, cornea protects the ocular surface by pathogens.

- Acts as a main refractive element of the eye, directing the incoming light towards the lens, which in turn focuses it on the retina.

Conjunctiva

The conjunctiva is a mucous membrane that covers the inner surface of the upper and lower eyelids and extends up on the surface of the sclera until the limbus.

It is a highly reactive tissue protected by a powerful immune system: is rich in blood vessels, connected to the lymphatic system and contains numerous immunocompetent cells such as Langerhans cells and lymphocytes.

The conjunctiva is typically divided into three regions called palpebral conjunctiva (covering the inner surface of the eyelids), conjunctiva of the fornix (represented by recesses forniceali upper and lower) and bulbar conjunctiva (the region between tubes and the limbus that covers the sclera) (17).

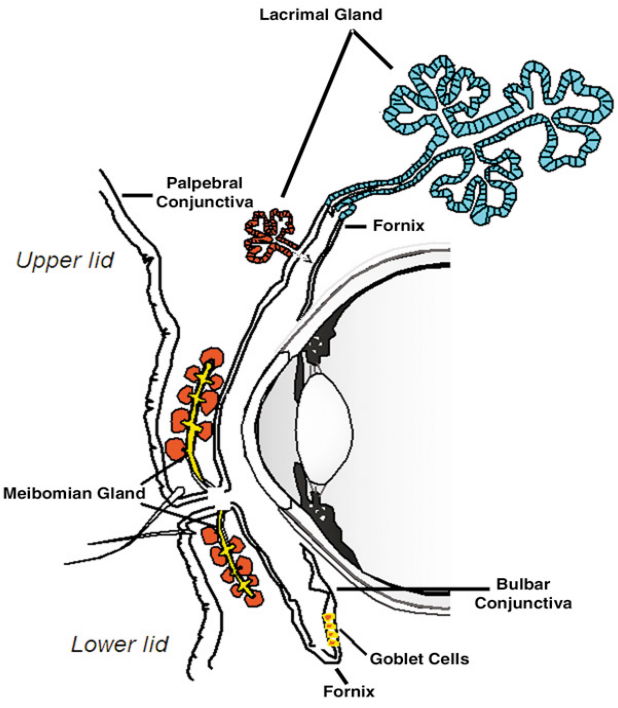


Fig 11. Schematic representation of the three major subdivisions of the conjunctiva.

Palpebral conjunctiva: starts at the level of the muco-cutaneous junction of the eyelid margins and includes the marginal region (extending from the tarsal glands and continues on the inner surface of the eyelid up to the subtarsal groove, a shallow groove marking the lower limit of the tarsus), the tarsal region (it is highly vascularized and strongly adhered to the underlying tarsus) and the orbital (extends to the fornices, which mark the line of transition of the eyelids to the eyeball).
The palpebral conjunctiva is constituted by a cuboidal epithelium that becomes keratinized beyond the margin of the eyelid, where the surface is not covered with fluids but is continuously exposed to air.

Forniceal Conjunctiva: is formed by a columnar epithelium and represents the transition zone between palpebral and bulbar conjunctiva.
Is divided into upper and lower conjunctival fornix, and can be folded into folds; in the lateral part of the upper fornix flow into the excretory ducts of the lacrimal glands.

Bulbar conjunctiva: covers the anterior sclera and continues with the limbal epithelium. The epithelium of the bulbar conjunctiva is attached to a thin basement membrane, discontinuous at several points, via relatively few hemidesmosomes.

From a structural point of view, the conjunctiva is divided into the following layers:

Epithelium: consists of several cell layers characterized by different organization and stratification respect to the cornea and a different production of mucins (for example, the MUC5AC); this epithelium allows the movement of the eyelid on the cornea and is vascularized. Conjunctival epithelial cell

morphology differs in different areas: the tarsa zone is cuboidal stratified, become columnar to the fornix and cuboidal again on the eyeball. The apical cells of the conjunctival epithelium did not look flat but have numerous microvilli and numerous vesicles in their cytoplasm containing mucins responsible for the formation of the glycocalyx. The distinctive characteristic of this epithelium is that contains goblet cells, intercalated in it (representing approximately 5.10% of the cells above-baseline): goblet cells are unicellular glands originate from the conjunctival epithelial cells that synthesize, store and secrete high molecular weight glycoproteins, mucins, essential for maintaining the integrity of the ocular surface (especially MUC5AC).

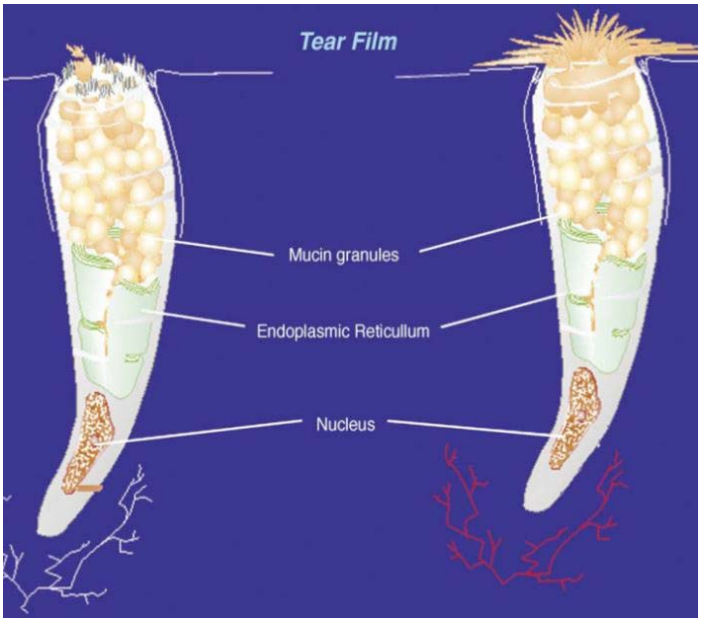


Fig 12. Schematic representation of goblet cells

Mucin deficiency is, in fact, involved in various diseases of the eye such as Stevens-Johnsons syndrome, neurotrophic keratitis, ocular cicatricial pemphigoid, dry eye syndrome, etc...

The nucleus and cytoplasmic organelles of the goblet cells are located toward the basal membrane, while the secretory granules are accumulate in the apical region conferring to these cells the characteristic shape of a goblet.

Basement membrane: consists mainly of collagen type IV and binds the conjunctival epithelium to the underlying sclera.

Sub-conjunctival layer: at the fornix conjunctiva and a large part of the bulbar conjunctiva, in place of the epithelium follows the subconjunctival layer, is a layer of fibrous connective tissue which gives considerable mobility to underlying layers (stroma) of conjunctiva (19).

Stroma or Sclera: conjunctiva is based on a fibrovascular connective tissue called sclera that provides structural support to the eye. Stroma varies in thickness and density: in tarsal conjunctiva the substantia propria is slim and compact while in fornix is thick and poorly attached to the globe.

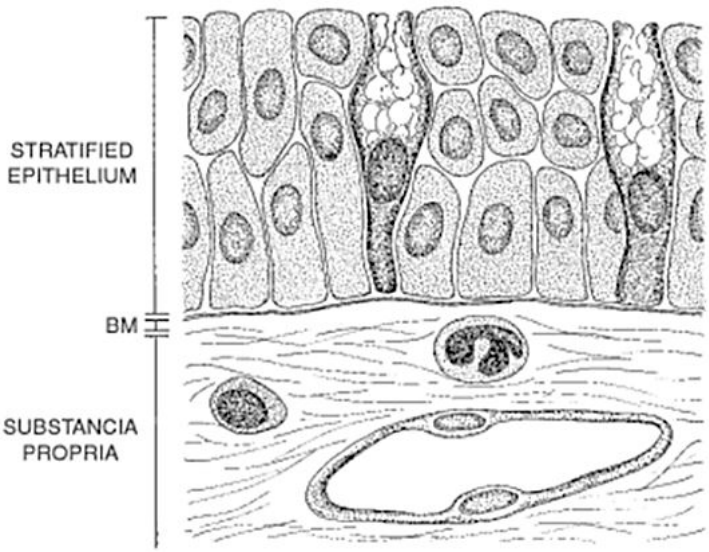


Fig 13. Conjunctival Structure

Substantia propria is constituted by superficial layers of connective tissue poorly interconnected and from deeper layers of fibrous tissue containing blood vessels, lymphatics and nerves.

Like in cornea, the stroma of sclera is composed primarily of collagen densely packed within a matrix of proteoglycans mixed with sporadic elastic fibers and fibroblasts.

However, unlike cornea, the fibrils of collagen in the sclera show a wide variation in terms of diameter and length and lamellae are extensively interwoven: this organization of the fibers leads to an increase of the dispersion of light rays that is responsible for the white opaque appearance of sclera (18).

The conjunctival epithelium has different characteristics from the corneal epithelium:

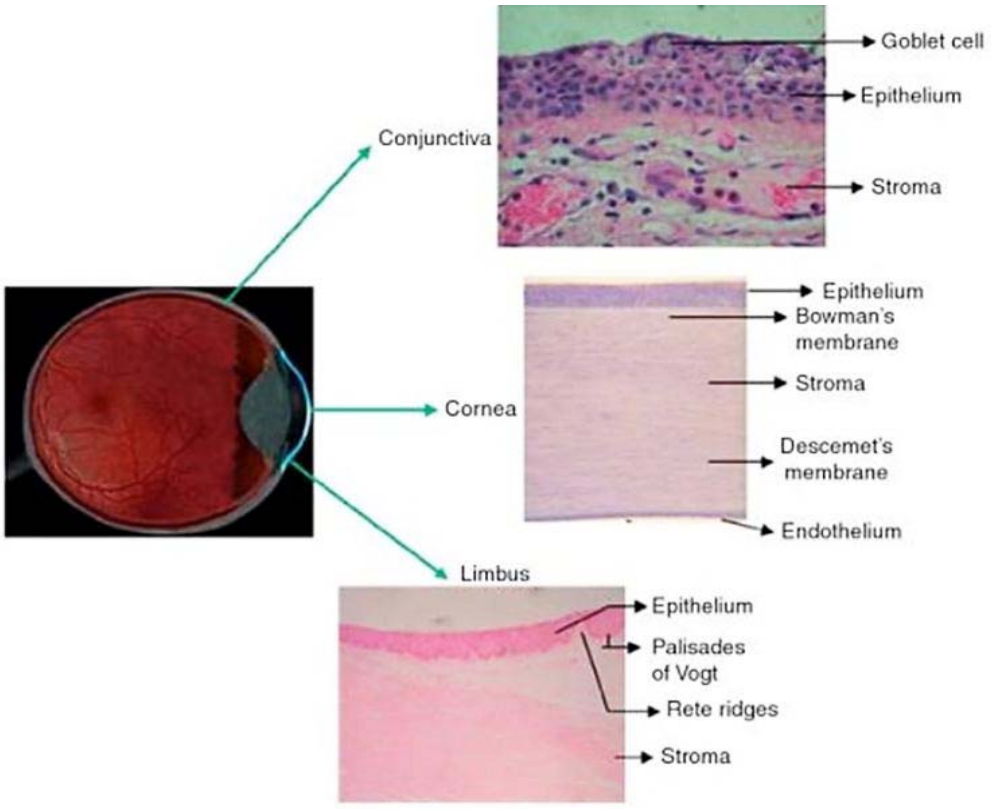


Fig 14. Histological differences between the conjunctiva, cornea and limbus

- cornea is a transparent, smooth, reflective, reflective surface without blood vessels, and the conjunctiva is a translucent, irregular and vascularized;

- cornea is devoid of goblet cells, while the latter are numerous in the conjunctiva;
- The corneal epithelium is often 5-6 cell layers with a regular progression from the basal cells to wing cells to those superficial cells on a avascular stroma, while the conjunctiva consists of 6-9 layers of cells arranged in an irregular manner on a well-vascularized stroma;
- The cornea receives nutrients and oxygen by diffusion from the anterior chamber, from the tear film and from the limbal vessels, while the conjunctiva is supplied directly by its own blood vessels;
- conjunctival stem cells, are uniformly distributed in the conjunctiva, are bipotent because they are capable of giving rise to epithelial cells and cells muciparous conjunctival epithelium, while the corneal stem cells, localized in the limbal region, are unipotent because they differentiate exclusively in corneal epithelial cells.
- Unlike conjunctiva, corneal epithelium is avascular and this feature is guaranteed by the balance between angiogenic factors and anti-angiogenic: the corneal epithelial cells, in fact, express the VEGF (Vascular Endothelial Growth Factor) but the action of this factor is blocked by the simultaneous presence in the tear film of a soluble form of its receptor VEGFR that sequesters endothelial growth factor preventing the stimulation of proliferation and migration of endothelial cells towards the cornea.
- These differences must be taken into consideration in the study of the pathophysiology of cornea and conjunctiva because they allow to explain some of the events that occur in the ocular surface of eyes suffering from specific diseases (for example, the migration of blood vessels in the cornea following damage to epithelium limbus-corneal) and to understand better the role that stem cells may be local in the resolution of these diseases.

Tear Film

Tear film is the main defense of the corneal surface from microbial invasion as well as from chemical damage, toxic or foreign bodies. In addition, provides growth factors and vitamins that are important for the proliferation and repair of the corneal epithelium and conjunctiva.
Several hypothetical models have been proposed to describe the composition of the tear film.

The first models showed the tear film as consisting of three distinct layers (3):

- **Internal mucous layer**: it is composed of mucins secreted by goblet cells of conjunctiva (MUC5AC) and the transmembrane mucin (MUC1 and MUC4) which extend from the apical surface of the corneal and conjunctival epithelial cells to form the glycocalyx. The mucins of glycocalyx make entire ocular surface hydrophilic allowing the aqueous layer to spread over the eye, in this way mucins provide protection minimizing the trauma on ocular surface during blinking (without glycocalyx the tears did not stick accurately on eye and the epithelial cells can be damaged).

- **Aqueous intermediate layer:** is secreted by primary and accessory lacrimal glands and is composed of electrolytes (sodium, potassium, magnesium, calcium, chloride, bicarbonate and phosphate ions: they

are responsible for the osmolarity of tears, keep the pH relatively constant and play an important role in maintaining epithelial integrity), proteins (lysozyme, lactoferrin, lipocalin, defensins, immunoglobulins, serum albumin, etc ...), enzymes, growth factors (EGF, TGFα, HGF, bFGF, PDGF, etc ...), vitamins (the vitamin A is essential for the integrity of the ocular surface epithelia), antimicrobial substances, cytokines and hormones [6]. The aqueous layer is responsible for creating an appropriate environment on ocular surface for the epithelial cells, carrying essential nutrients and oxygen to the cornea that is not vascularized and remove from it cellular debris, toxic elements and foreign objects: changing the composition of the aqueous layer,can affect the health, proliferation, maturation and movement of cells of the ocular surface.

- **External lipid layer:** is produced by the meibomian glands (or tarsal glands). Each gland consists of a rectilinear duct, which crosses the tarsus in its entirety, with numerous lateral diverticula, which opens onto the eyelid margin free through a tiny orifice. The oily secretion produced by these glands is distributed on the lid margins, creating thus a lipid layer on tear film when palpebral open. The main function of this layer is to prevent the evaporation of tears and ensure the stability of the film lacrimal.The lipid layer is constituted by two phases: an outermost relatively thick layer containing non-polar lipids such as wax esters and triglycerides, and an inner thin polar layer consisting primarily by phospholipids.

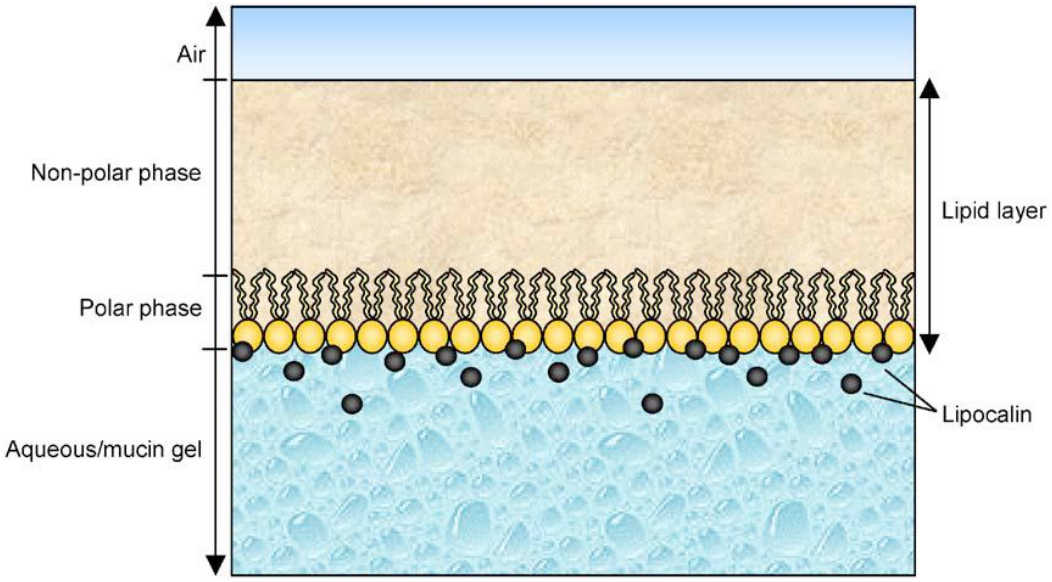


Fig 15. structure of lipid layer of the lacrimal film

However, considering the solubility and the hydrophilicity of mucins in the aqueous solution, it is unlikely that can exist a clear separation between the mucous layer and aqueous layer of the tear film.

The newer models suggest, therefore, that the aqueous layer is actually a mixture of lacrimal fluids and mucins without a precise distinction between of those.

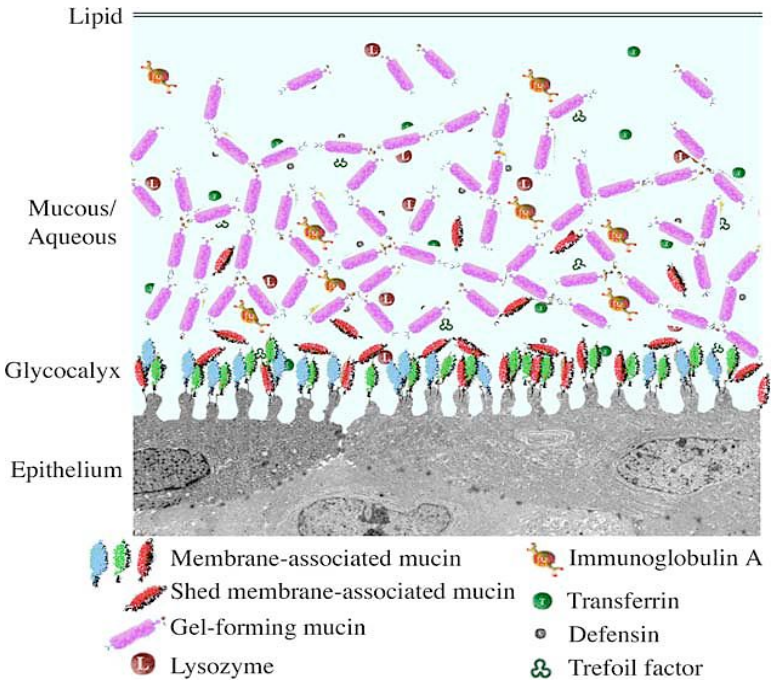


Fig 16. Structure of lacrimal layer

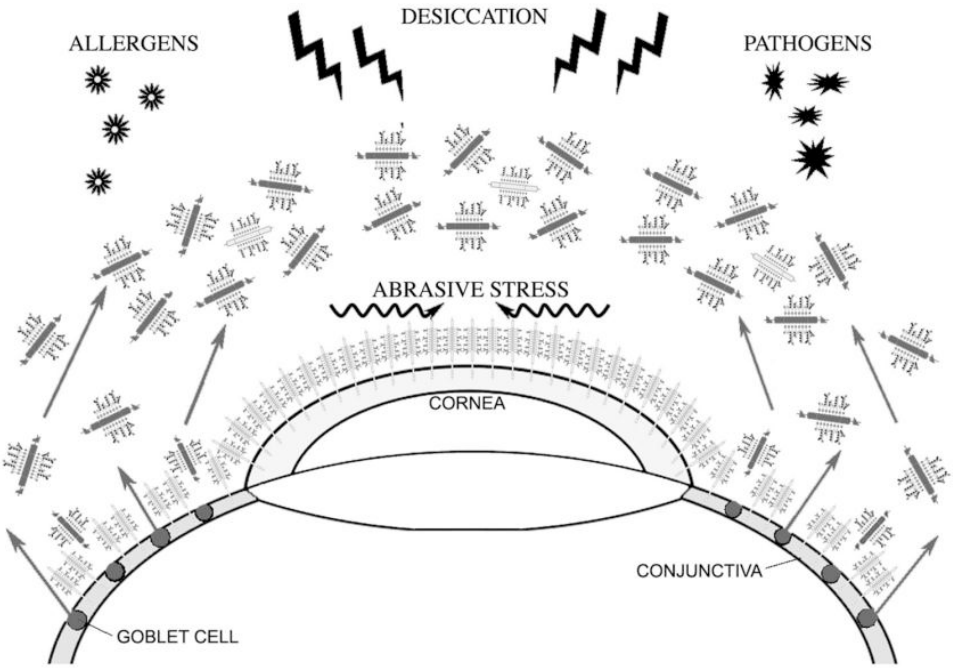


Fig 17. Protective functions exercised by the mucins of tear film

Mucin is a glycoprotein that has, within its protein backbone, of consecutive repetitions of amino acids rich in serine and threonine, which provide sites for O-glycosylation.

Each gene has a unique sequence for mucin "tandem repeat" and its alleles vary in the number of repetitive elements resulting in polymorphism within the same individual and between individuals (2).

The extensive O-glycosylation of the mucins begins with the binding of an N-acetyl-galactosamine (GalNAc) to a serine or threonine on tandem repeats from part of a family of glycosyl-transferase called polypeptide-GalNAc-transferase (pp-GalNAc-ts).

Extension of the sugar chain by the sequential addition of carbohydrates is obtained by the action of different glisosil-transferase which are cell-specific and tissue-specific.

As a result of this important glycosylation, more than 55% of the mass of the ocular mucins is composed of carbohydrates (5).

Mucins are important structural and functional components of the tear film and are produced by corneal epithelium, and from the conjunctival epithelium and goblet cells.

Goblet cells are, unicellular glands containing numerous granules in the apical region filled of MUC5AC in a manner that be secreted on the ocular surface (7).

MUC5AC molecules are tightly packed into the secretory granules because the repulsion forces between mucins created by anionic carbohydrates are neutralized by the highly cationic microenvironment of the granules (levels of ions calcium into the secretory vesicles are three times higher than those of 'external environment), but with its secretion the cationic neutralization is lost and this allows to hydrate and expand the mucins in the tear film (10).

In humans have been identified 18 different mucins divided into two classes depending on the presence or absence of a transmembrane domain in the carboxy-terminal region of the molecule:

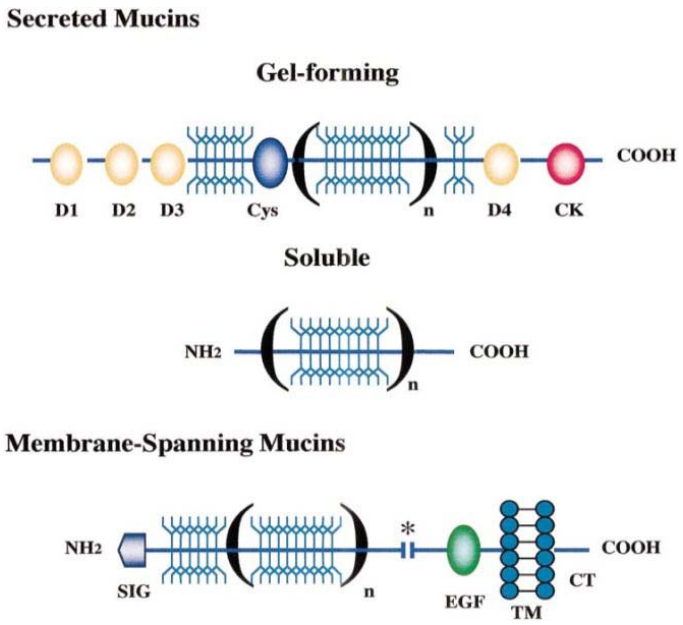


Fig 18. Comparison of the structural motifs of mucins secreted and those associated with the membrane

Mucins membrane-associated: (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21): these mucins contain a small cytoplasmic tail, a hydrophobic transmembrane domain that anchors to cell membrane, and an extracellular domain highly glycosylated that contributes to formation of glycocalyx on apical surface of corneal and conjunctival epithelial cells. The cytoplasmic tail of these mucins is highly conserved across mammalian species and appears to be able to interact with certain intracellular proteins: for example, the tyrosine residues in the cytoplasmic domain of the MUC1 that can be phosphorylated and, as those of MUC12, may participate a cascade of signal transduction by interacting with proteins containing the SH2 domain that recognizes phosphotyrosines.

Mucins Secreted: are incorporated in the mucous layer of the tear film and, depending on their ability to form polymers, are further divided into two subcategories, **soluble mucins** (MUC7 and MUC9) and **gel-forming mucins** (MUC2, MUC5AC, MUC5B, MUC6, MUC19). The latter are characterized by a large central sequence "tandem repeat" flanked by domains rich in cysteines (homologous domain D of the so-called von Willebrand factor) which allow the polymerization of individual molecules of mucin through formation of disulfide bridges (this allows the formation of macromolecular structures with an average molecular weight greater than 40MDa).

It is also interesting to note that the genes for these mucins are grouped in the same locus on chromosome 11p15.5 (9).

The soluble mucins are the smallest mucins known, they lack the domains rich in cysteines and are, therefore, found as monomers.

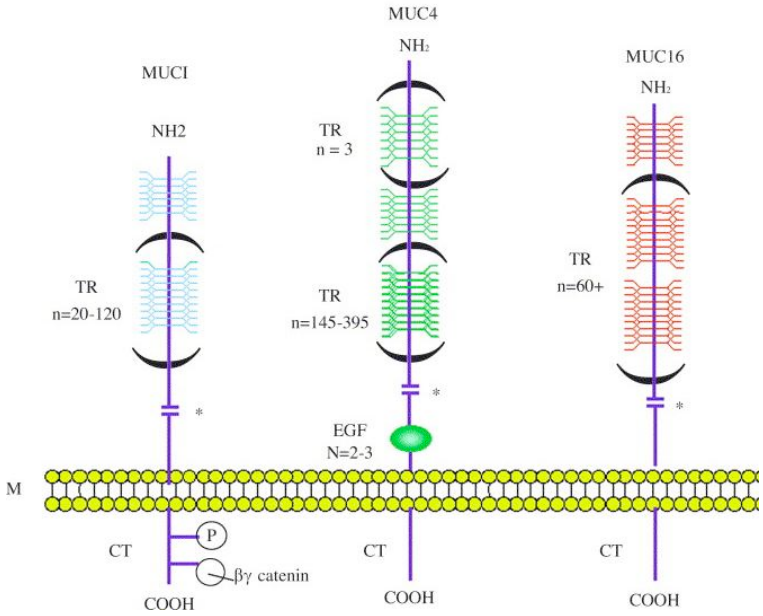


Fig 19. Mucins associated to the cell membrane

Corneal and conjunctival epithelia express at least three types of transmembrane mucins: the MUC1, the MUC4 and MUC16. These mucins are concentrated on the ends of the apical microvilli of suprabasal cells, where they form a dense glycocalyx interface epithelium-tear film but they can also be released and find them in tears.

Mucins MUC1 and MUC16 are present in both corneal and conjunctival epithelia and interact through their cytoplasmic tails with cytoskeletal proteins. The MUC4 is, instead, prevailing in the conjunctival with a gradual decrease towards the central corneal epithelium (5).

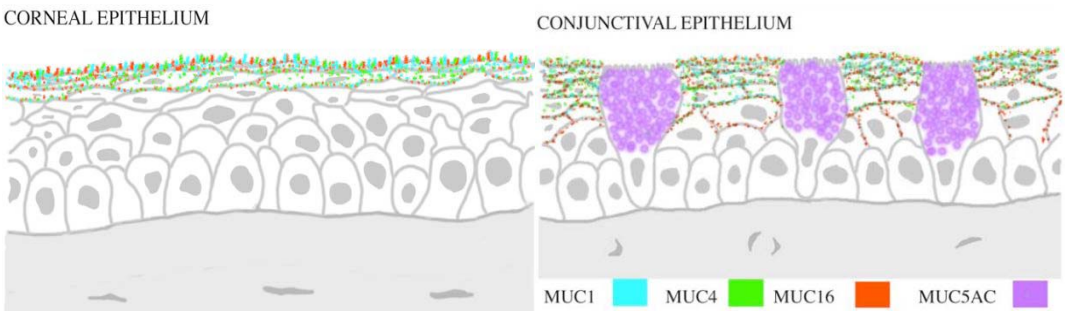


Fig 20. Distribution of mucins in corneal and conjunctival epithelium

Among the mucins present in the tear film there are, MUC5AC (is the most abundant and is produced, stored and secreted by goblet cells interspersed in the conjunctival), MUC2 (present at low concentration) and MUC7 (produced by the tear glands and from the conjunctiva).

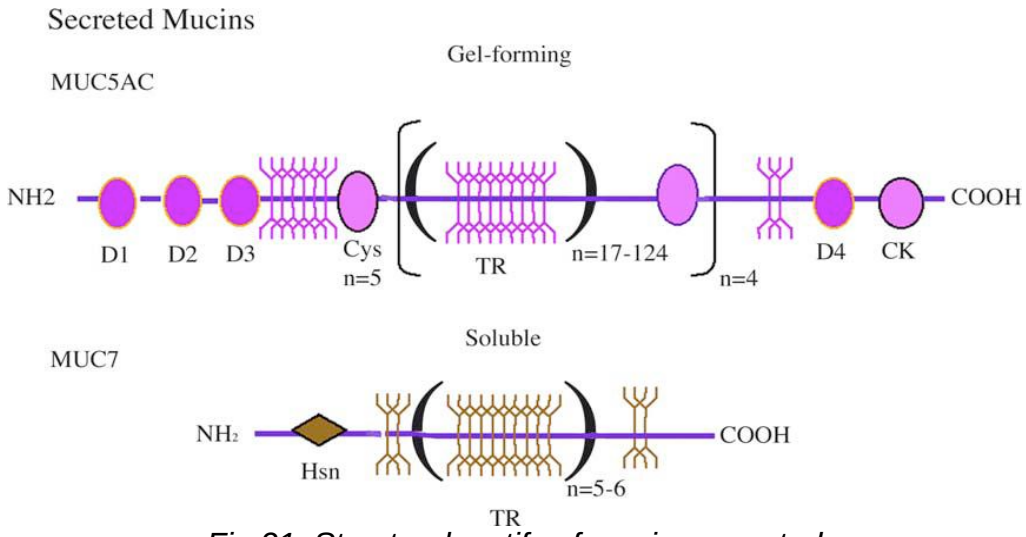


Fig 21. Structural motifs of mucins secreted

In addition MUC5AC playing an important role in maintaining the ocular surface moist, stabilizes the tear film, retains the defensive molecules secreted by the lacrimal glands and provides a physical and chemical barrier that protects the ocular surface from pathogens, dehydration, chemical, mechanical and thermal injuries.

The two major classes of mucins perform different functions (5):

- **Mucins Secreted:**

1. Removal of pathogens, allergens and cellular debris: The gel-forming mucins have the ability to trap allergens and cellular debris by facilitating their removal from the ocular surface, as a result of their extensive glycosylation, these mucins also show a large number of epitopes that can act as receptors

for ligands present on the surface of certain microorganisms and viruses and prevent engraftment to epithelial cells.

2. Lubrication: Thanks to their ability to retain water, mucins secreted can form a highly hydrated gel which stabilizes the tear film, generates a refracting and smooth surface with high optical quality over the cornea, lubricates epithelial surfaces by reducing friction during wink and prevents dehydration

3. Antimicrobial activity: it was noted that a peptide of 20 amino acids of MUC7 possesses a potent antimicrobial and antifungal activity, so it is possible to assume that also other mucins may have similar properties.

- **Mucins membrane-associated:**

1. Dis-adhesive Function: the secreted and transmembrane mucins that are highly glycosylated , and so have negative charges that prevent them to interacting with each other. This repulsion allows the movement of mucins secreted by goblet cells on mucins associated with conjunctival and corneal epithelial apical membranes, with consequent prevention of possible microtraumas to the ocular surface via the reduction of abrasive stress during blinking and preventing the adhesion of epithelial surfaces to overlap (for example, the corneal epithelium and the palpebral conjunctiva) during wink or sleep.

2. Formation of a chemical and physical barrier that prevents the penetration of pathogens into the ocular surface.

Stem Cells of the Ocular Surface

The surface epithelia are renewed constantly during the life of an organism (for example, human epidermis is renewed every month or so, while the corneal epithelium every 9-12 months) thanks to presence of stem cells and progenitor cells that are the unique proliferating cells in a normal adult tissue.

Adult stem cells are those cells in an adult tissue that have the function to generate and regenerate the cells of that tissue throughout the life of the organism.

Stem cells have the unique ability to self-renew and to generate progenitor cells "committed", known as "transient amplifying (TA) cells", which differentiate into cell lineages of the tissue of origin after a limited number of cell divisions.

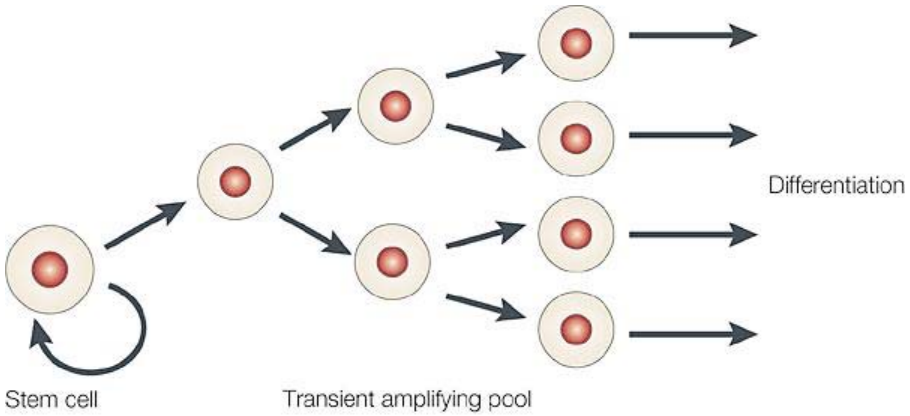


Fig 22. Self-renewal and differentiation of stem cells

The replication of a stem cell usually gives rise to another stem cell, which remains in this maturational state, and to TA cell that multiplies rapidly but has a limited proliferative capacity (asymmetric division); however, in some

circumstances (for example, in the presence of a wound to be repaired) the stem cell go under a self-fate symmetric division, generating two identical stem cells, or to a differentiative symmetric division, through which the stem cell gives rise to two "transient amplifying cells "addressed to terminal differentiation.

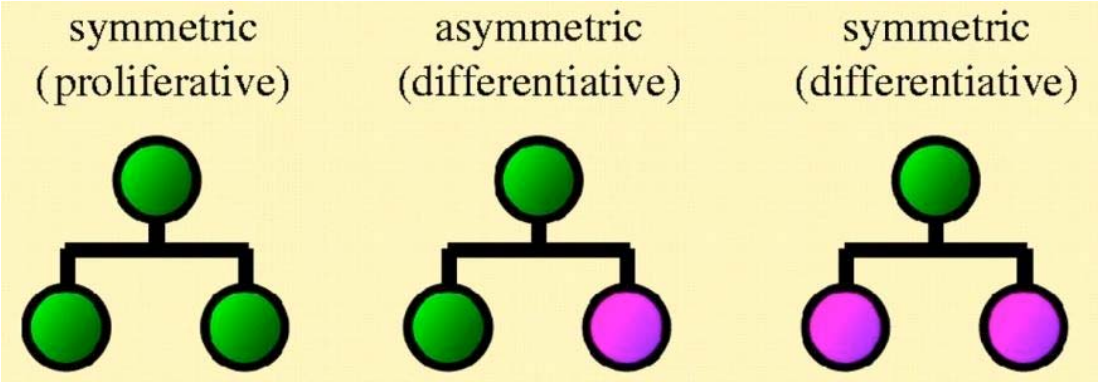


Fig 23. Modality of stem cell division

The most important function of TA cells is to increase the number of differentiated progeny produced by each division of a stem cell, thus allowing the stem cells to proliferate sporadically, at least in conditions of normal tissue homeostasis.

Adult stem cells have been identified in several tissues of the human body and show some special features:

- **Multipotency:** adult stem cells can generate different cell types of the tissue of origin, however, in some tissues these cells are uni-or bi-potent, capable to giving rise to only one or two cell types;
- **Self-renewal:** is the ability of a stem cell to self-renew generating a cell identical to itself;
- **Mitotic quiescence in vivo**: under normal tissue homeostasis, adult stem cells rarely proliferate and are identified as **"slow-cycling cells"**

(an exception is the intestinal stem cells that divide frequently, approximately every 24 hours);

- **High proliferative ability in vitro:** indicates the ability to produce many cell generations in vitro and, therefore, to reach out more lifespans before senescence;
- **Active telomerase :** this feature protects the stem cells from senescence and death to which they are, instead, the destine of the other proliferating cells;
- **Clonogenicity:** indicates the ability of a stem cell to form a colony in culture, or a clone formed by cells identical to each other;
- **Relatively undifferentiated state:** adult stem cells are already directed to differentiate into cell types of the tissue to which they belong (and not to "lineages" other cell tissues), but still do not express the differentiation markers typical of terminally differentiated cells of that tissue.

The identification of stem cells or is entirely based on the evaluation of their proliferative capacity in vitro or on the identification of slow-cycling cells that retain [^{3}H] TdR or BrdU in vivo.

However, not all epithelial stem cells are slow-cycling and experiments "label-retaining" can only be conducted in laboratory animals which can lead to differences between species.

These problems can be solved by analyzing at clonal level the proliferative capacity of human epithelial stem cells in vitro. In fact, when a clone is formed from a single cell, its growth potential can be evaluated by the type of colonies resulting from a single cell plating and the clone can be distinguished to one of three categories (27) (28):

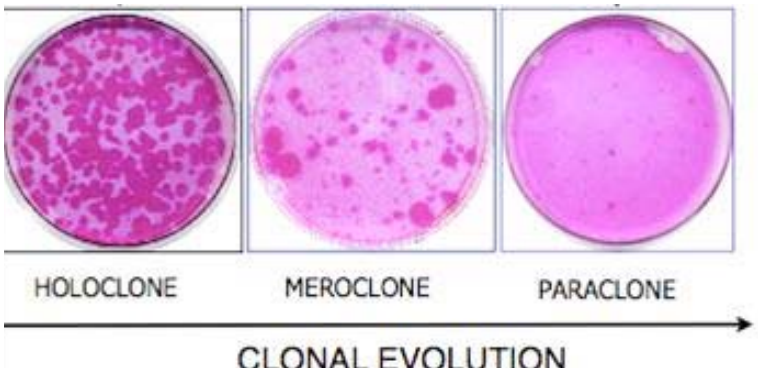


Fig 24. CFE representatives of the three types of clones identified by clonal analysis

- **Holoclones:** these colonies are classified as large (10-30mm^2 after twelve days of culture) and round with smooth and regular perimeter, consisting entirely of small cells with little cytoplasm. The forming cells by holoclones are considered stem cells because they have the highest proliferative capacity (in the case of the skin, for example, may undergo 120-160 duplication and a single holoclone is able to generate the entire epidermis of a human being), less than 5% of colonies of a holoclone are terminally differentiated (abortive colonies), are able to self-renew, possess telomerase activity, can generate all the lineages of the epithelial tissue of origin and continuously repair the damage to epithelial tissue of belonging.
- **Meroclones:** are large colonies with irregular perimeter formed by small cells with different growth potential, which represent a state of transition between olocloni and paracloni. The meroclone is constituted by TA cells capable to go under 20-60 cell divisions before senescence and the amount of terminals colonies that it generates is greater than 5% but less than 95%.
- **Paraclones:** small colonies (less than 5mm2 area on the twelfth day of culture) with irregular and jagged perimeter and made up of large cells classified as paracloni. The cells of this clone are "transient amplyfing cells" that can go under 15 cell divisions before senescence and

generate only abortive colonies (more than 95%) containing terminally differentiated cells.

Holoclones, meroclones and paraclones, previously identified in skin, constitute also the proliferative compartment of the human ocular epithelium. By clonal analysis was, in fact, evaluated the clonogenic capacity (ability of a cell to form a colony), the proliferative potential (ability to produce cell generations) and the potential for differentiation of different regions of the human eye epithelium, and it was observed that the clonogenic cells with high proliferative capacity (typical of stem cells) are segregated in the limbus (**corneal stem cells**) but also uniformly distributed in the bulbar conjunctiva and of the fornix (**conjunctival stem cells**) (14).

Limbal epithelial stem cells

The limbal basal cells are considered stem cells of the corneal epithelium because of different experimental evidences (25).

- Slow-cycling cells (identified experimentally as "label retaining cells"), a characteristic of stem cells, have been found in vivo only in the basal layer of the limbus and the cornea;
- The limbal basal cells are the only capable to form holoclones in vitro, while the latter can't be isolated from the cornea;

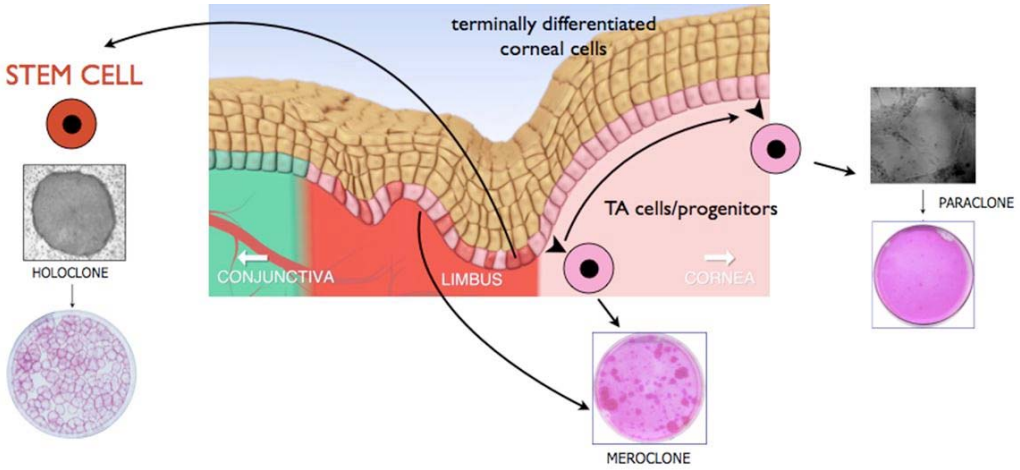


Fig 25. Clonal types generated by cells derived from the limbal basal layer

- The keratin K3, a marker of differentiation of the corneal epithelium, is not expressed in the limbal basal cells but is present in the suprabasal cells and in all other layers of the corneal epithelium, indicating that the basal limbal cells are relatively undifferentiated;
- Under identical conditions of growth in vitro, the keratin K3 is not expressed by epithelial cells of bulbar conjunctival, while it is present in the cultures of limbal and corneal cells, allowing to exclude that the conjunctival cells, adjacent to the limbal region, are the precursors of the corneal epithelium;
- The cells of the limbal region show a longer "life-span" (length of total life in culture) in vitro compared to those of the central cornea;
- Cells of peripheral region of the cornea undergo a centripetal migration towards the central or peripheral cornea;
- The destruction of the limbal basal cells (for example, for thermal burns, chemical, radiation, etc. ....) compromises the wound healing ability of central corneal epithelial resulting in "**conjunctivalizzation**" of the cornea, a condition known as "**Limbal stem cell deficiency**"(LSCD), characterized by neovascularization, chronic inflammation, corneal opacity and loss of vision;



Fig 26. Reconstruction of eye surface characterized by LSCD

- Only the transplantation of a limbal epithelium, but not conjunctival, can replace a damaged corneal epithelium and restore the transparency of the cornea.

The human limbal stem cells support the continuous renewal of the corneal epithelium during the entire life of the organism, the process of differentiation of the stem cells generate progenitors TA that migrate in a centripetal manner on the basal layer of the cornea, where actively proliferate toward postmitotic cells on suprabasal corneal epithelium.
The post-mitotic cells are identifiable as wing cells, which further mature in terminally differentiated cells of the corneal epithelium surface which then fall off and are lost in tear film (21).

Conjunctival stem cells

Stem cells are uniformly distributed in the epithelium covering the eye bulb and the fornix, have the ability to generate conjunctival keratinocytes and goblet cells suggesting the bipotent ability of these stem cells.
In vitro studies have demonstrated that stem cells generate conjunctival goblet cells at specific stages of their life in culture: the first peak of production of goblet cells is originated after 45-50 cell doublings, while the

second peak, most significant, occurs at 10 -20 cell divisions before senescence.

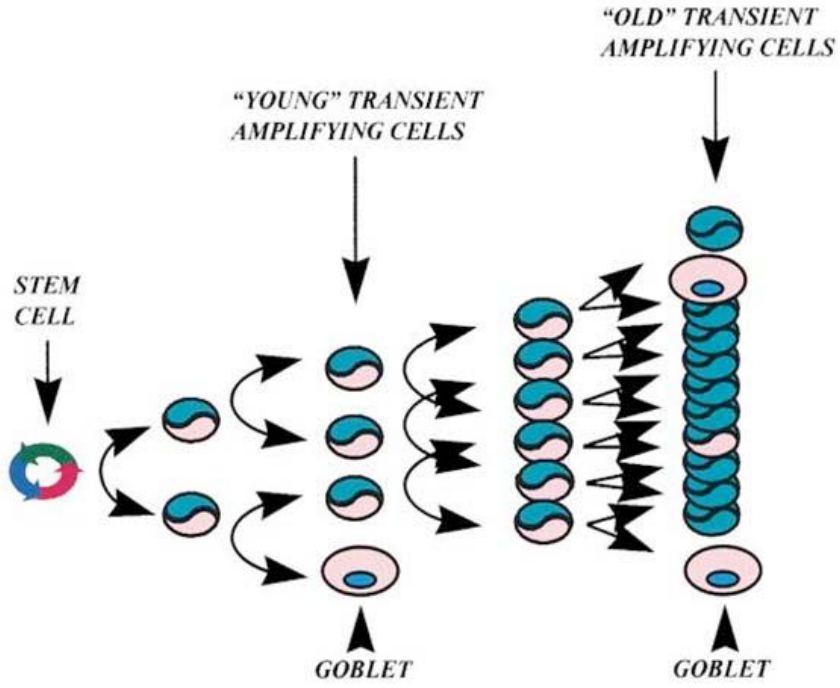


Fig 27. Schematic description of a model for goblet cells generation

Both "young" progenitors (transient amplifying cells) that give rise to early meroclones still able to undergo 35-60 doublings and "old" (late) meroclones those cells that undergoing 20-35 cell divisions before senescence, are able to generate goblet cells, but the quantity produced by late meroclones is markedly increased and this fits with the suprabasal location of goblet cells in vivo.

While growing colonies have a goblet cells content of 200-500 cells per cm^2, confluent conjunctival sheets had >5.000 goblet cells/cm^2. We have calculated that this value corresponds to a gblet/keratinocyte ratio of 1/30 a ratio not far from that found in vivo in resting bulbar conjunctiva.

Indeed, goblet cells elicit their function in the suprabasal layers, where they secrete the mucin responsible for the formation of the tear film (14).

Markers of Stem cells and of Differentiation

The identification of stem cells of the epithelia ocular surface is based on the evaluation of certain proteins expression that have been observed to be preferentially expressed by holoclones rather than meroclones/paraclones and that can be considered markers of stemness.
Markers analyzed:

ΔNp63α: the p63 gene generates transcripts of "full-length" (TAp63) and N-terminally truncated transcripts (ΔNp63) initiated by different promoters, each transcript is alternatively spliced to encode three different p63 isoforms indicated as α, β and γ. The p63 gene products are essential for the morphogenesis and the regeneration of stratified epithelia. In particular has recently been shown that ΔNp63α sustains the proliferative potential of the stem cells of many stratified epithelia (16)
ΔNp63α is necessary for the maintenance of the proliferative potential of limbal stem cells proliferative potential of stem cells, while the two isoforms ΔNp63β and ΔNp63γ are likely to play a role in epithelial differentiation specifically during the process of corneal regeneration.
Transcription factor ΔNp63α is likely to identify the stem cell population of the human limbus but not TA cells that covering the corneal surface and is abundantly expressed by epidermal and limbal holoclones, but is undetectable in paraclones.

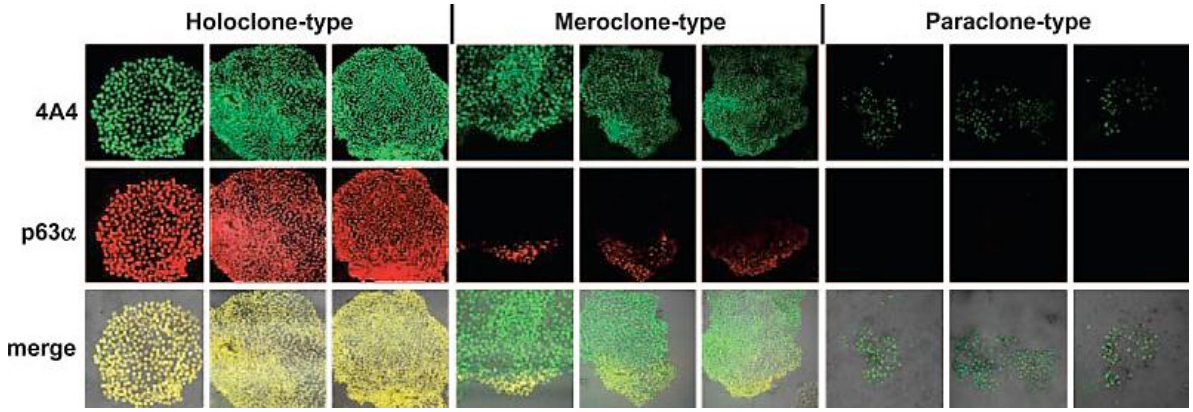


Fig 28. The expression of p63 isoforms of three clonal types.

ΔNp63α is a stemness marker and its identification at holoclones by immunodetection is an exclusive and simple method for determining the presence of an adequate number of stem cells restricted only to culture limbal grafts (29).

C/EBPδ: the transcription factor "CCAAT/enhancer binding protein δ" (C / EBPδ) regulates the cell cycle by inducing a G0/G1 arrest and this effect is specific for epithelial cells.

C/EBPδ is a member of a highly conserved family of leucine zipper transcription factors expressed in a variety of tissues and cell types and involved in the control of cellular proliferation and differentiation, metabolism and inflammation. At least six members of the family have been isolated and characterized (C/EBPα–C/EBPζ), for instance, it has been shown that C/EBPα and -β induces cell cycle exit in normal keratinocytes and positively regulates the program of squamous differentiation in the epidermis and accordingly, that C/EBPα and -β are contained in the suprabasal layers of both human limbal and corneal epithelium (26).

C/EBPδ, instead, is co-expressed with ΔNp63α in the same patches of basal cells (approximately 10%).on one hand C/EBPδ induces mitotic quiescence(through a positive regulation of p27Kip1 and p57Kip2) and self-renewal of limbal stem cells; on the other, it preserves their proliferative

potential (essential for stem cell–dependent tissue regeneration) through a positive regulation of ΔNp63α.

Bmi1: is a member of the polycomb group transcription factors played a crucial role in the cell identity, in the self-renewal of stem cells and in the cell cycle of haematopoietic neural stem cells and in limbal stem cells. A defined number of mitotically quiescent limbal stem cells co express Bmi1, ΔNp63α, and C/EBPδ under normal homeostasis. Co expression of Bmi1, ΔNp63α, and C/EBPδ therefore identifies limbal holoclones and is part of the genetic program maintaining stem cell identity.

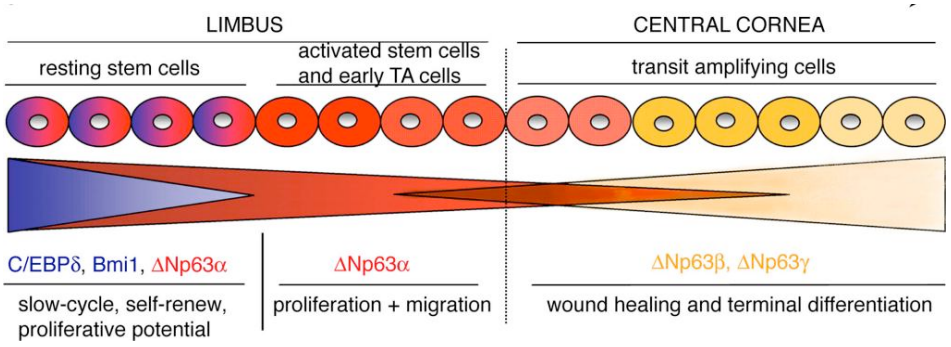


Fig 29. Clonal Evolution, cell proliferation and cell migration in corneal wound healing

Under stress conditions, such as those induced by a corneal damage, a fraction of such stem cells switches off C/EBPδ and Bmi1 but maintains ΔNp63α necessary for proliferate and migrate to the central cornea to restore and regenerate the corneal epithelium.

Under this stress conditions, activated stem cells, however, lose their self-renewal properties, enter into the TA compartment, and progressively lose ΔNp63α expression. TA cells switch on ΔNp63β and ΔNp63γ, which might regulate terminal differentiation and stratification during the regeneration of the damaged corneal epithelium. These data indicate that co-expression of BMI1, C /EBPδ and ΔNp63α identifies the limbal stem cells mitotically

quiescent in vivo and the human limbal holoclones in vitro, but not the meroclones nor paraclones.

Variety of differentiation markers have also been proposed to distinguish the SCs from differentiated cells because the suprabasal epithelia of the cornea and limbus showed strong immunoreactivity for these markers in vivo and their expression increases from Holoclone to meroclone to paraclone in vitro.

These markers include:

Cytokeratins: belong to a family of about 30 protein, have an molecular mass between 40kDa and 68kDa, each of which indicated with a "K" followed by a number, are epithelial-specific intermediate filaments, can be divided into two subfamilies: acidic type I K 9-20 and neutral to basic type II K 1-8. Each cytokeratin is a product of a distinct gene.

Their function is not only to maintain the shape of a cell and to protect the cell from mechanical stress, but they are also important for differentiation and specialized functions, including protection from apoptosis, stress and injury, the intracellular transduction as signals and malignant transformation (59).

When the cells of the corneal epithelium differentiate show a expressed variation in the type of keratins: mainly keratins K5 and K14 are expressed by cells of the basal layer, while the basic keratin K3 and acidic keratin K12 are characteristics of the suprabasal cell layers and are, therefore, considered markers of differentiation of the corneal epithelium.

The cytokeratin K19 is strongly expressed in the conjunctival epithelium and poorly expressed in the corneal epithelium. This difference expression has allowed the use of immunostaining with specific antibodies in the diagnostic technique (impression cytology) to define the severity of the conjunctivalization in patients with LSCD.

More pronounced differences between the superficial layer of the cornea and the conjunctiva were found with cytokeratin 7, as we have confirmed in our laboratory.

The absence of this cytokeratin by both the basal and suprabasal of the central cornea but its strong presence in the apical layer of the bulbar conjunctiva.

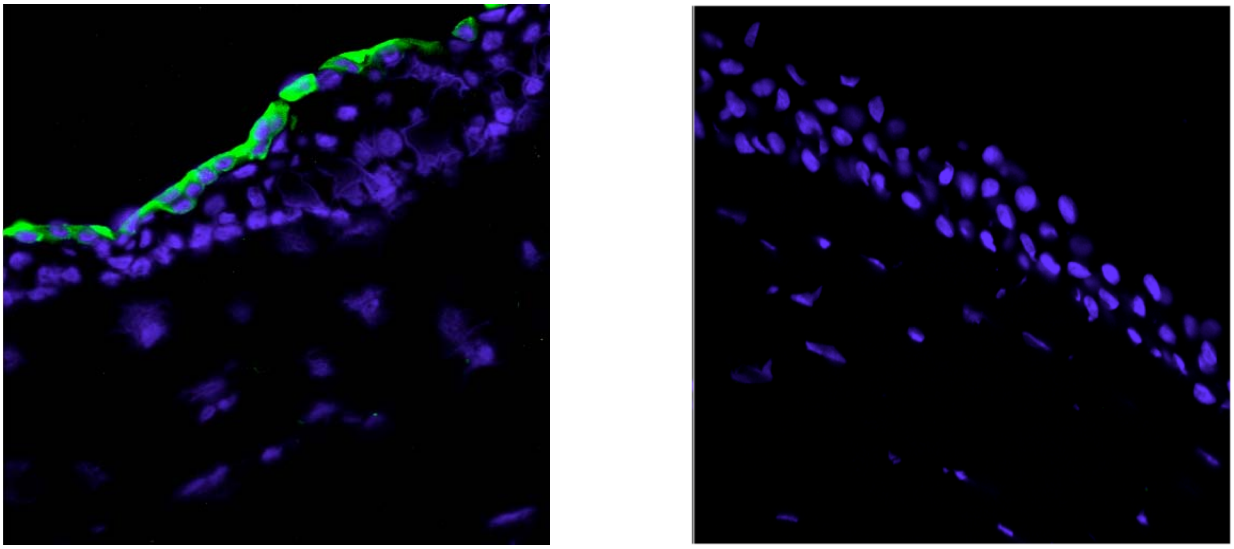


Fig 30. Fluorescent Immunostaining for K7 (green) in bulbar conjunctival epithelium (left) and central corneal epithelium (right).

14-3-3σ (also called HME1 or stratifin): belongs to a large group of highly conserved and homologous dimeric proteins, expressed in
every mammalian tissue.

This protein elicit their biochemical functions by binding to a variety of cellular proteins containing specific phosphoserine motifs, and therefore have been associated with a diverse number of biochemical processes. For instance binding of 14-3-3σ to serine-phosphorylated Raf is essential for Raf kinase activity, and hence for the Ras/MAPK signalling cascade whereas 14-3-3σ binding to the serine-phosphorylated death agonist, BAD, interferes with apoptosis.

14-3-3σ is abundantly expressed in the suprabasal layers of human epithelia and its down regulation is accompanied by the maintenance of telomerase activity and allows keratinocytes to escape senescence by impairing clonal

evolution. Suggesting that downregulation forcing the keratinocytes in the stemness state (24).

Diseases of the ocular surface and the role of the Nerve Growth Factor

The human ocular surface can be damaged by many different diseases (genetic, autoimmune/trophic, traumatic, etc ...) that may damage corneal stem cellss or conjunctival stem cells.

Some of these diseases, Although different in origin and prognosis, are united by the reduced number of goblet cells and at decreased levels of mucins, in particular the Goblet-specific MUC5AC.

Recent studies have also shown that these diseases are improving following the administration of Nerve Growth Factor (NGF), a growth factor identified primarily for its ability to promote the survival, development and maturation of neurons.

These results have led to greater caution against these diseases and the role that NGF can play in healing processes.

Limbal and Corneal Pathologies

Several ocular and systemic diseases, such as neurotrophic keratitis (NK), diabetes mellitus and multiple sclerosis as well as some triggers such as viral infections, chemical burns, corneal surgery and abuse of topical anesthetics can cause corneal neurotrophic ulcers because these pathologies damage the sensory innervations of the cornea. Experimental evidence, have shown that the loss of corneal sensory innervations leads to a(35, 36, 38):

- decrease number of corneal stem cells, decreased metabolic and mitotic rates in the corneal epithelium, the absence of spontaneous healing and consequent break of the epithelium.
- corneal epithelial thinning and loss of microvilli of the superficial cells.
- Progressive corneal damage, with epithelial defects, vascularisation, opacification, ulceration and ultimately, perforation.
- decrease number of goblet cells of conjunctival epithelium and loss of microvilli of the superficial cells.

In the presence of corneal ulcers the standard treatments consist of covering the eye with a patch or soft contact lens, tarsoraphy and constructing of a conjunctival flap, but they are often ineffective, and the outcome is often loss or severe impairment of vision.

in vitro study demonstrated that nerve growth factor (NGF) is produced, stored and released in human corneal epithelial cells and also this epithelium express the high-affinity NGF receptor (TrkA) and low affinity receptor (p75) through which NGF

exert its action, via an autocrine/paracrine circuit which supports survival and an equilibrate function of the epithelial cells.

Instead the NGF induces proliferation and differentiation of epithelial cells, stimulates fibroblasts migration, differentiation and collagen production, and restores corneal sensitivity, suggesting the possibility to use this factor for the treatment of the patients with neurotrophic ulcers.

Recent clinical studies have showed that topical administration of NGF healed the ulcers and improved corneal sensitivity and visual acuity without local or systemic side effects. Furthermore, the resolution of the disease is not associated with the severity of the ulcers or to their perforation in the corneal stroma, nor the age of the patient or at the causes of the ulcers (34, 39).

Corneal sensitivity improvement in patients treated with NGF and, in particular, in those affected by neurotrophic Keratitis, suggests that administration of NGF allows a functional recovery of corneal sensory innervation, which plays a key role in maintaining the tropism and the function of the corneal epithelium through the release of trophic factors such as NGF. Corneal sensory nerves exerts a profound influence on the integrity of the epithelium and their damage leads to breakage of the corneal epithelium and exfoliation of its cells.

Because the exogenous NGF is able to restore the integrity and corneal sensitivity, you can assume that the progressive corneal damage that occurs in patients with corneal sensory nerve dysfunction is caused by a deficiency in the synthesis, in the release and/or use of endogenous NGF.

Conjunctival Pathologies

The pathologies of the ocular surface in which the expression, distribution and structure of mucins are altered, are those which involving the dryness or keratinization of the corneal and conjunctival epithelium. These diseases are the dry eye syndrome, ocular cicatricial pemphigoid, ocular allergies, Stevens-Johnson Syndrome, vitamin A deficiency, etc. .., all characterized by loss of goblet cells and alteration and/or disappearance of mucins "gel-forming" or those mucins associated with cell membranes.

In some disease, overproduction of mucin is due to the presence of activated T-cells (60)

Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. Dry eye is recognized as a disturbance of the *Lacrimal Functional Unit* (**LFU**), an

integrated system comprising the lacrimal glands, ocular surface (cornea, conjunctiva and meibomian glands) and lids, and the sensory and motor nerves that connect them (61). Perturbation of the LFU is considered to play an important role in the evolution of different forms of dry eye.

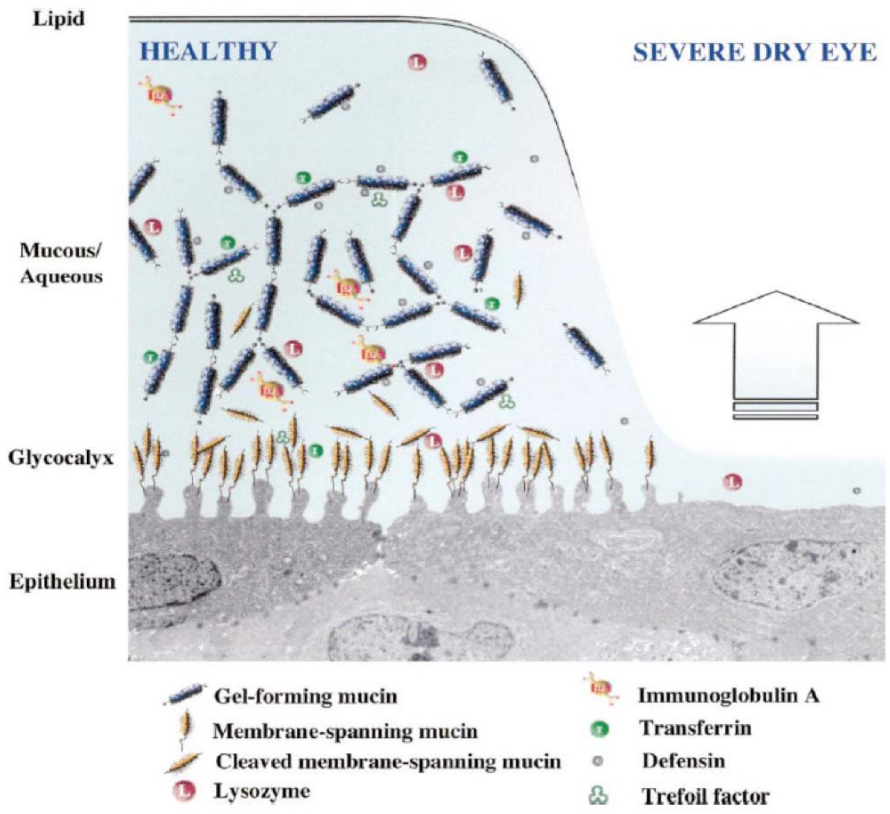


Fig 31. Lacrimal structure of healthy and affected subjects

The major classes of dry eye, are still held to be aqueous tear-deficient dry eye (**ADDE**) and evaporative dry eye (**EDE**), in the etiopathogenic classification. ADDE has two major subclasses, SS dry eye (SSDE) and non-SS dry eye. The most severe symptoms of dry eye diseases are seen in patients with *Sjögren Syndrome* (SjS). Sjogren dry Eye syndrome is an exocrinopathy in which the lacrimal and salivary glands are targeted by an autoimmune process; other organs are also affected. The lacrimal and

salivary glands are infiltrated by activated T-cells, which cause acinar and ductular cell death and hyposecretion of the tears or saliva.
There are two forms of SS, and classification criteria have recently been harmonized in a European-American collaboration.

- Primary *SS* consists of the occurrence of ADDE in combination with symptoms of dry mouth, in the presence of auto-antibodies, evidence of reduced salivary secretion and with a positive focus score on minor salivary gland biopsy.

- *Secondary SS* consists of the features of primary SS together with the features of an overt autoimmune connective disease, such as rheumatoid arthritis, which is the most common, or systemic lupus erythematosis, polyarteritis nodosa, Wegener's granulomatosis, systemic sclerosis, primary biliary sclerosis, or mixed connective tissue disease.

The precise triggers leading to autoimmune acinar damage are not known in full, but risk factors include genetic profile,78 androgen status79 (a low androgen pool favouring an inflammatory environment within the target tissues), and exposure to environmental agents, ranging from viral infections affecting the lacrimal gland to polluted environments (61).
Non-Sjogren Syndrome Dry Eye is a form of ADDE due to lacrimal dysfunction, where the systemic autoimmune features characteristic of SSDE have been excluded. The most common form is age-related dry eye.

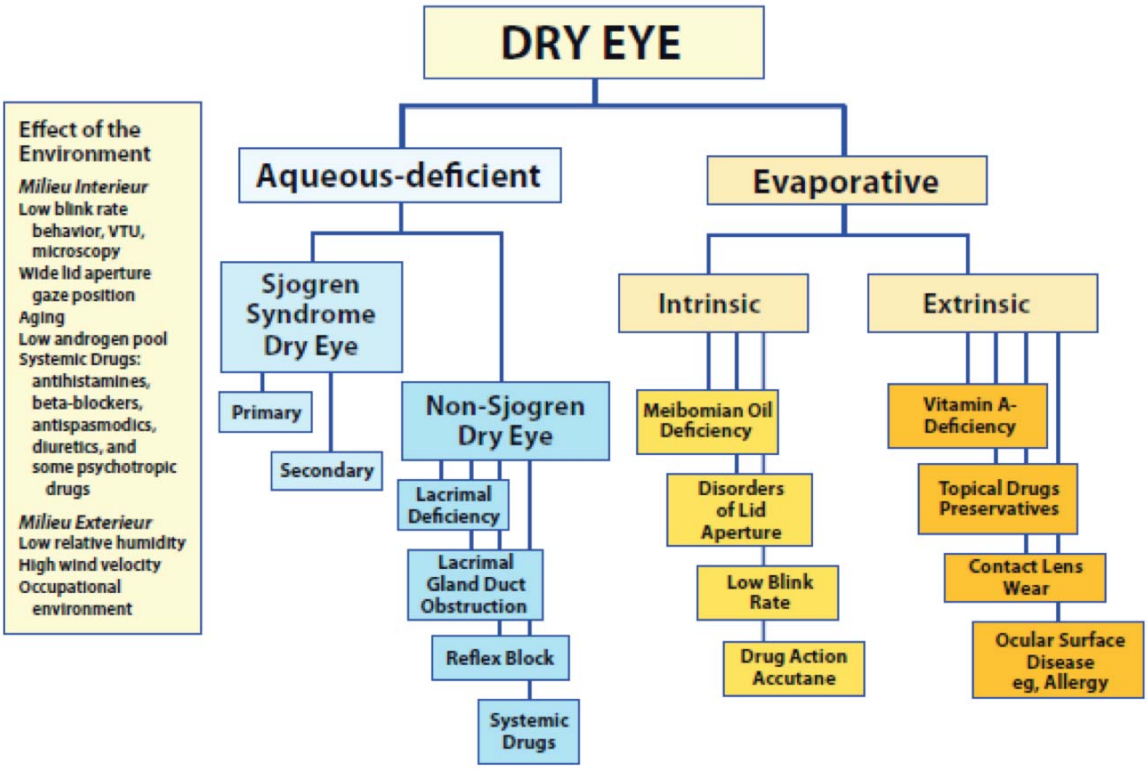


Fig 32. Major Etiological causes of dry eye

Over the past couple of decades, a variety of mouse strains, have been developed to study the immuno-pathological nature of SjS. Based on results of studies using NOD mice and various gene knock-out (KO) congenic partner strains of NOD, proposed that the development and onset of autoimmune exocrinopathy in this model of SjS is comprised of two mutually distinct steps:

- an initial lymphocyte-independent phase exhibiting intrinsic problems in exocrine tissue homeostasis or differentiated function.
- The subsequent tissue-specific immunological attack by activated T-cells, B-cells and their products against the targeted salivary and lacrimal glands.

Consistently with this hypothesis, in patients with SjS, a significant reduction in goblet cells and MUC5AC mRNA and protein expression has been described suggesting a crucial role of this mucin in dry eye disease of the ocular surface. The importance of goblet cells as major producers of ocular surface mucin is well established with critical emphasis placed on the number of functional goblet cells in the conjunctiva and on the amount and rate by which they synthesize mucin. The stimulation of the parasympathetic with acetylcholine and vasoactive intestinal peptide (VIP) and sympathetic nerves that surround the conjunctival goblet cells (CG) can induce goblet cells secretion. Another potent stimulus of goblet cell secretion is the purinergic agonist (UTP and ATP) released from nerves, platelets, damaged cells or bacteria. In particular, ATP stimulates airway goblet cell secretion through protein kinase C (PKC) pathway (62).

Growth factors are other candidates that could play a major role stimulating goblet cell secretion. Several types of growth factors have been identified in tears and in the conjunctiva inducing EGF, VEGF, TGFα and β It was reported that the -3752/-3426 region of MUC5AC promoter was able to confer TGF β mediated repression of reporter transcription, via the Smad 3/4 signalling pathway (63).

Another growth factor involved in epithelial turnover is the Nerve growth factor (NGF), considering that NGF potentially affects all components of the ocular surface, it might play a pivotal role in a variety of diseases involving several components of the ocular surface including conjunctiva (inflammation, self renewal, mucin secretion) and lacrimal glands (tear production). In all human ocular surface epithelial cells, including limbal stem cells, express both NGF receptors trkANGFR (tyrosine kinase) and p75NTR (member of the tumor necrosis factor receptor family.

The Nerve Growth Factor is the best characterized element of a family of growth factors structurally and functionally related, known as Neurotrophins, which include the Brain Derived Neurotrophic Factor (BDNF)", the Neurotrophin 3 (NT3), the Neurotrofin 4/5 (NT4/5) and the Neurotrophin 6 e 7 (NT6 and NT7 genes have been identified only in fish and do not seem to have orthologs in mammals) (46).

The neurotrophins play an important role in the survival, development and death in the neurons of the nervous system.

The NGF was discovered around 1950 by Rita Levi Montalcini for its ability to promote the survival and growth of sensory and sympathetic neurons and the differentiation of neurons in the central nervous system.

The discovery of the Nerve Growth Factor

In a 1948 article, Elmer Bueker reported the results of an experiment consisting of the grafting of fragments of mouse sarcoma (S180) into the body wall of a 3 day chick embryos. The histological study of the embryos fixed 3-5 days later showed that the tumor fragment was innervated by sensory nerve fibers emerging from adjacent dorsal root ganglia and that these ganglia volume appeared to be greater compared with that of homologous ganglia of the contra lateral side not grafted with S180.

These results showed that the histochemical properties of the fast-growing mouse sarcoma offered a favorable field for growth of sensory fibers.

However, the histological studies showed that the nerve fibers branched randomly between tumoral cells without establishing synaptic connections with them.

Rita Levi Montalcini decided to repeat this experiments with the same technique, this time using fragments of Sarcoma 37 (another tumor of identical origin), considerably more invasive than Sarcoma 180.

This tumor, known as Sarcoma 37, caused the same effects of Sarcoma 180, however, in chicken embryos subjected to graft, it was observed that sympathetic ganglia non directly connected with the tumor and, therefore, with neoplastic tissues, were enormously enlarged.

In addition, spare nerve fibres produced by sympathetic ganglia invaded organs without synaptic contacts with their cells and even blood vessels. These observations allowed him to speculate that the S37 effect was due to the ability of cancer cells to release a humoral factor able to influence the differentiation and growth of nerve cells susceptible to its action.

In 1954, the collaboration with biochemist S. Choen led to the identification of a nucleo-protein fraction extracted from tumors able to stimulate nerve growth and this subdivision was called 'Nerve Growth Promoting Factor and, as a result, Nerve Growth Factor (NGF).

To test whether the biological activity was performance by the entire nucleo-protein fraction or by one of the two components (protein or nucleic acid), Cohen further purified the tumor factor using snake venom as containing the enzyme phosphodiesterase that degrades nucleic acids.

If nucleic acids were an essential component of NGF, their enzymatic degradation should cancel the biological activity of factor neoplastic, while if on the contrary this activity had continued would have meant that the real responsible for the effect on neuronal growth had to be the protein component.

In fact, the examination of the cultures of the basal ganglia grown in the presence of the NGF-acting tumor extract and of snake venom, showed an unexpected result: a marked increase in the density of the fibrillar 'halo' around the ganglia cultured rather than those grown in the presence of a tumor factor only.

Further studies demonstrated that this was due to the fact that the NGF factor was present in greater amounts in snake venom compared to mouse tumor

extract: injections of NGF, extracted and purified from the venom of snakes, in chicken embryos caused an effect identical to that obtained by grafting fragments of S180 and S37, proving that the factor extracted from venom and tumor were the same.

In 1958 it was discovered another rich source of NGF in submandibular salivary glands of adult male mice. These results were soon followed by purification and identification by Cohen of this salivary factor in a protein molecule of evaluated mol. weight of 44.000.

In 1959 a specific antiserum to salivary NGF (AS-NGF) likewise abolished the formation of the in-vitro fibrillar halo. The results suggested testing the effects of daily injections of small amounts of this antiserum in neonatal rodents. The experiments determined the sympathetic ganglia atrophy and death of nerve cells, providing the unequivocabile evidence of the essential role NGF plays in the survival of these cells.

From 1960 onwards began to be studied the mechanisms of action of NGF, relations with the receptors, the various functional roles, the chemical identity, the genetic structure, the interaction with the central nervous system, with the immune and with the endocrine, to identify the family of growth factors by which the NGF participates: the neurotrophins.

Structure and function of neurotrophins

The neurotrophins and their genes share homologies in structure and sequence: the protein product of each gene contains a signal sequence and a pro-domain followed by neurotrophin mature sequence, whereby each protein must be processed by proteolysis to generate the mature form.

The genes for the four mammalian neurotrophins encode glycosilated precursors (approximately 30-35 kDa), the pro-neurotrophins, which are cleaved by convertase enzymes, such as Furin and Convertase 1 and 2 (PC1

and PC2), to generate the mature forms of neurotrophins, monomers of about 12-14 kDa. After being properly processed, neurotrophins form homodimers functionally active through formation of non-covalent bonds (49).

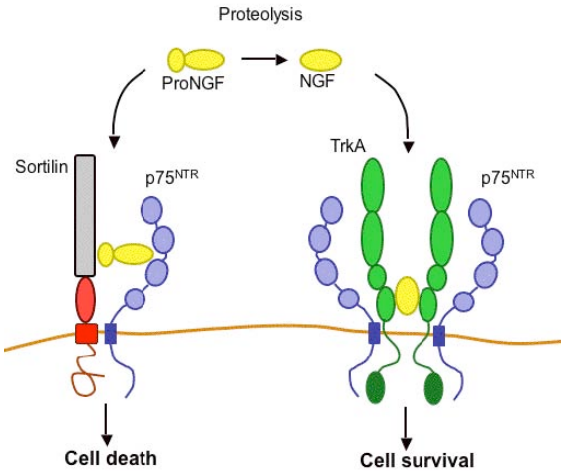


Fig 33. NGF is synthesized as a precursor (pro-NGF) and undergoes processing to generate mature NGF with different biological activity.

In particular, NGF is encoded by a gene located on the short arm of chromosome 1 and consists of two polypeptide chains of 13 kDa each one, and consisting of 118 amino acids: each monomer has three disulfide bonds that give a strong stability to the three-dimensional structure of the molecule and the two monomers, united by weak bonds, forming a dimer of 26.5 kDa molecular weight.

Some studies have shown that even the pro-neurotrophins can carry out biological functions: the precursor pro-NGF, binds p75NTR receptor with greater affinity than the NGF mature and activates the apoptotic process more effectively.

The discovery that NGF can be secreted as pro-NGF, which activates preferentially the receptor p75 to mediate apoptosis, or as mature NGF, which activates the TrkA receptor to promote survival, suggests that the result of neurotrophin biological action depends on the connection between the two forms and may be adjusted by proteolytic cleavage (50).

In fact, every pro-neurotrophin is able to bind receptor p75NTR but not Trk receptors, while following the maturation process each neurotrophin remains able to ties and activate p75NTR but exhibits an affinity more specific for the three Trk receptors, to which the NGF binds preferentially TrkA, BDNF and NT4/5 bind TrkB and the NT3 ties TrkC (55).

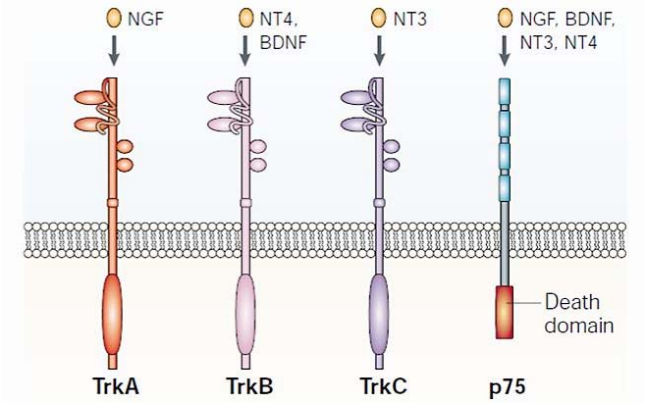


Fig 34. Schematic representation of the interaction between neurotrophins and their receptors (Trk and p75).

The neurotrophins were initially identified as critical factors for the survival, growth, differentiation and death of sensory neurons, able to adjust the growth of axons and dendrites, synaptic processes and neurotransmitter release, but now are recognized as capable of mediating many functions also in cell types not neuronal as:

- Immune Cells: NGF increases expression of the receptors for IL2 in Natural Killer cells (NK), suppresses apoptosis and enhances the functions of macrophages, neutrophils and eosinophils; promotes the granulocytic differentiation of hematopoietic stem cells. NGF and TrkA receptor are strongly expressed in lymphoid organs, indicating their involvement in the process of differentiation of immune cells, NGF also induces B-lymphocytes proliferation and their differentiation into

plasma cells and finally modulates the proliferation and maturation of T-lymphocytes. [45]

- Epithelial Cells: the human keratinocytes synthesize and secrete NGF biologically active and they also express the TrkA receptors and p75 with consequent formation of an autocrine loop able to adjust their proliferation and their differentiation. NGF regulates the migration of epithelial cells and protect them from apoptosis
- Fibroblasts: these cells synthesize, secrete NGF and express TrkA receptor. The fact that Nerve Growth Factor stimulates the proliferation of epithelial cells and the migration of fibroblasts, confirms his involvement in the healing of the wounds because fibroblasts are the main effectors of the repair processes for their ability to migrate in the injured area, proliferate, produce the components of the extracellular matrix and differentiate into my-fibroblasts for the wound margins contraction.

- Endothelial Cells: these cells produce NGF and expressing the corresponding receptor TrkA, which activate the migration and proliferation of endothelial cells by speeding up the processes of neo-angiogenesis during the healing of wounds

The signal transduction systems that mediate the different biological functions of the neurotrophins are activated by the interaction of these with two classes of membrane receptors, the receptors tyrosine kinases Trk (Tropomyosin-Related Kinase) and the receptor for the neurotrophins p75 (p75 NTR) belonging to the family of receptors TNF (Tumor Necrosis Factor).

While the Trk receptors are responsible for the transduction of signals for survival and growth mediated by neurotrophins, the receptor p75 can act as a co-receptor Trk, enhancing the activity, or it can autonomously activate signal

transduction pathway that culminate in the induction of apoptosis or in promoting the survival (46).

Trk receptors and p75NTR

TrkA was initially identified as an oncogene derived from the fusion of the tropomyosin with a tyrosine kinase unknown, for which the corresponding proto-oncogene was called "tropomyosin related kinase (trk) ". Subsequently, were identified for their high homology with TrkA, also the receptors TrkB and TrkC, all expressed in cell populations neuronal and able to bind preferentially specific neurotrophins.[54]

Trk receptors belong to the super-family of receptor tyrosine kinases and all three receptors TrkA, TrkB and TrkC share the same architecture, with their extracellular portion formed from a cluster rich in cysteines (domain 1), three repetitions rich in leucine (domain 2), A second cluster rich in cysteines (domain 3) and two domains flu-immunoglobulin (domain 4 and domain 5).

Binding studies and deletion on the three receptors indicates that the domain 5 is the one responsible for the bond with the neurotrophins.

The extracellular portion of these receptors is linked, through a single propeller trans-membrane, at the domain tyrosine kinase intracellular: the kinase domains of the Trk receptors are highly homologous with a sequence identity of greater than 75, while the extracellular portions, members of the binding of different neurotrophins, show more differences (the sequence identity is of 50-55%) (47).

The three genes "trk" encode full-length receptors but the alternative splicing leads to the formation of different isoforms: for example, the alternative splicing generates the TrkA receptors TrkB free of a short amino acid sequence in their extracellular portion, of the receptors TrkB and TrkC which lacking the kinase domain and intracellular receptor TrkC with an insert in kinase domain, all defective variants in signal transduction.

The binding of the homodimers of neurotrophins involves the dimerization of the Trk receptors and auto-phosphorylation of their intracellular domains, with consequent formation of phospho-tyrosinic motives which act as binding sites for signalling molecules that regulate growth and cell survival by activating the pathway of signal transduction involving Ras-MAPK, phosphatidil-inositol 3' kinase (PI3K) and phospholipase C gamma (PLCγ).

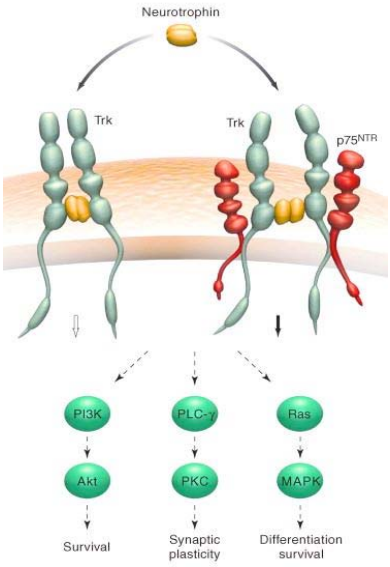


Fig 35. Activation of several signal transduction cascades after NGF stimulation and the main NGF-mediated biological activities.

The receptor p75 is encoded by the gene p75NTR, located on the long arm of chromosome 17, such as a precursor of 427aa that following the removal of the peptide signal generates a trans-membrane receptor with an extracellular portion devoted to the binding of the ligand and a cytoplasmic domain containing a "death domain", a structural motif typical of receptors inducing apoptosis.

The receptor p75 was, in fact, the first receptor identified by the superfamily of Tumor Necrosis Factor (TNF) which includes about 25 receptors such as Fas, TNFR1 (Tumor Necrosis Factor Receptor1), TNFR2, RANK and CD40.

The extracellular portion of p75NTR contains four domains rich in cysteines (CRDs) and numerous sites of N- and O-glycosylation, while the intracellular domain contains a site of palmitoylation, two potential binding sites to TRAF (TNFR-Associated Factor, acts as a bridge between the receptors and TNFR protein intracellular signalling), a potential site for the activation of "G-proteins", a binding site PDZ domains and a death domain.

The members of the TNFR family, in fact, don't have an inherent enzymatic activity but activate the signal transduction cascade interacting with different adaptor proteins cytoplasmic and this explains the presence in intracellular portion of p75 in many regions of protein-binding signal.

In the case of the receptor p75, the products of alternative splicing comprise a free variant of extracellular domains CRD2-4, unable to bind the neurotrophins, and a soluble form consisting of only extracellular portion of p75 able to binding their ligands.

The biological activity mediated by this receptor depends on the type of neurotrophin linked and by the simultaneous presence or absence of the TrkA receptor on the cell surface: in TrkA cells negative the binding of NGF to the receptor p75 leads to apoptosis, while in the presence of the TrkA receptor, p75 increases the binding affinity of the TrkA receptor for the NGF by creating a high affinity binding site and promoting cell survival.

The cell death mediated by the binding of pro-neurotrophin receptor p75 (in association with a co-receptor called sortilina) is explained by the important role played by apoptotic process in properly define the target group for the innervation during development but is also associated with inflammatory processes that involve the nerves damaged.

Unlike the Trk receptors that show a high specificity for the respective ligands, the receptor p75 binds all the neurotrophins in the same relatively weak nanomolar affinity.

TrkA receptors and p75 are indicated respectively as "high-affinity receptor" and "low-affinity receptor", however, binding studies have revealed that the two receptors interact independently with the neurotrophins with a similar dissociation constant Kd of approximately 10-9M, a higher value than that characterizes the high-affinity binding sites (Kd 10-11M).

The receptor p75 is characterized, in fact, by a quick rate of association and dissociation for the NGF and this explains the low binding affinity to this ligand. The receptor TrkA instead, shows a kinetic of dissociation very slow, suggesting a greater affinity for the NGF, but the low speed of binding of the Nerve Growth Factor receptor TrkA involves an affinity for this ligand comparable to that of the receptor p75. However, when the two receptors are co-expressed on the cell surface, it form a high affinity binding site characterized by a speed of binding of NGF 25 times greater than that shown by TrkA receptor in the absence of p75: it seems that the presence of the receptor p75 confer to TrkA an increased capacity of response to low concentrations of the neurotrophin. [48]

Hypothesis to explain this phenomenon are two:

- Model of the presentation of the ligand: the first hypothesis does not require a direct interaction between the two receptors but argues that the receptor p75 binds the NGF by increasing the local concentration and facilitating the recognition on the part of the TrkA receptor

Model of the conformational change: according to this hypothesis, the receptor p75 interacts with TrkA producing a conformational change that increases the affinity for the NGF and, consequently, the formation of the high affinity binding.

TrkA receptors and p75 have different functions in cell populations neuronal and not neuronal:

RECEPTOR	FUNCTION
TrKA	• Training of high affinity binding site • Ligand internalization • Axon Growth • Activations of PLCγ, PI3K and MAPK • Survival and differentiation of neuronal cells • Survival, proliferation, and cell migration
p75NTR	• Training of high affinity binding site • Modulation of the kinase activity of the Trk receptors • Hydrolysis of sphingomyelins with production of ceramide, go-between of the apoptotic process • Increase the specificity of the Trk receptors for the ligands primary • Induction of apoptosis • Cell Migration (Schwann cells) • Increase secretion of enzymes degrading extracellular mold • Cell survival NFkB-mediated

Pathways of cellular signalling activated by NGF

The pathways of signal transduction pathways induced by the activation of the Trk receptors mediated by neurotrophins regulate positively the proliferation and cell survival, the growth of axons and dendrites, the remodeling of the cytoskeleton, the formation and function of synapses, etc.

The activation of Trk receptors by ligands involves several conserved residues phosphorylation of tyrosines present in the cytoplasmic domain: in man, the important binding sites in TrkA receptor for the interaction with

signal intracellular proteins are tyrosine 496 (Tyr496) in the region juxta-membrane and Tyrosine 791 (Tyr791) at the end of the cytoplasmic domain. After binding with neurotrophins, the dimerization and auto-phosphorylation, the Trk receptors recruit adaptor proteins as Shc (Src homology-containing protein) and FRS2 (Fibroblast Growth Factor Receptor Substrate 2) and effector proteins such as PI3K, PLCγ and Ras, activating three possible signalling pathways:

Pathway signaling through Ras: regulates the proliferation and cell differentiation through the activation of the cascade of MAPK (Mitogen-Activated Protein Kinase). The Shc protein binds to the Tyr496 phosphorylated forms of the TrkA receptor and is in turn phosphorylated, recruiting Grb2 (adapter protein that acts as a bridge between Shc and SOS because it binds the phospho-tyrosines of Shc through domain SH2 and a region rich in proline of SOS through domain SH3) and SOS at the plasma membrane. SOS is a guanidil exchange factor (GEF) that allows for the replacement of GDP with GTP in Ras protein allowing the activation of the latter: Ras-GTP can, therefore, activate the cascade of the MAP kinases from the serine-threonine kinase Raf that phosphorylates and activates the threonine-tyrosine kinase Mek that, in turn, Phosphorylates and activates the serine-threonine kinases Erk, which migrates into the nucleus and initiates the processes of gene transcription important for proliferation and cell growth. Also the protein FRS2 can bind the Tyr496 phosphorylated and seems to be associated with the complex Grb2/SOS to prolong the signaling cascade of the Ras/ERK.

Pathway signaling through PI3K: this signal transduction pathway plays an important role in cell survival TrkA-dependent. The protein PI3K is formed of a catalytic subunit indicated as p110 and a regulatory subunit called p85:

following the binding of a neurotrophin receptor Trk, there is the formation of the reasons phospho-tirosinici in the intracellular portion of the receptor itself, which are recognized by the domain SH2 subunit p85 and this interaction leads to the detachment of the two subunits of PI3K with consequent activation of the catalytic portion p110. the latter phosphorylates membrane phospholipids leading at the formation of phospho-inositidi PI(3,4,5)P3 that are recognized by PH domain of the serine-threonine kinase PDK1 (Phosphoinositide-Dependent Kinase-1) and protein Akt, allowing the recruitment at the plasma membrane. Akt ,activated by PDK1 can, in turn, phosphorylate and inhibiting some target proteins such as the pro-apoptotic protein Bad, caspase 9 and the inhibitor of NFκB, IκB, thereby promoting cell survival.

Pathway signaling via PLCγ: tyrosine Tyr791 phosphorylated on TrkA, and similar residues on TrkB and TrkC, act as binding sites for the PLCγ, which is phosphorylated and activated by kinase domain of the receptor. The PLCγ activated hydrolyzes the residues of diphosphate phosphatidyl-inositol PIP2 of the plasma membrane in inositol triphosphate (IP3) and diacyl-glycerol (DAGS): the first increases the levels of Ca^{2+} intracellular leading to the activation of certain proteins regulated by this second messenger as the calmodulin and some kinases; the DAG activates Protein Kinase Cδ (PKCδ), important for cell growth.

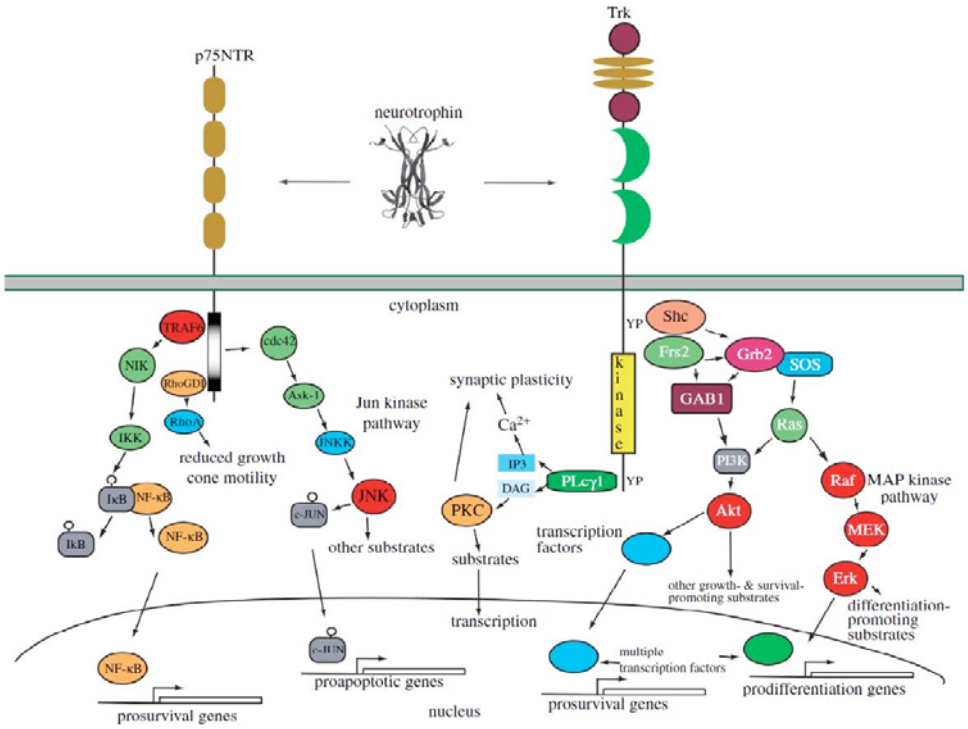


Fig 36. Signalling pathways after NGF stimulation through its receptors.

The neurotrophin connection to receptor p75NTR actives distinct signalling pathways that may promote survival or death cell .

The signalling pathways pro-survival activated by p75 involve the adaptor proteins TRAF (TNF Receptor-Associated Factor) and RIP2 (Receptor-Interacting Protein 2) that mediate signals anti-apoptotic through NFκB and Akt, while tracks pro-apoptotic involve an increase of kinase JNK (promote apoptosis by increasing the levels of the protein p53), the turnover of sphingomyelins with production of ceramide (stimulates the apoptosis because it inhibits the signalling pathways of survival mediated by Erk and PI3K) and the association with many adaptor proteins such as Neurotrophin-Receptor Interacting Factor (NRIF), Neurotrophin Associated Cell Death Executor (NADE), etc. , that directly promote the cell cycle arrest and apoptosis through the activation of caspases.

Materials and Methods

Primary cultures of human conjunctival and corneal epithelial cells and NGF treatment

Conjunctival and limbal epithelial cells were obtained from healthy volunteers at the time to cataract surgery and cultivated on a lethally irradiated feeder layer 3T3-J2 cells. Briefly biopsy was treated with trypsin (0.05% trypsin and 0.01% EDTA) at 37°C for approximately 80 minutes. Cells were collected every 20 minutes, to obtain an average of 17.3 x 103 cells/mm2. Cells were plated (1.5 x 104/cm2) on lethally irradiated 3T3-J2 cells (2.4x104/cm2) and cultured in 5% CO2 and humidified atmosphere in DME and Ham's F12 media (2:1 mixture) containing FCS (10%), insulin (5 mg/mL), adenine (0.18 mM), hydrocortisone (0.4 mg/mL), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin/streptomycin (50 IU/mL). Epidermal growth factor (10 ng/mL) was added at 10 ng/mL beginning at the first feeding, 3 days after plating. Cultures were then fed every other day. Sub-confluent primary cultures were passaged at a density of 6 x 103 cells/cm2 and cultured as above. Conjunctival cells were subject to specific characterization for NGF, trkANGFR, and p75NTR expression (see confocal and relative real time PCR). NGF concentration was evaluated by a specific ELISA in culture medium of conjunctival epithelial cells at different times.

To investigate the effect of NGF on proliferative compartment,

exogenous NGF at concentration of 100 ng/mL (human recombinant NGF from Scil) was added to the culture medium (modified at a low FCS concentration of 1%). Cells (300–2000) from the biopsy and from each cell passage of serially cultivated mass and clonal cultures were plated onto 3T3 feeder layers and cultivated as above. Colonies were fixed 12 days later, stained with rhodamine B, and scored under a dissecting microscope. Values are expressed as the ratio of the number colonies on the number of inoculated cells. All colonies were scored whether progressively growing or

aborted (total colony forming efficiency and percentage of aborted colonies values).

Determination the Colony-forming Efficiency (CFE)

Cells (500-1500) from each cell passage of serially cultivated mass were inoculated into each of two indicator dishes containing irradiated 3T3-J2 cells. Colonies were fixed 12 d late and stained with Rodamine B.

The number of cell generations was calculated using the following formula: x = 3.322 log N/No, where N equals the total number of cells obtained at each passage and No equals the number of clonogenic cells. Clonogenic cells were calculated from CFE data, which were determined separately in parallel dishes at the time of cell passage. The informations obtained after CFE analysis are the following: Adhesion capacity of clonogenic cells, ratio between differentiated cells and proliferative clonogenic cells, regeneration capacity of the tissue (the colony is stratified in the middle), Migration (diameter) and the number of abortive colonies is an early index of how many cells are differentiating.

Cell Line Culture

The limbal epithelial cell line (HCL-ECs) was a kind gift of Ilene

Gipson. HCL -ECs were cultured in keratinocyte serum-free medium (K-SFM; Gibco-Invitrogen Corp., Rockville, MD) containing 25 x g/mL bovine pituitary extract (BPE) and 10 ng/mL EGF in six-well plates (5 x 104 cells/cm2) at 37°C in a 5% CO2, followed by growth in a 1:1 mixture of K-SFM and low calcium dF12 (DMEM-F12; Euroclone).

HCL-ECs were subject to specific characterization and silencing with specific siRNA for TrkA and P75 expression.
The PC12 cell line was derived from a transplantable rat pheochromocytoma. We use them as a positive control for the NGF functionality. These cells respond reversibly to NGF by induction of neuronal phenotype when pated on Collagen IV coated culture flasks.

Immunofluorescence

Conjunctival and Limbal Cryo-sections included in OCT (optimal cutting temperature) and cultures cultivated on cover-glasses were fixed with (PFA 3%), permeabilized (0.5 % Triton X-100 in PBS), coated with 2% BSA for 30 minutes at 37°C. The following antibodies were used: rabbit anti-p63α (Primm), rabbit anti-TrkA (Epitomics), mouse anti-p75 (Millipore), mouse anti-Ki67 (Leica). Secondary FITC-labelled antibodies (Alexa-fluor 488) were obtained by Invitrogen. Confocal analysis was performed with confocal laser-scanning microscopy (LSM 510, Zeiss).

Real-Time PCR

Total RNA was extracted from $2x10^6$ epithelial cells with miRNasy Mini kit (Qiagen). RNA quality was analyzed by spectrophotometer analysis (Nanodrop) and 1% agarose gel electrophoresis. cDNA were synthesized from 100 ng of RNA and PCR reaction was perform using 40 cycles in 7900 HT fast real Time PCR System (Applied Biosystem).

Primer sequence are:

hGAPDH_F: GAAGGTGAAGGTCGGAGTC
hGAPDH_R: GAAGATGGTGATGGGATTTC

hp63Tot_F: GGCTGTTCCCCTCTACTCGAA
hp63Tot_R: CATGAGCTGAGCCGTGAATTC

hBmi-1_F: CTGGTTGCCCATTGACAGC
hBmi-1_R: CAGAAAATGAATGCGAGCCA

hp63alpha_F: GACCACCATCTATCAGATTGAGCAT
hp63alpha_R:TGCTCAGGGATTTTCAGACTTG

hCEBPd_F: TGCAGTTTCTTGGGACATAGGA
hCEBPd_R33 :CACGTTTAGCTTCTCTCGCAGTT

h14-3-3-sigma_F:GAGAGAGCCAGTCTGATCCAGAAG
h14-3-3-sigma_R:GAAGGCTGCCATGTCCTCAT

hKrt19_F:GGTCATGGCCGAGCAGAA
hKrt19_R:TTCAGTCCGGCTGGTGAA

Western Blotting

Total proteins were extracted from $2x10^6$ epithelial cells with RIPA buffer, following the condition supply by the manufacturer (Sigma). Equal protein amounts (80μg) were fractionated on 8% SDS-page resolving gel.

Electroporeted proteins were transfer to Hybond membranes and were probed with the following antibodies: rabbit anti-Δp63α (Primm), rabbit anti-TrkA (Epitomics), mouse anti-p75 (millipore), mouse anti-Bmi1 (Millipore), mouse anti-p634A4 and rabbit anti-C/EBPδ (Santa Cruz Biotechnology), mouse anti-GAPDH (Abcam), anti-14-3-3σ (abcam) and k19 (epitomics). Immunoreactions were performed using HRP coniugated secondary antibodies (1:10000) (Santa Cruz Biotechnologies) and develop by ECL techniques (SuperSignal West Pico, Pierce). Quantification of bands was done using Image J software.

Histological procedure

Conjunctival cultures were fixed with PFA (3%) three days after the confluence and were stained with Alcian blu-periodic acid-Schiff kit according to the manufacturers instruction (Bioptica, Milan). Quantification of Goblet cell was done using the bright-field microscopy (Axiocam Zeiss). We performed this technique for the conjunctival cells after elutriation which are seeded on poly-L-lysine chambers. We performed hematoxylin-eosin and giemsa staining in the organotypic over-confluent conjunctival culture.

Sandwich-ELISA

The supernatants of the two conjunctival organotypic cultures "treated" and "untreated" retrieved on the 5th day and 9th day of culture, were diluted according to a 1:1 ratio with a carbonate-bicarbonate buffer pH9.6 , distributed in special 96-well plates and allowed to dry by incubation at 42 °C in order to allow the absorption of the components of the supernatants on the bottom of the wells . Then the samples were rehydrated by washing in 1X

PBS and incubated with PBS1X-BSA2 (blocking) at room temperature for 1 h, spent which was added to each well the specific primary antibody for the MUC5AC (sc-33667; Santa Cruz Biotech) diluted 1:700 in PBS-Tween 0.05 % . After incubation for 1 h at room temperature, were carried out washes in 1X PBS, added the secondary antibody conjugated to HRP enzyme and carried out a further incubation at room temperature for 1 h.
To the wells was added, finally, the TMB solution containing the colorimetric substrate for the peroxidase and the samples were incubated for 15-30min at 37 °C: at the end of the incubation was added a solution of STOP, optic density (OD) was detected at 470 to 570 nm in a microplate ELISA reader (GLOMAX).

Proliferation Assay

Cell proliferation was investigated at each experiment by counting either cells after brief enzymatic digestion (trypan blue exclusion test), or cells stained with the mouse anti-human ki67 antibody (1:20, Leica) a nuclear proliferating factor ki67 recognizing all the cell cycles except G0. We used the Click-iT® EdU cell proliferation assay to provide a superior alternative to traditional methods for detecting and quantitating newly synthesized DNA. Click-iT® assays use a modified nucleoside, EdU (5-ethynyl-2´-deoxyuridine), that is incorporated during DNA synthesis in a quick click chemistry reaction (Invitrogen) and next we quantified the positive cells for EdU with Imagej.

Inhibition and silencing of the receptors TrkA and p75

Small molecule oxindole derived (GW 441756 Axon Medchem) was used for inhibit TrkA at 0.23 μM and monoclonal antibody p75 was used as biological

blockade (clone ME20.4 millipore). For transient silencing we used the smart pool of human NTRK1 (4914) and human NGFR (4804, Thermo scientific). We used lipofectamine RNAiMAx (invitrogen) for the transfection of these small RNAs.

Elutriation

Confluent secondary conjunctival cultures in two different growth state sub and over-confluent were trypsinized and cells were suspended in the elutriation buffer (see reference 63) the counter-flow centrifugation-elutriation was carried-out at room temperature in a Beckman centrifuge equipped with an elutriation rotor (Avanti J-26XP) and a Sanderson elutriation chamber. A peristaltic pump was used for the counter-flow. When the cell suspension was loaded in the Sanderson's chamber, the rotor speed was 1000 r.p.m. and the counter-flow rate was set at 5 ml/min. These conditions allow cells to separate inside the chamber. Subsequently while the rotor speed was maintained at a constant rate, the counter-flow rate was increased stepwise to 6,5-10-15-25-50 ml/min. For each step 150 ml elutriate was collected. The cells elutriated from each fraction and each growth state were centrifugated, resuspended in KNO and was separated and seeded (1000 cells) in indicator dish for CFE analysis and (25.000 cells) on chamber slide with poly-L-lysine for goblet quantification.

Feeder Depletion

Depletion of feeder cells (3T3-J2) with the Feeder Removal MicroBeads (miltenyi) have been developed for the efficient depletion of mouse feeder cells from co-cultures with human cultures cells. Each conjunctival strain treated and untreated with NGF was depleted form 3T3-J2 cells for obtaining

an pure human epithelial population for further analysis like Microarray, Facs and RT-PCR.

Flow Cytometry Detection of Surface Antigens For Cells

Pellet from 30.000 cells for each samples was centrifugated at 4500 r.p.m. for 5 minutes and resuspended by pipetting in 25μl of PBS-FBS 2%-EDTA 5mM and 1μl of primary antibody was added and mixed by vortexing. After 30 minutes at +4°C the samples washed with 500μl of PBS-FBS 2%-EDTA 5mM, centrifuge at 4500 r.p.m. for 5 minutes and resuspend each sample in 350μl of PBS-FBS 2%-EDTA 5mM. The antibody used for FACS analysis are, TrkA-FITCH (Millipore), p75-PE (BD) and Feeder-FITCH (miltenyi).

Microarray Analysis

Human conjunctival cells from 5 different donors were sub-cultured on 3T3 feeder cells, plated at the density of 8.000/cm^2 and in keratinocyte medium (see culture medium) in the presence or absence of NGF added every another day. After 4 days of treatment both NGF-treated and untreated controls were trypsinized, feeder-depleted (as described before). For all the conjunctival cells samples feeder contamination was lower than 1%, as detected by flow cytometry. For each sample 0.7×106 conjunctival cells were washed twice with phosphate-buffered saline, lysed in Qiazol reagent (Qiagen, Hilden, Germany) and frozen at −80°C. Total cellular RNA was extracted using miRNeasy Micro kit (Qiagen) according to the supplier's recommendation.

For both NGF-treated and untreated Control (Cntrl) conjunctival cells, obtained from 5 independent experiments, polyadenilated mRNA was converted in biotinilated aRNA according to the GeneAtlas 3' IVT Express Kit protocol supplied by Affymetrix (Santa Clara, CA, USA). Similarly, Affymetrix Human Genome U219 GeneAtlas® hybridization, staining, and scanning were performed according to the Affymetrix GeneAtlas™ Hybridization, Wash, and Stain Kit for 3' IVT Array Strips instruction manual.

Microarray analysis was performed on CEL files using Partek® Genomics Suite™ (Partek Inc., MO, USA) (http://www.partek.com). The microarray data were processed using Robust Multi-Array Analysis (RMA) for background adjustment and signal normalization. Batch effect removal was applied for the different samples, to remove individual variations.

ANOVA was then performed to identify significant differentially expressed transcripts. More in detail, the criterion of ≥2 relative fold change at a false discovery rate of ≤5% was defined in order to select differentially expressed transcripts between NGF-treated and untreated conjunctival cells.

Organotypic Conjunctival epithelium

Conjunctival cells co-cultivated in the presence of a layer of 3T3 feeder cells previously inactivated by γ-irradiation (6000 rads) and seeded on fibrin gel (Tissucol Baxter) composed by two solutions: fibrinogen and thrombin. When the conjunctival cell cultures (treated and untreated) reached the over-confluent state (multi-layer stratification) we separate in two equal parts. One part was included with OCT for immunofluorescence analysis and the other part we treated with dispase solution and after complete dissolution protein extracts obtained.

Clonal Analysis

The growth potential and the clonogenic capacity of individual multiplying limbal epithelial cells is best evaluated by clonal analysis. After limiting dilution single cells was seeded onto 96- multi well plates (total 288 cells) containing lethally irradiated feeder-layer. After seven days of cultivation, each well is searched for the presence of a clone under an inverted microscope. 26 clones is then dissociated with trypsin to obtain a single cell suspension, which for each clone 1/4 is inoculated into several indicator dishes (usually 100mm Petri dishes) and the remain 3/4 is divided into two equal parts and seeded in two 6-multi well after 12h we change the concentration of FBS10% to FBS1% and we start to treat with 100ng/ml NGF (one part) and cell cultures after reached sub-confluent we extract the protein for further analysis.

The indicator dishes are fixed after 12 days of cultivation, stained with Rhodamine B and examined under a dissecting microscope, then three types of colonies are distinguished: 7 Holoclones (fewer than 5% of colonies abort, 3 Paraclones more than 95% of abortive colonies and 16 Meroclones with intermediate percentage.

Immuno-cytochemistry Analysis

Quantitative immuno-detection of ΔNp63α after cytospin preparation of limbal and conjunctival cells (treated and un treated) and immonofluorescence staining with ΔNp63α (primm). Compared to conventional techniques,

Quantitative Fluorescent Immunohistochemistry (Q-FIHC) allows the automated quantification of protein signals from a low number of cells (500 cells). p63α-bright selected on basis of diameter and mean ΔNp63α of fluorescent intensity value. On the basis of long lasting results from Pellegrini G. and De luca M. group, only cultures that contain more than 3% Δp63α-bright cells with a small diameter (6-12μm) are used to prepare grafts with clinical success.

RESULTS

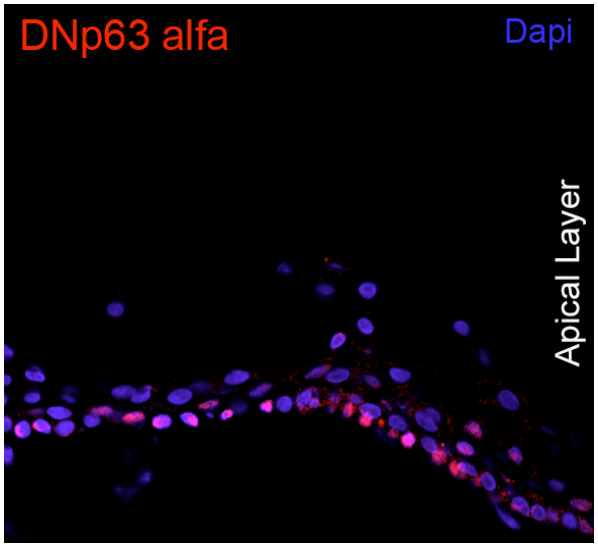
Bulbar conjunctival culture cells and treatment with NGF

Expression of TrkA, p75 receptors and ΔNp63α in human bulbar conjunctival cells.

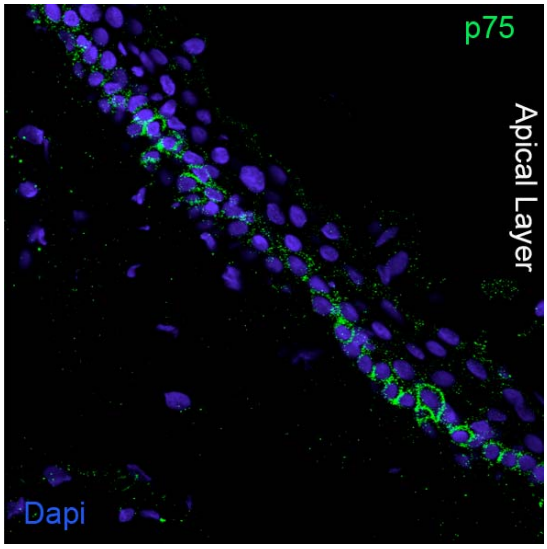
Immunofluorescence analysis of bulbar conjunctival sections indicates, as previously described (65), that this conjunctival region in vivo expresses the two receptors which mediate the effects of NGF, TrkA and p75 (Fig 37B and C). p75NTR receptor is expressed in patches of basal cells while trkANGFR has diffuse expression.

We also demonstrate that basal cells of this region express ΔNp63α (Fig 37A) which sustain the proliferative potential of basal epidermal keratinocytes and identify limbal stem cells both in vivo and in vitro (*Pellegrini et al*.,2001).

A.



B.



C.

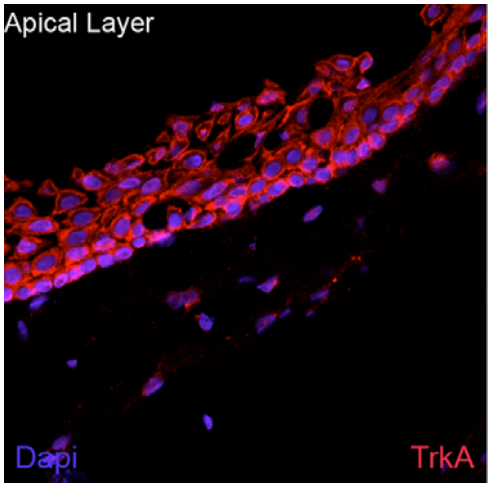
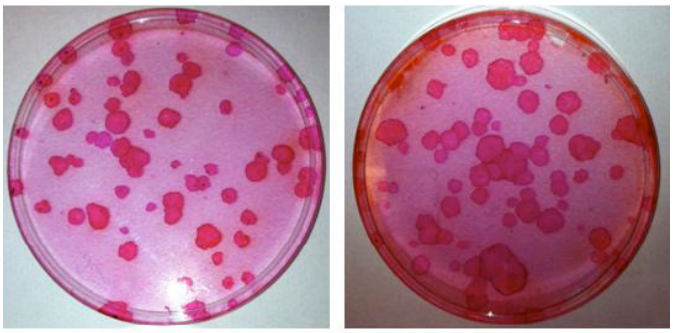


Fig 37. **Expression of TrkA and p75 in human conjunctiva**. Immunofluorescence analysis of human conjunctiva. Sections were stained with **A.** ΔNp63α (red) **B.**p75 (green) and **C.** TrkA (red).

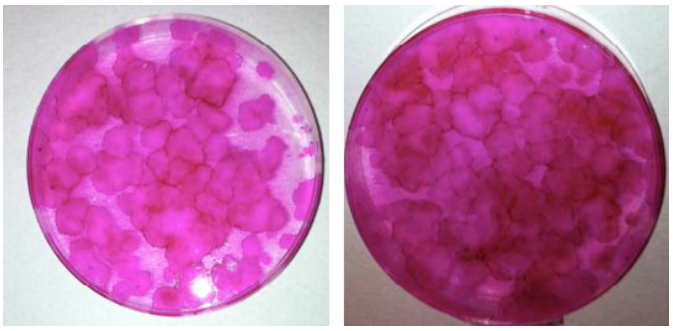
Evaluation of Clonogenic Ability and Growth Potential after NGF treatment.

In order to evaluated the clonogenic ability of four biopsies taken from different donors, we plated 500 cells from each serial passage onto lethally irradiated 3T3-J2 cells, and stained 12 days later with rhodhamine B. At the second passage we treated the dishes with 1% of FBS and we added to the culture medium 100 ng/ml of NGF in order to investigate its effect on proliferative compartment.

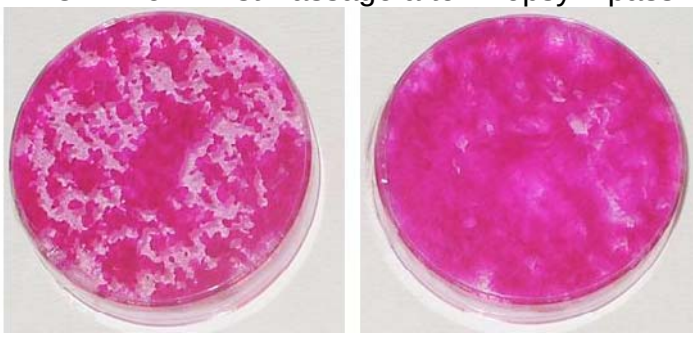
The analysis of the colony-forming efficiency of control samples compared to treated samples have showed an increase number of the clonogenic cells after exposure to NGF in all passages analized (Fig 38). This result suggests a possibile role of this growth factor for the stimulation of conjunctival epithelial cells clonogenicity.



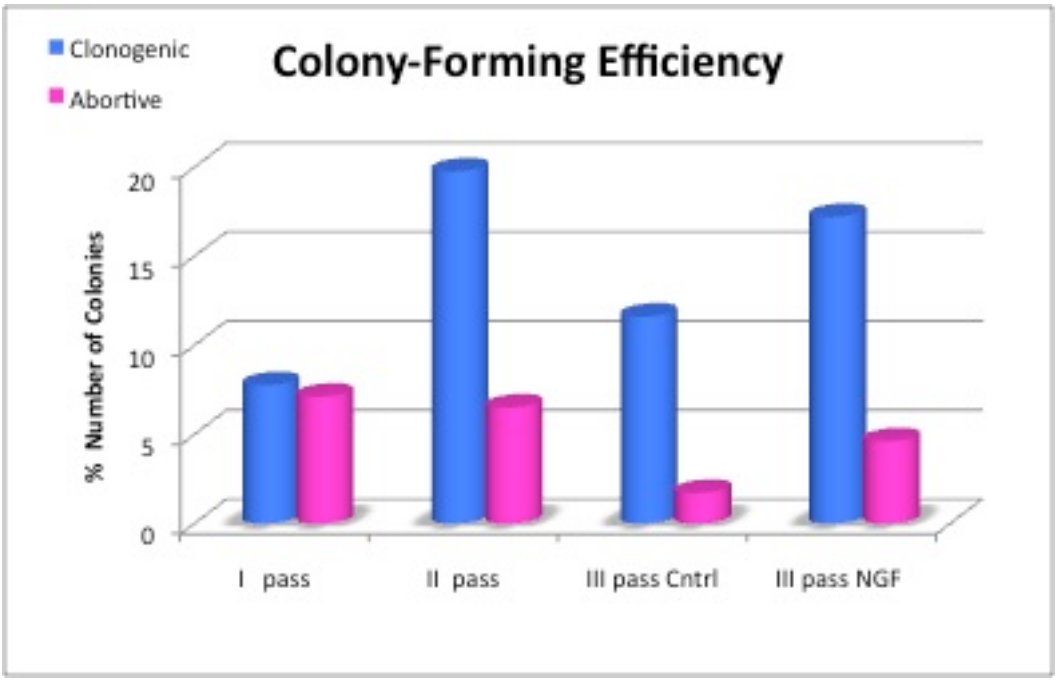
A. CFE from Biopsy



B. CFE from First Passage after Biopsy II pass



B. Left dish is the control indicator and the right dish is treated with NGF IIIpas



D.CFE assays and evaluation of clonogenic and abortive colonies

Fig 38. Indicator dishes plated with conjunctival cells and Colony Forming Efficiency Analysis (CFE). **A** and **B** show the CFE of the first two passages. **C** The dishes of the control and treated samples were cultured with 1% of FBS. All the CFE showed here are representative for all four strains. **D.** CFE are expressed as the ratio of the total number of colonies on the number of inoculated cells.

In vitro expression of TrkA and p75NTR in conjunctival cultures.

In conjunctival cultures, the cells of this epithelium express the two receptors, TrkA and p75, like the in vivo immunostaining. We found that TrkA has a diffuse expression in both untreated and treated cultures (Fig 39). p75 is expressed by a discrete number of cells and its expression increases after treatment. The expression pattern of these receptors after treatment with 100 ng/ml with NGF indicates that this concentration of NGF does not saturates the receptors and it can be optimal for observe both positive and negatives modulations in our system.

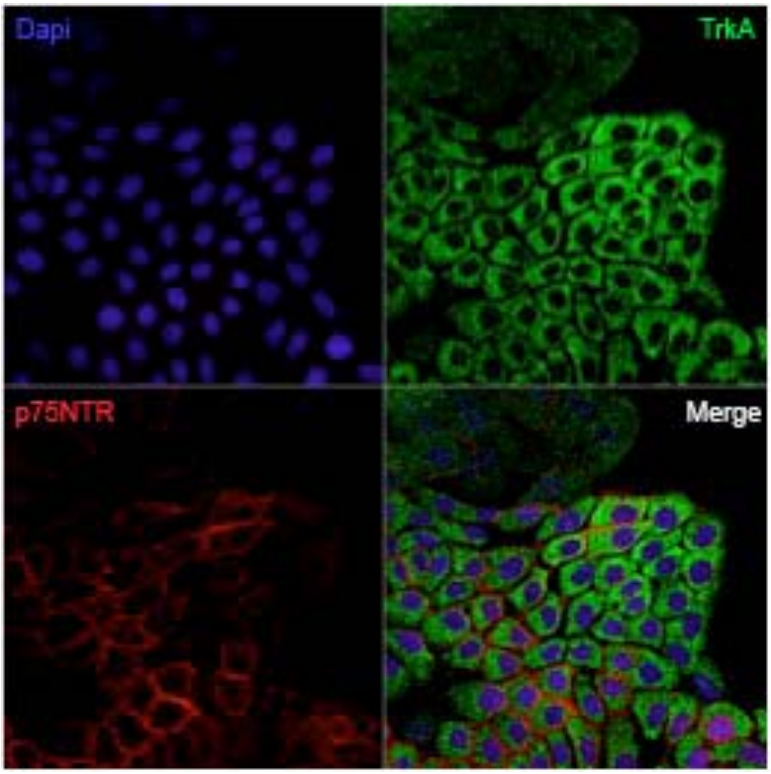


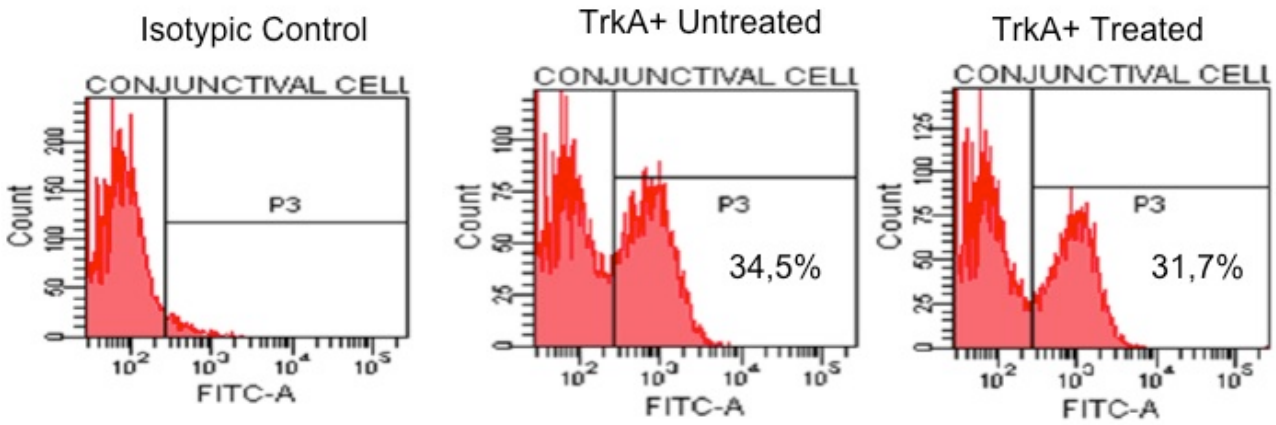
Fig 39. Double immunostaining with TrkA (green) and p75NTR (red) of conjunctival untreated cultured cells stained.

Cytofluorimetric and western blot analysis of untreated and NGF treated cultured cells at II passage showed that the expression of TtkA is little modified after exogenous stimulation with NGF. The little decrease observed by cytofluorimetric analysis is probably due to TrkA endocytosis; with immunofluorescence we observed a diffuse expression. For highlight the effect on epithelial cells we depleted the feeder-layer in order to obtain a pure conjunctival population. The amount of feeder is shown in the following table.

	Strain1		Strain 2		Strain 3		Strain 4	
	CONTR	NGF	CONTR	NGF	CONTR	NGF	CONTR	NGF
FEEDER +	0,1%	0,3%	0,7%	0,2%	0,1%	0,3%	0,6%	0,8%
TRKA +	34,5%	31,7%	20,2%	24,6%	35,8%	31,3%	14,5%	9,1%

Table 1. Percentage of feeder layer contamination and TrkA expression in four strains monitored by cytofluorimetric analysis.

A.



B.

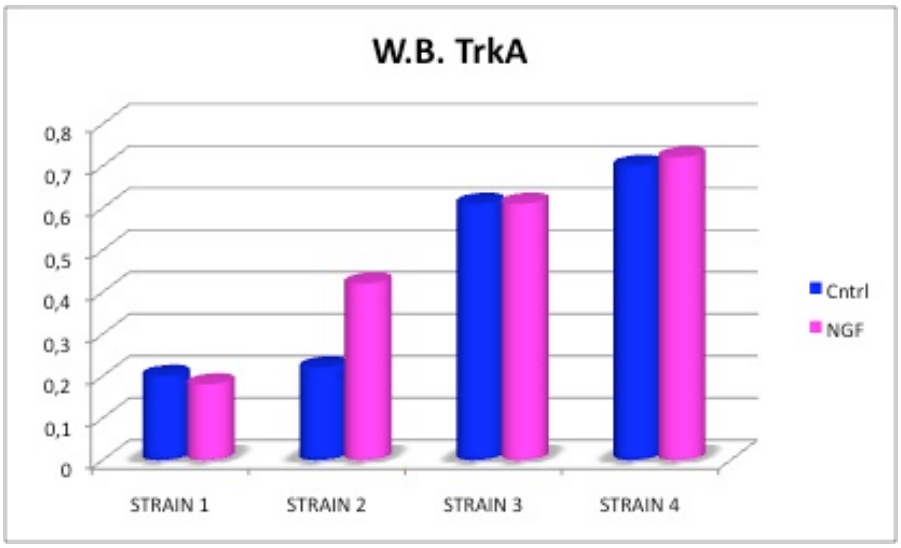
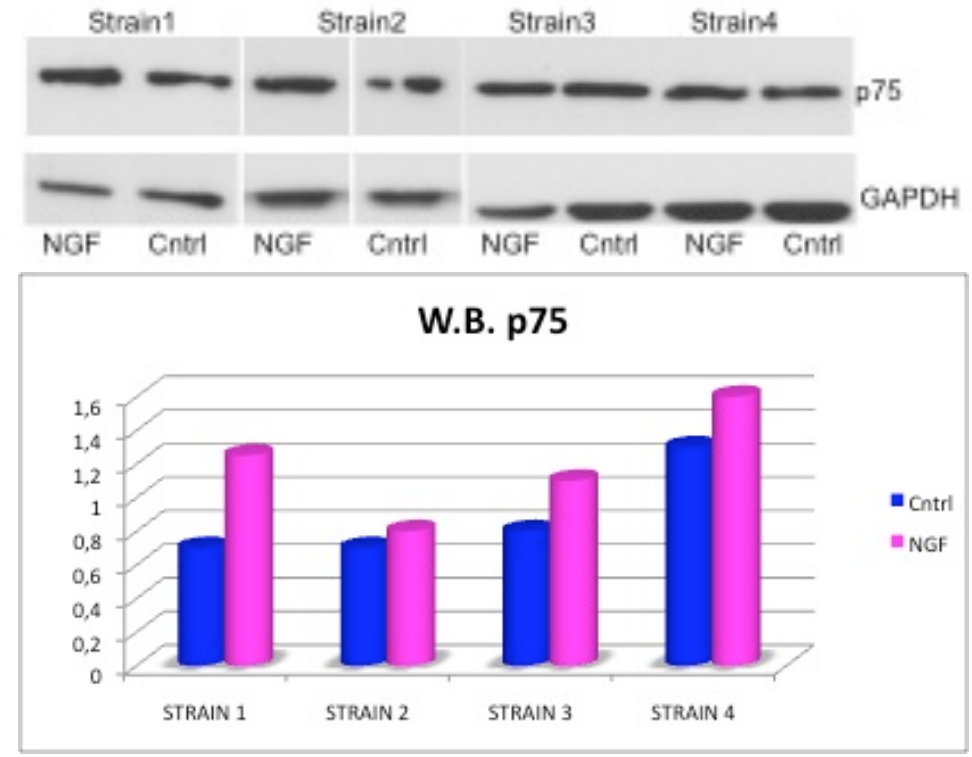


Fig 40. Cytofluorimetric and Western Blot analysis **A.** Histograms of TrkA receptor in strain1 untreated and treated with NGF. **B.** Western Blot analysis of the expression of TrkA. Protein samples were normalized with GAPDH.

The expression of p75 is increased after treatment with NGF in most of the strains. This event is observed by measuring mRNA and protein levels by western blot and RT-PCR respectively (Fig 41). With immunofluorescence staining (Fig 39.) we observed the same results in the treated cells.

A.



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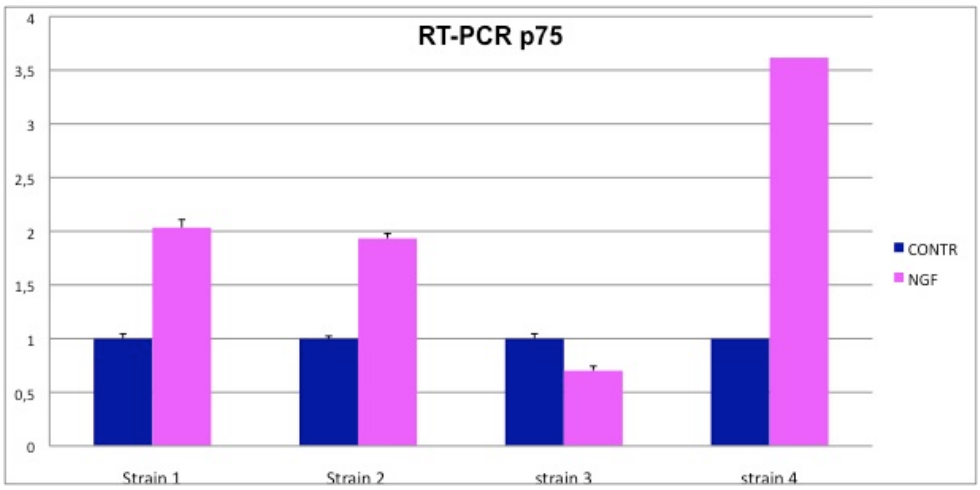


Fig 41 Western Blot and Real Time-PCR analysis. **A**. Western Blot analysis of p75 and its quantification by imageJ. Samples were normalized using GAPDH. **B.** Quantification of p75 mRNA expression by Real-time PCR level of GAPDH were measured as loading control for mRNA .

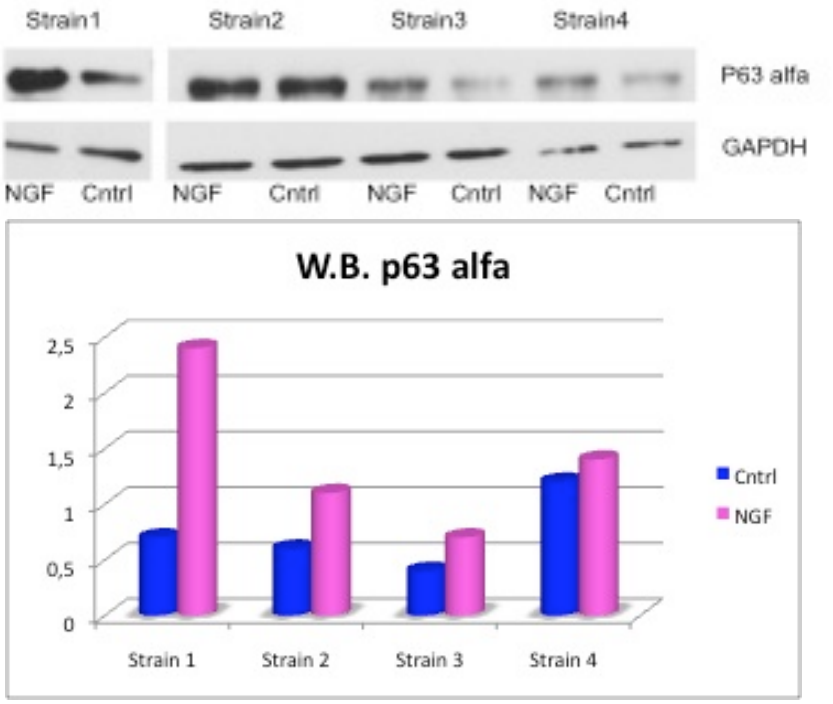
Analysis of the proliferation and stemness markers

The stemness markers like ΔNp63α, Bmi1 and C-EBPδ have been analyzed with different techniques. We observed an increase of protein and mRNA levels of p63α and p63 tot (4A4) after NGF treatment (Fig 42). These

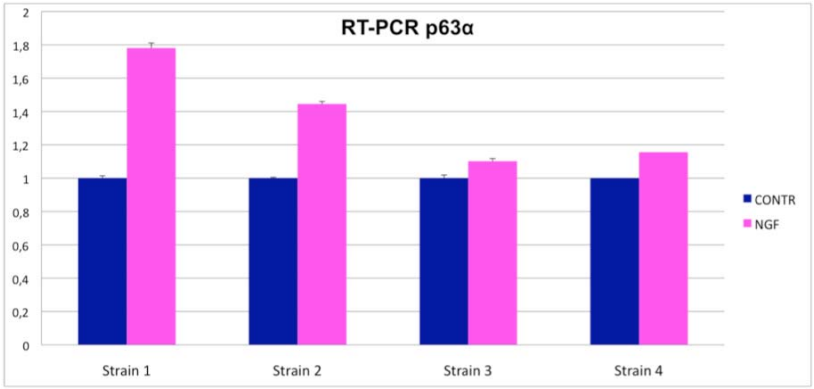
transcription factors are associated with the proliferative potential of the progenitors and/or stem cells. The expression of Bmi1 and C/EBPδ, remain constant or increase moderately; these proteins are responsible for mitotic quiescence and self-renewal.

The increase expression of the protein and mRNA levels of p63α and p63tot is quantitatively variable in relation to analyzed strain.

A.



B.



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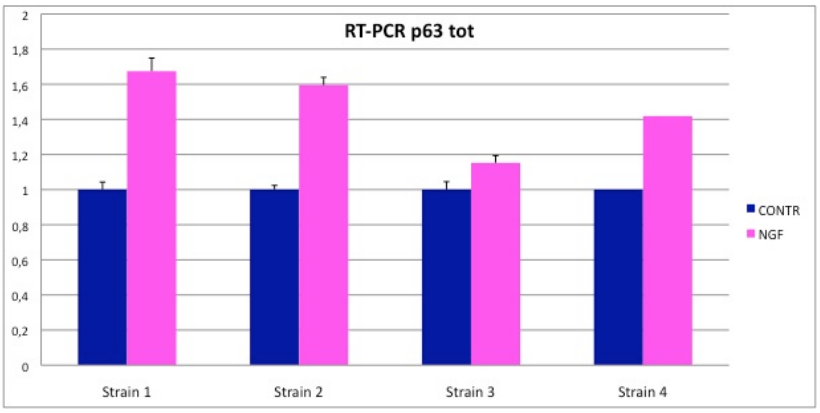
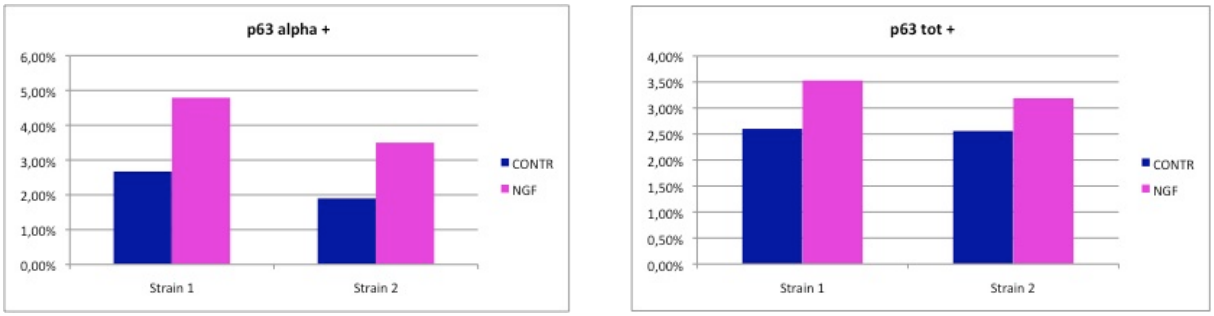


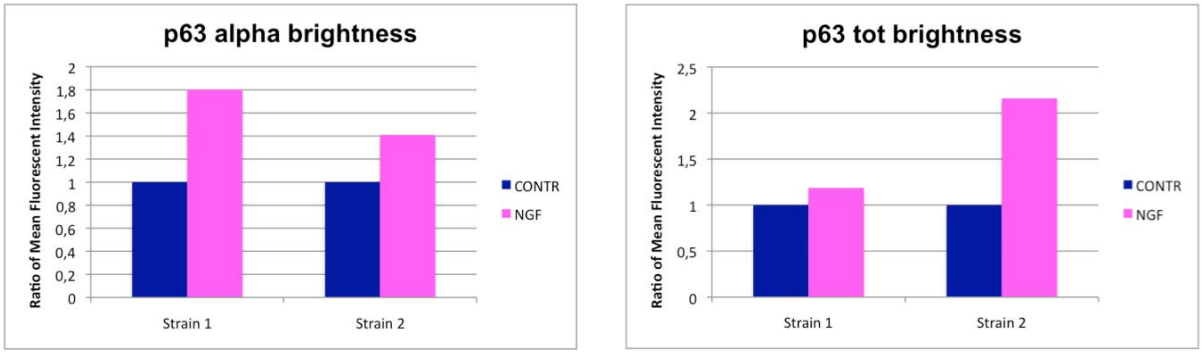
Fig 42. Western Blot and Real Time PCR analysis. **A**. Western Blot analysis of p63α and its quantification. **B** and **C.** Quantification of ΔNp63α and all ΔNp63 isoforms (p63 tot) mRNA expression by Real-time PCR, level of GAPDH were measured as loading control for protein and mRNA levels.

The analysis of cytopsin preparations with bi-parametric automated immunofluorescence has highlighted an increase in the stem cells number in cultures, after treatment with NGF (Fig 43), as indicated from the intersection analysis between the expression of p63α and/or p63 tot and the dimensions of cells.

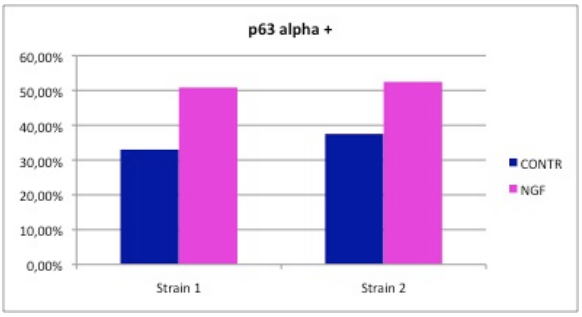
A.



B.



C.



D.

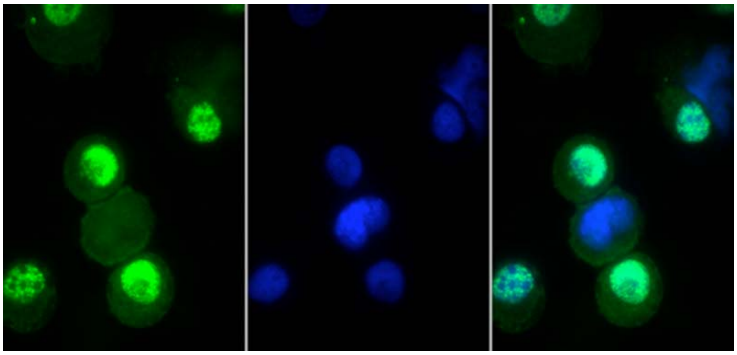


Fig 43. Immuno-cytochemistry Analysis **A.** Percentage of stem/progenitor cells **B.** Ratio of mean fluorescent intensity of p63α and p63 tot. **C.** Percentage of p63α positive cells. **D.** immunostaining of p63α (green) and Dapi (blu) in cytospin preparations.

It should be highlighted that small variations in the expression of this transcription factor have been made in relation to different permanence of epithelia in vivo (22).

Samples treated with this growth factor showed a moderate increase number of cells expressing the nuclear antigen Ki67 if compared with untreated culture (Fig 44).

A. Cntrl **B.** NGF

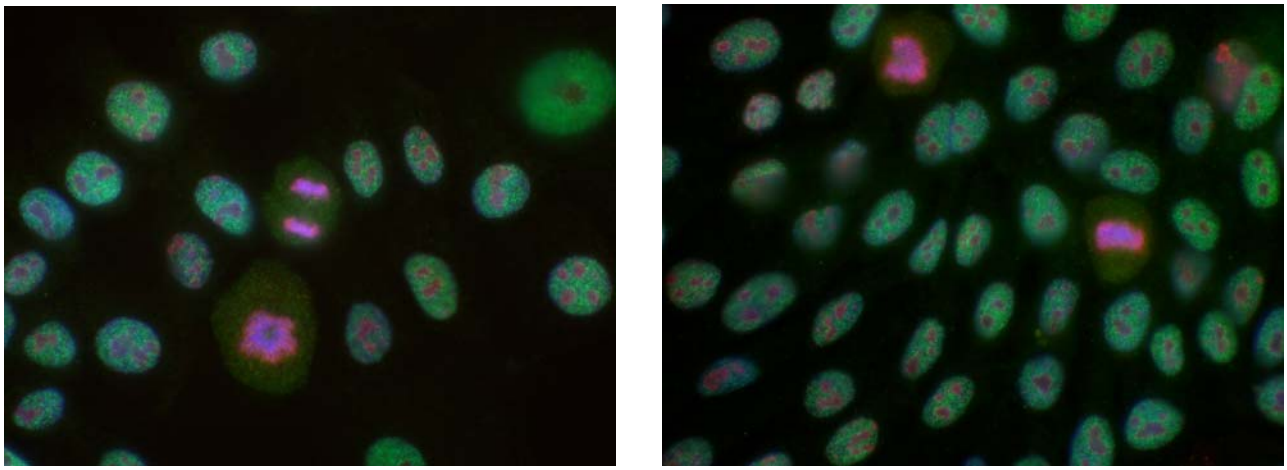
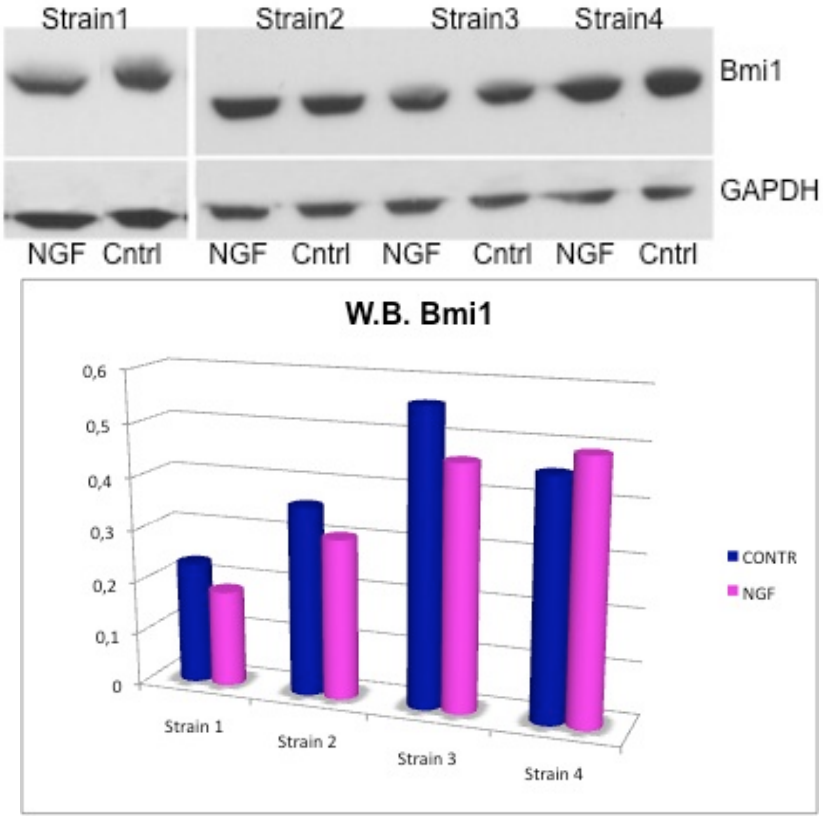


Fig44. Double immunofluorescence of conjunctival cultured cells **A.**untreated and **B**.treated, stained with ΔNp63α (green) and Ki67 (red).

Protein and mRNA expression of mitotic quiescence and self-renewal markers like C/EBPδ and Bmi1 remain constant or increase moderately after NGF treatment (Fig 45).

A.



B.

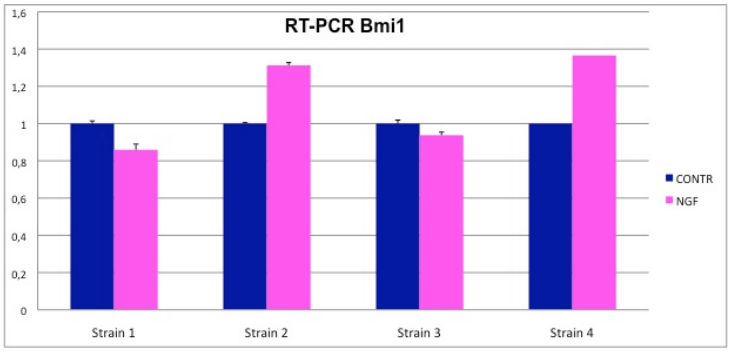


Fig 45. Western Blot and RT-PCR analysis **A.** Western blot for Bmi1 and its quantification. Protein samples were normalized with GAPDH **B.** Bmi1 mRNA in cultures data were normalized to specific house-keeping mRNA (GAPDH).

NGF induces an increase of C/EBPδ mRNA as showed by relative real-time PCR (Fig 46).

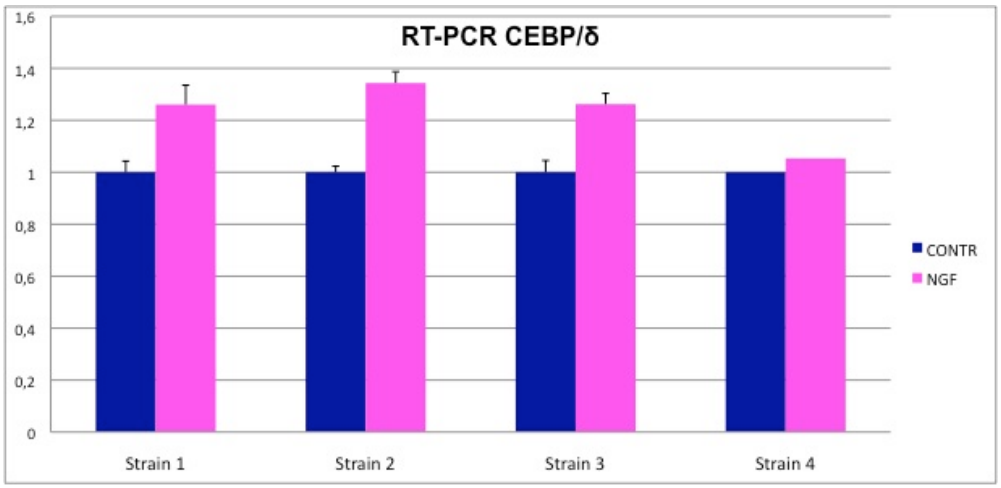
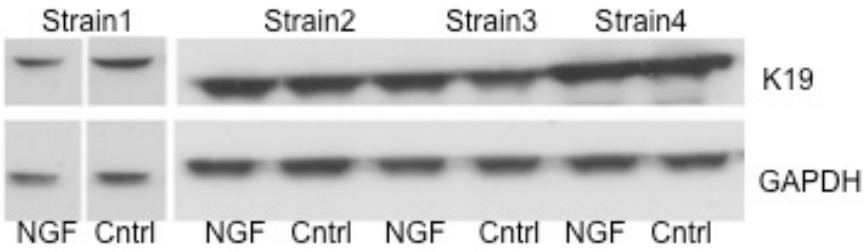


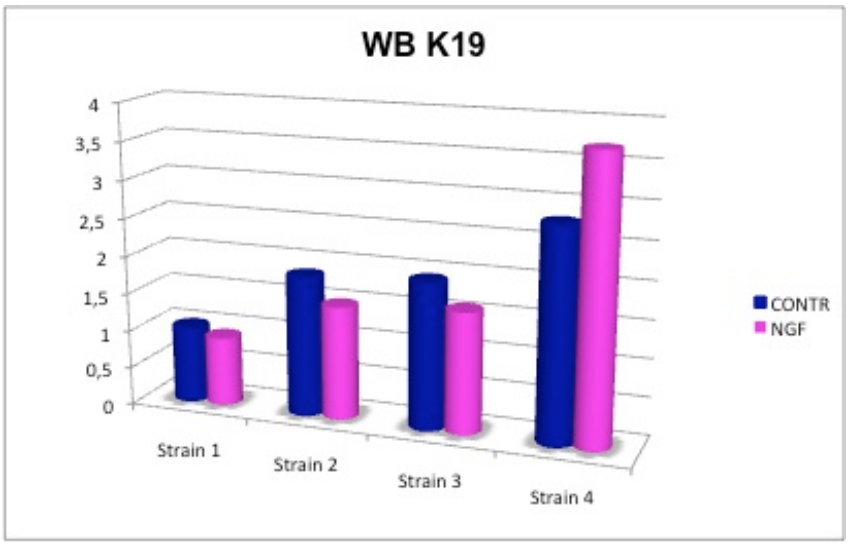
FIG 46. RT-PCR analysis. C/EBPδ mRNA in cultures data were normalized to specific house-keeping mRNA (GAPDH).

Expression of differentiation markers, 14-3-3σ and K19.

Protein and mRNA expression levels of epithelial differentiation markers, 14-3-3σ and K19, are variable between the four strains. their pattern of expression remains stable or increase moderately after treatment with NGF (Fig 47).

A.





B.

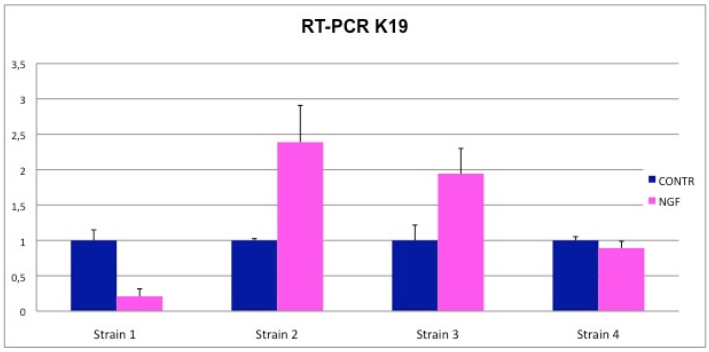
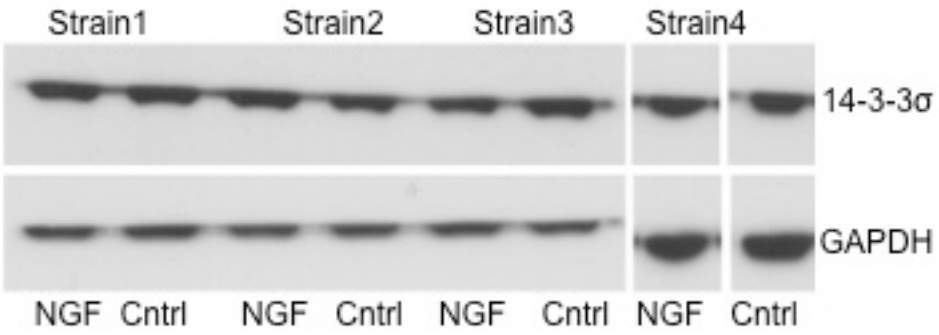
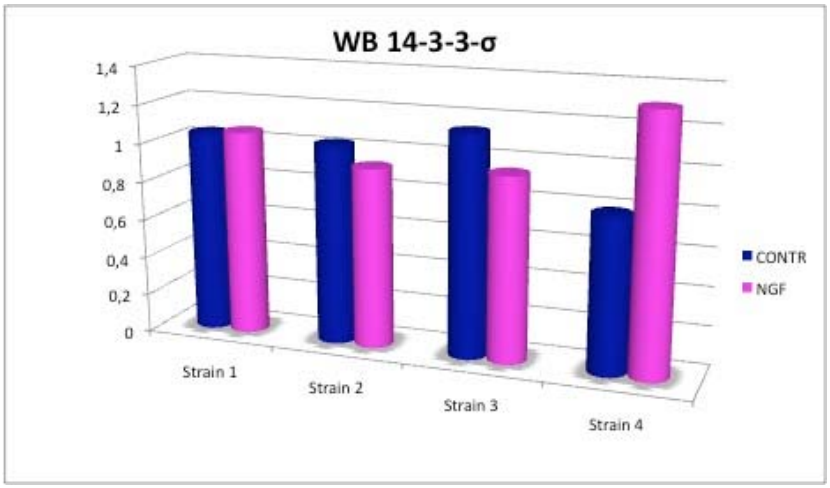


Fig 47. Western Blot and RT-PCR analysis **A.** Western blot of K19 and its quantification. **B.** K19 mRNA quantification, level of GAPDH were measured as loading control for protein and mRNA levels .

Protein and mRNA expression level of 14-3-3σ are increased more in the forth strain which contained more abortive colonies (data not show from CFE analysis), than in the other strains.

A.





B.

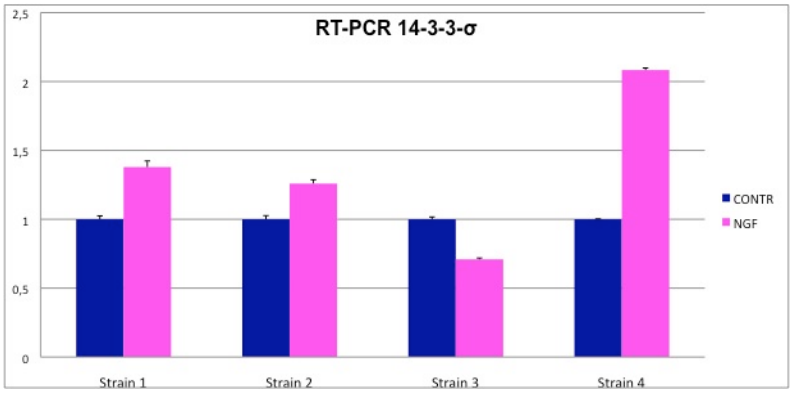
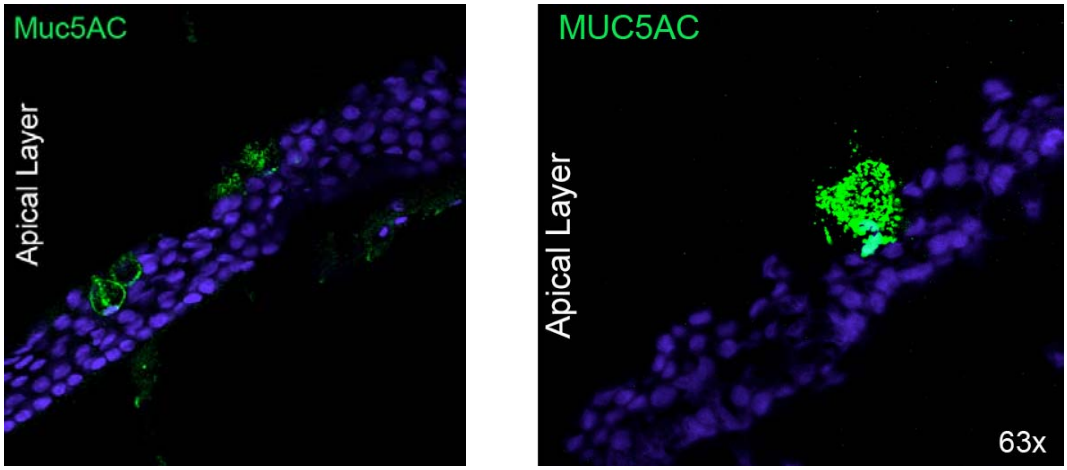


Fig 48. Western Blot and RT-PCR analysis A. Western blot for 14-3-3σ and its quantification**.** 14-3-3σ mRNA quantification, level of GAPDH were measured as loading control for protein and mRNA levels

Goblet Cell Differentiation and Mucin Production after NGF treatment.

Goblet cells are normally formed in apical layer of the entire human conjunctiva and express the specific mucin5AC (Fig 49).

A.



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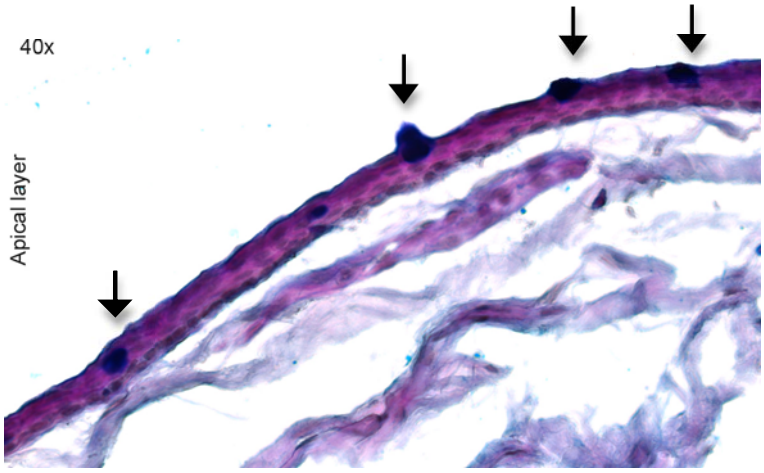
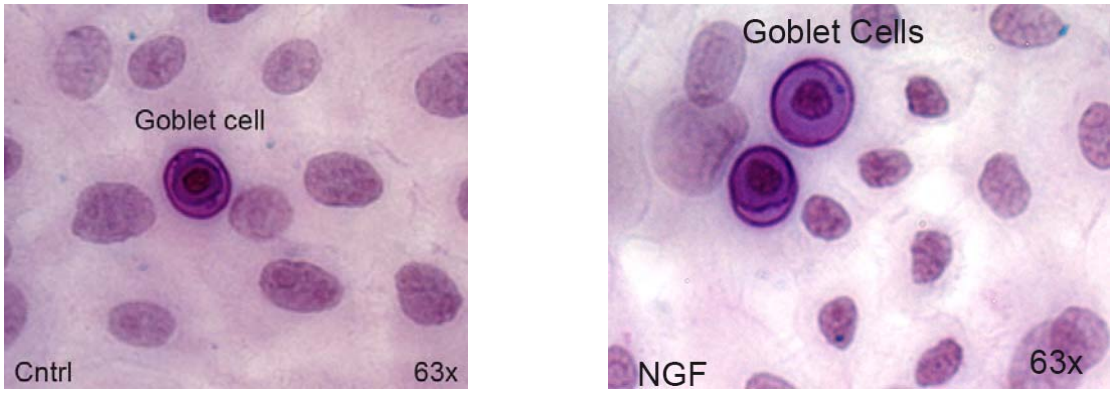


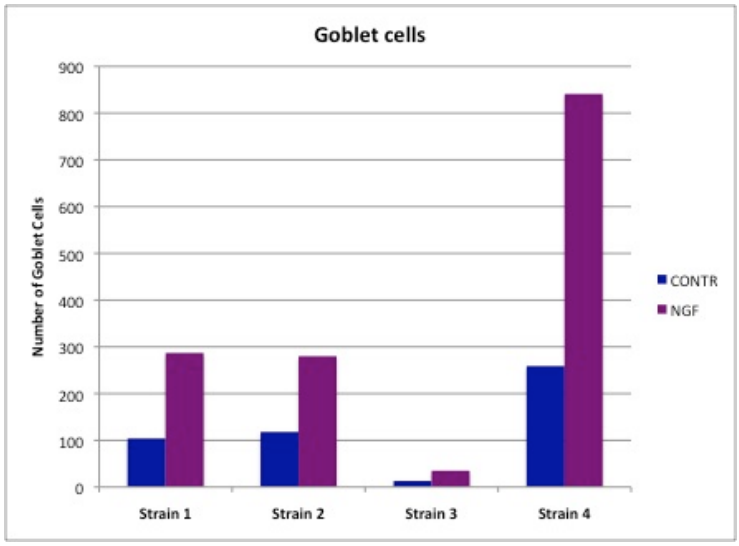
Fig 49. Immunostaining for Goblet and Muc5AC positive cells**A.** Immunostaining with Muc5AC (green) in sections of resting bulbar conjunctival. **B.** Immunohistochemical analysis with PAS-Alcian Blue for Goblet cells detection (arrows indicate the goblet cells).

The most intriguing data of this thesis is that NGF can induce the differentiation of conjunctival epithelial cells into goblet cells. We observed an increase number of goblet cells in cultures treated with the nerve growth factor comparing to the untreated samples. We revealed the presence of the goblet cells and quantified them by alcian/PAS.

A.



B.



C.

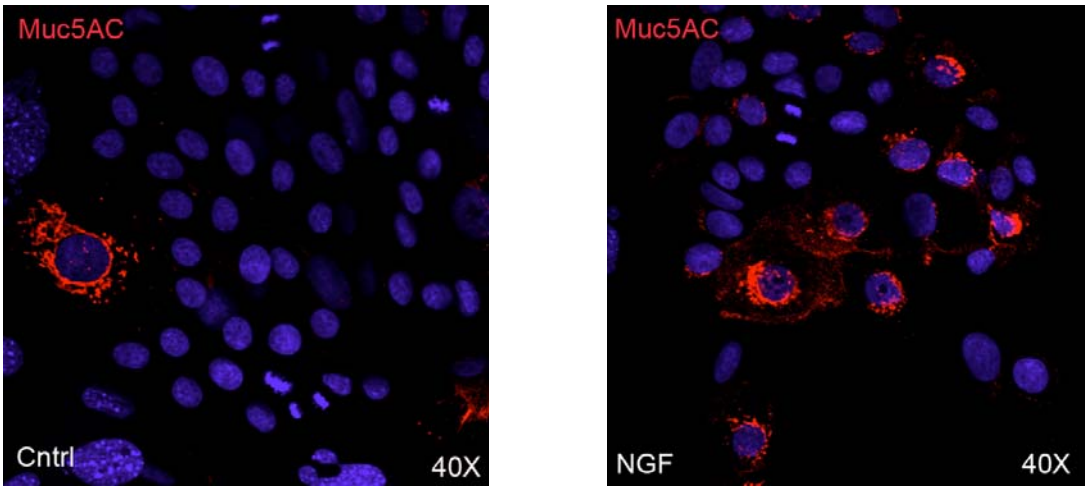


Fig 50. Immunostaining and quantification of Goblet cells **A.** Goblet cells identified by PAS and **B.** Goblet cells quantification under light microscopy **C.** Mucin 5AC (red) positive staining were increased by NGF exposure.

Furthermore we demonstrate that NGF not only induce an increase of goblet cells but also stimulates the synthesis of the "gel-forming" mucin 5AC which is essential for the stability of the tear film.

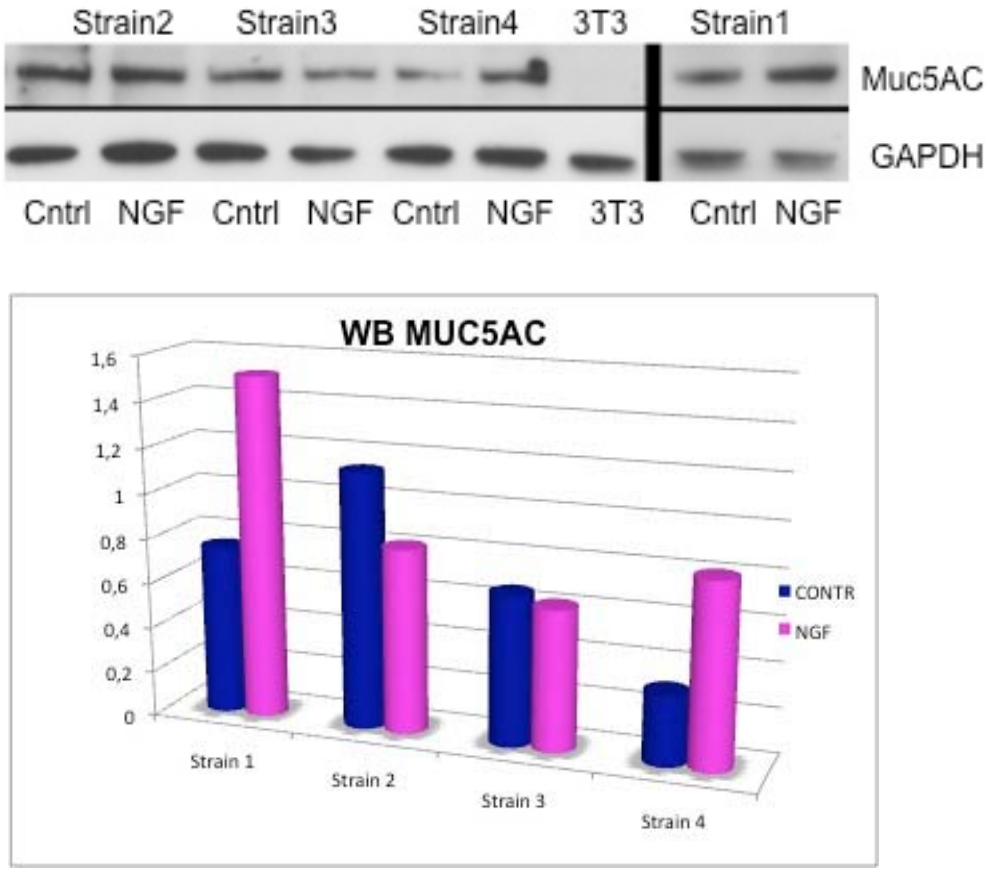


Fig 51. Western Blot shows an increase amount of cellular MUC5AC after treatment with NGF in two strains. Protein samples were normalized on the levels of GAPDH.

Microarray analysis

To further investigate the effects of NGF in conjunctival cultures, we effectuated microarray analysis at high quality RNA samples treated and untreated with NGF. The results of principal components analysis (PCA)

confirmed more variation on the genes expression profiles between the strains than between untreated and treated samples with NGF.

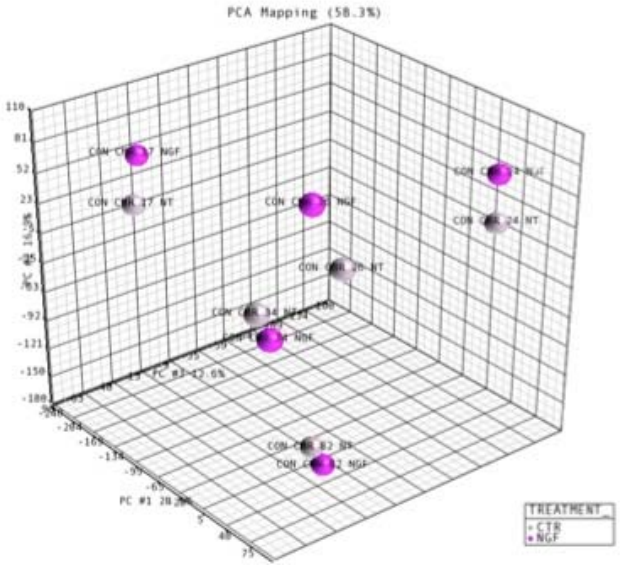


Fig 52. Principal Components Analysis (PCA) indicates variation between the strains analyzed but the absence of cultures untreated/treated

After this observation from the arrays analysis we hypothesize that could be a technical bias since goblet cells population is poorly represented in a conjunctival culture. Therefore we try to enrich our population with goblet cells. At this propose we used the elutriation method, which allow to separate the different cell fraction based on cell dimension and density. We decided to use this technique since goblet cells are different from keratinocytes for dimension, cytoplasmic/nuclear ration and morphology. We showed (Fig 53) that small undifferentiated cells can be separated from large and terminal differentiated cells. Moreover alcian-P.A.S. staining indicates that a specific cells fraction is highly enriched in mature goblet cells.

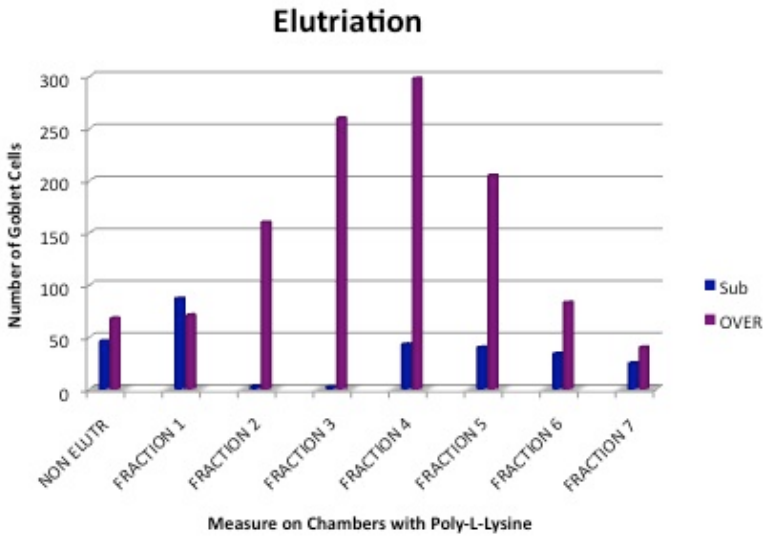


Fig 53. Alcian.PAS staining on elutriated cells, seeded on chambers slides with Poly-l-lysine. The forth fraction is the most enriched in goblet cells.

Since a variable trend between different cultures strains was observed, we decided to evaluate the modulation of goblet cells formation by alcian-PAS at three different stage of growth (sub-confluent, confluent and over-confluent) after treatment with NGF. The highest goblet cell production was observed after NGF treatment at over-confluent stage. This suggest that NGF can accelerate the "cell doubling clock" which leads a cojunctival epithelial cell to activate the differentiation pathway for goblet cells formation.

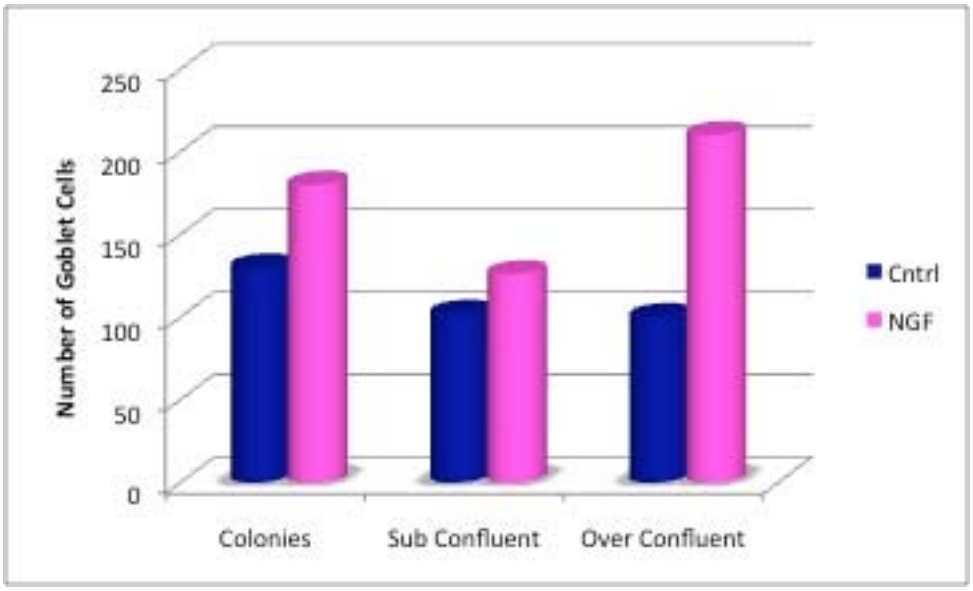
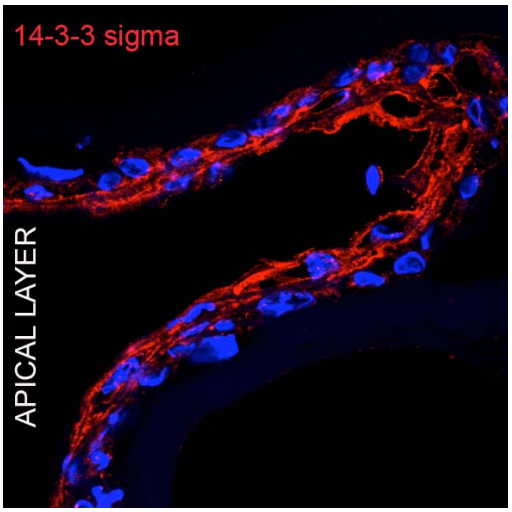


Fig 54. Goblet cell quantification after immunohistochemical staining with Alcian/PAS at three stage of confluence.

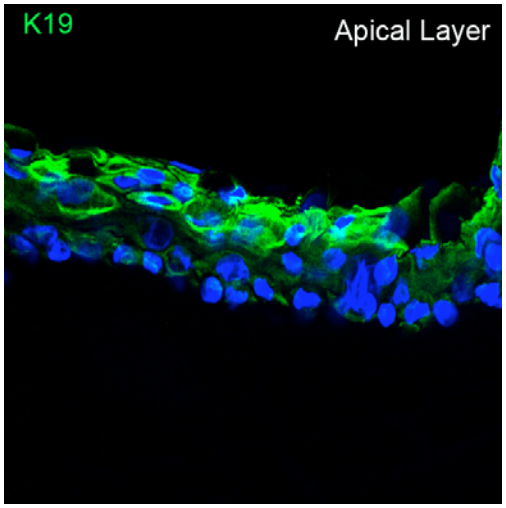
In addition we assessed if 1-2 mm of bulbar conjunctival biopsy is be able to generate this epithelium on fibrin support seeded with feeder-layer (fibroblast). Moreover we analyzed if 100ng/ml of NGF in organotypic culture is sufficient to induce a conjunctival cells to differentiate into goblet cells and to stimulate the production and release of mucin5AC.

Through immunofluorescence analysis we evaluated the epithelial layer organization and the expression of specific differentiation markers of the epithelium. Organotipic culture was included in freezing mounting medium and was lysated for further analysis after complete stratification. The analysis revealed (Fig 55) a comparable cell stratification in the presence or absence of NGF and a correct localization of differentiation markers and laminin 5.

A.



B.



C.

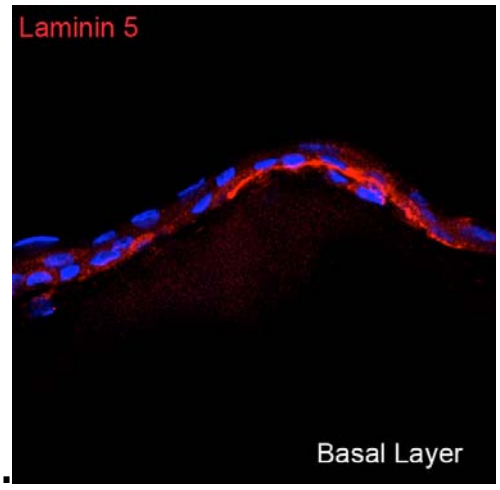
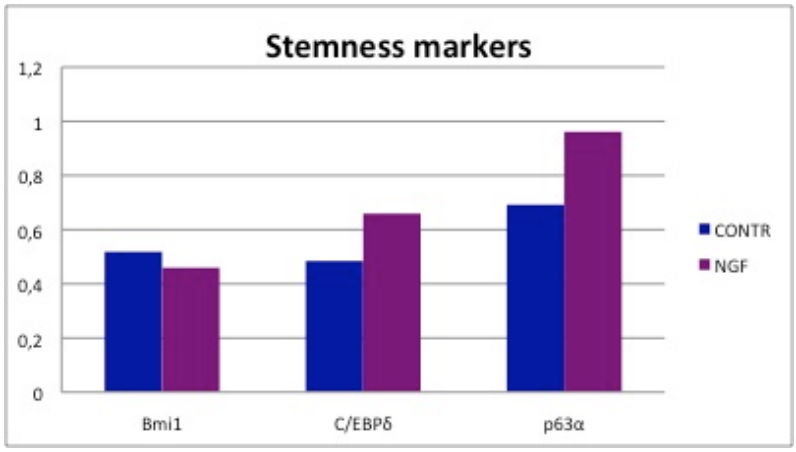
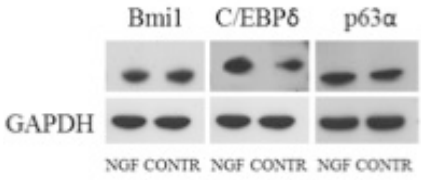


Fig 55. Immunofluorescence staining. In vitro reconstruction of conjunctival epithelium on fibrin support and evaluation of epithelia organization by specific epithelial markers. **A.** Immunofluorescence staining 14-3-3σ (red) **B.** Keratin 19 (green) **C.** Laminin5 (red) and Dapi (blu).

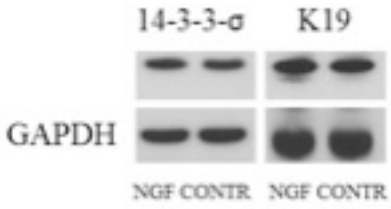
Through western blot analysis we observed a similar tendency between untreated and NGF treated samples in the expression of proliferative/stemness and differentiation markers. Both receptors are expressed suggesting their implication for the maintenance of epithelium homeostasis.

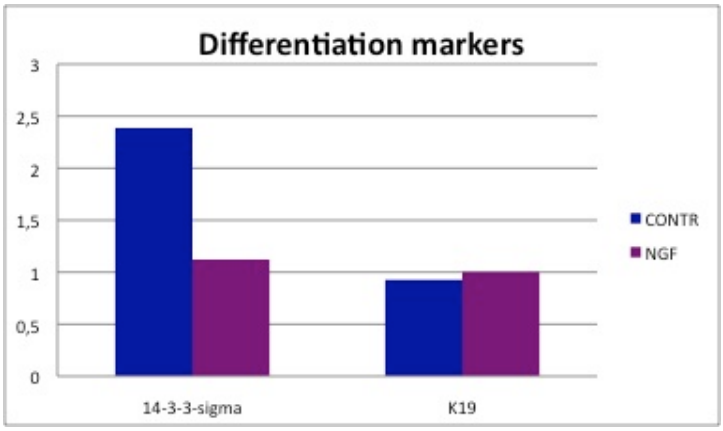
A.





B.





C.

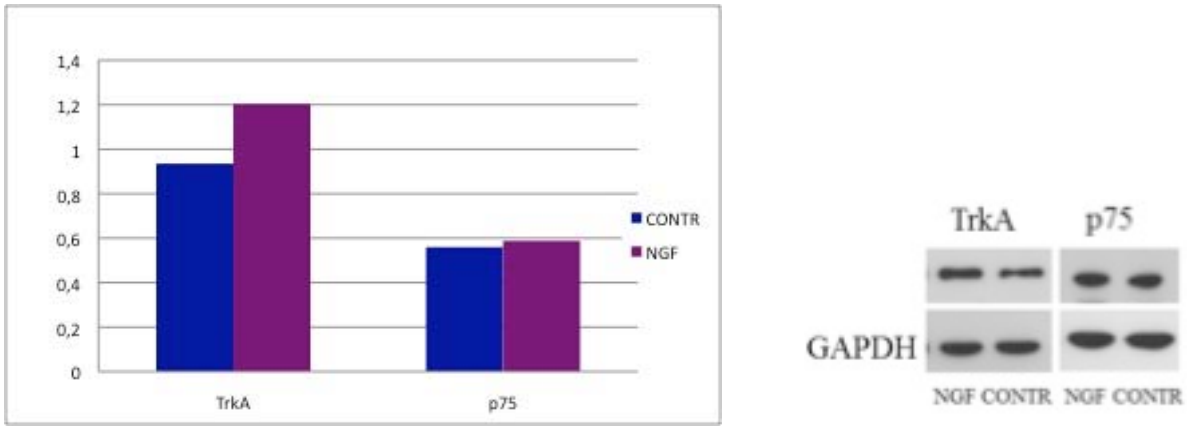


Fig 56. Western blot analysis on in vitro reconstructed conjunctival epithelial cells and treatment with NGF on biocompatible support. Quantification of **A.** stemness markers **B.** Differentiation martkers **C.** TrkA and p75 receptors. Samples were normalized on the expression level of GAPDH.

in addition immunohistochemical staining revealed goblet cells formation in the suprabasal layer of the organotypic epithelium (Fig 57).

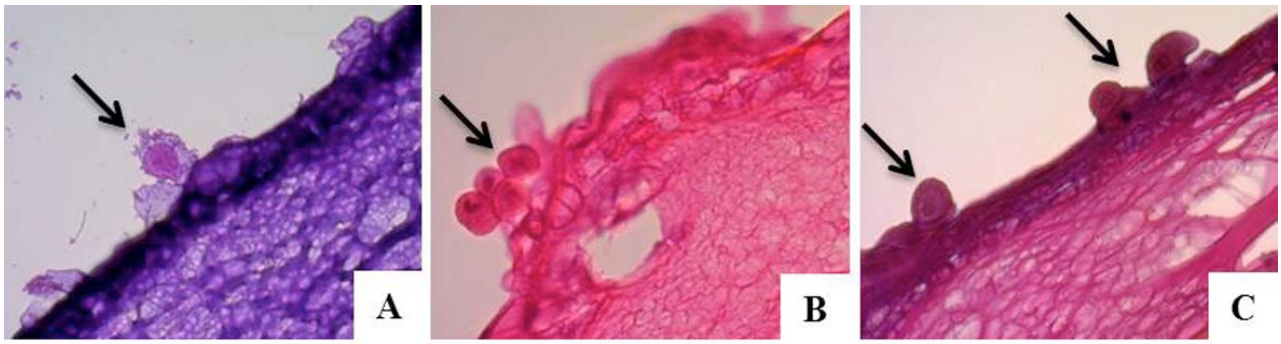


Fig 57. Histochemical staining with **A.** May Grunwald Giemsa **B.** Hematoxylin Eosin **C.** Alcian P.A.S. for Goblet cells detection which are indicated with the arrows.

Supernatant analysis by ELISA of the conjunctival organotypic culture confirmed the positive effect of NGF on the synthesis / release of MUC5AC. Moreover we observed that this activity depends on the concentration of exogenous NGF supplied to the conjunctival cells. It is interesting to note that

the doubling dose (from 100ng/ml to 200ng/ml) cancels the effect of release of MUC5AC by conjunctival cells, suggesting a negative "feedback" effect.

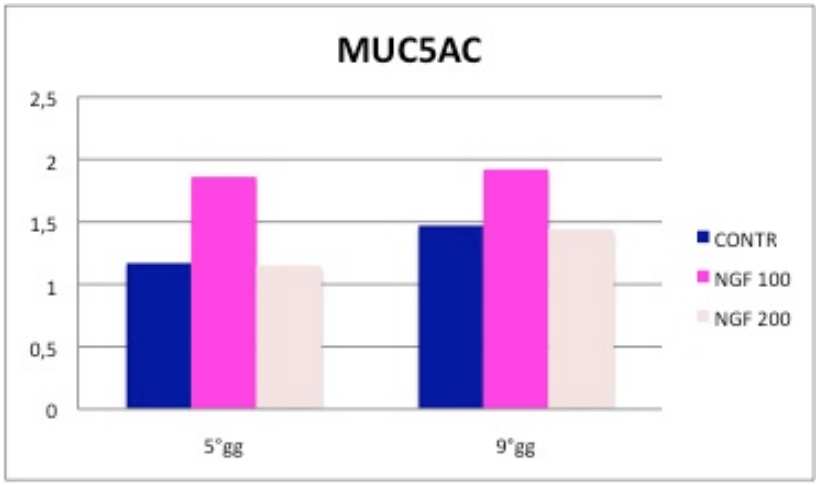


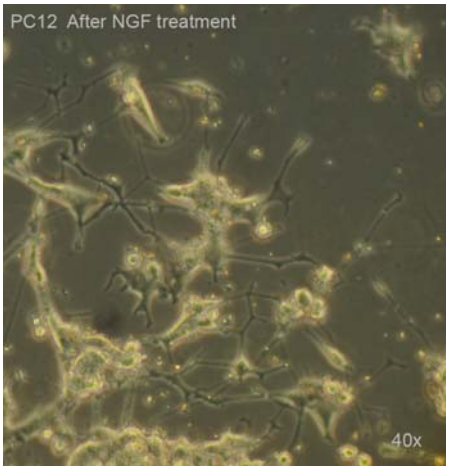
Fig 58. Sandwich ELISA analysis. Muc5AC protein secretion in the culture media after somministration of two different concentrations of NGF as detected by sandwich ELISA.

Therefore these data demonstrated the potential effect of NGF in the treatment of eye diseases characterized by a reduced number of goblet cells or by an instability of the tear film dye to a decreased production and release of MUC5AC.

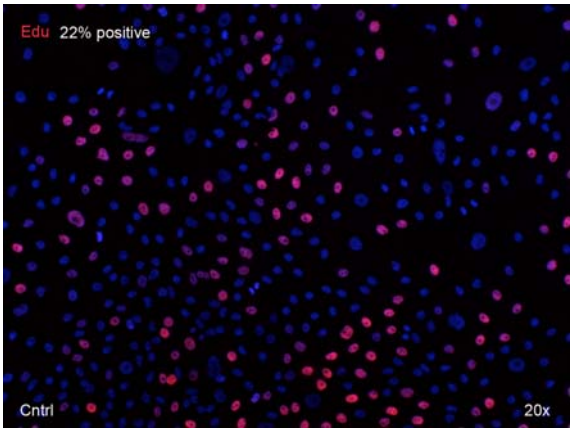
Limbal-Corneal Culture Cells and treatment with NGF

As a positive control (for all experiments) for NGF function we used PC12 cell line (rat pheochromocytoma). In parallel, we also treated with 100ng/ml NGF the corneal human cell line (HCLE, gift from Gipson, expressing specific receptors TrkA and p75). Figure 59A, indicates that NGF can stimulate PC12 differentiation and HCLE proliferation (Fig 59B).

A.



B.



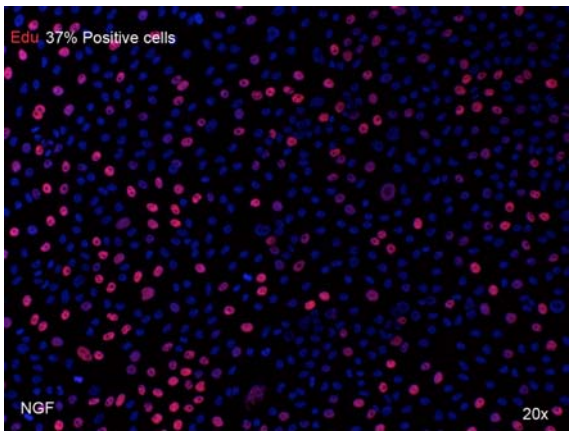


Fig 59. **A.** PC12 cell line differentiation after treatment and **B.** Human limbal Cell Line (HCLE) treated with NGF (right) increasing its proliferative potential 15% more than the cultures untreated (left) as shown after quantification of Edu (proliferative marker) with imageJ.

Human Limbal Epithelial cells behavior following NGF treatment.

The involvement of the NGF in inflammation and corneal healing demonstrated by in vivo studies (Lambiase et al; 2007) as well as the strong expression of TrkA receptor in limbal and corneal region and the expression of p75 in patches in the basal layer of limbus suggest that this receptor is expressed by the stem cells of the limbus and that NGF may play a role in the homeostasis of the resident cells of this epithelium.

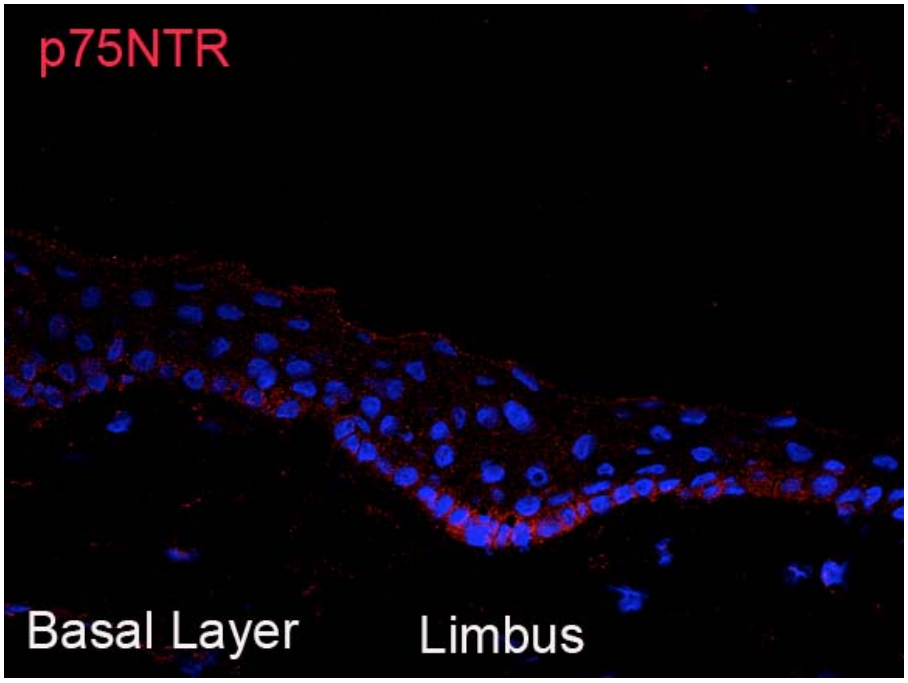
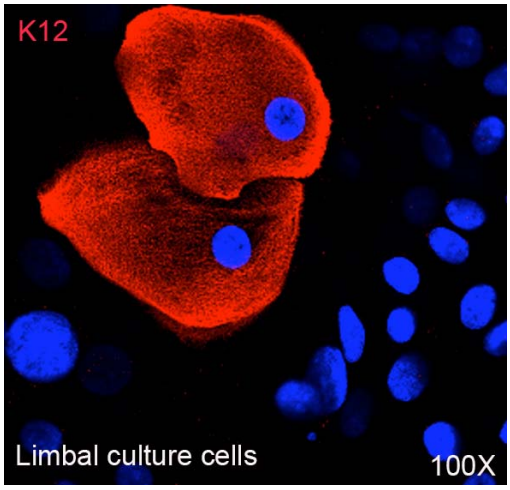


Fig 60. Immunofluorescence staining for p75 in Limbus. Expression of this receptor in patches of the basal limbal epithelium.

For this reason, we assessed the effects of the NGF on the limbal corneal epithelium**.**

The distinction between conjunctival and limbal cells in cultures is effectuated through the specific markers like k3/k12 that expressed only in stratified limbal-corneal cells.



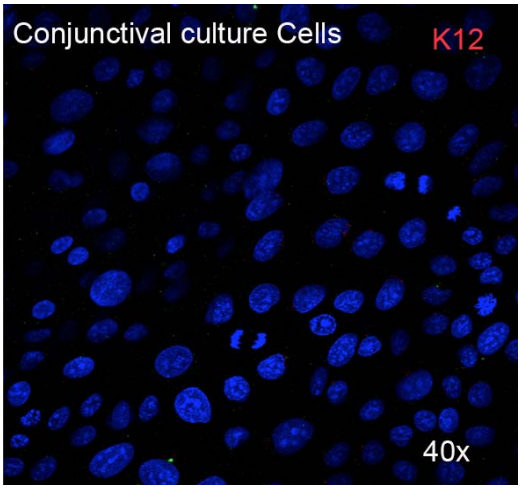


Fig 61.Immunofluorescence staininig in different epithelial cell cultures and their distinction by specific markers like keratin 12 specific of Limbal-corneal culture cells (right). Conjunctival culture cells are negative for this keratin in vitro (left).

In order to examine whether NGF modulates the limbal cells proliferation and when this effect is maximal we treated with 100ng/ml the limbal cells and collected them at different time point (6h, 15h and 24h). We evaluated the proliferation of cells at each time point by Edu (5-ethynyl-2-deoxyuridine) marker. We measured (by image j) Edu-positive cells and the highest peak of proliferation was after 15h.

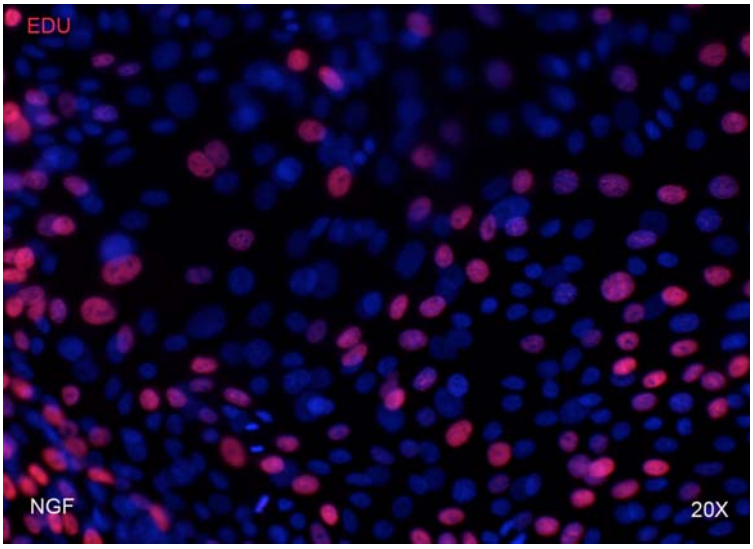


Fig 62. Immunofluorescence for Edu incorporation by Limbal cultures cells after treatment with NGF and immunostaining after 15h of their fixation with PFA 3%.

NGF TIME POINT	%EdU positive cells	proliferation increase
CTL 6h	31%	
NGF 6h	41%	10.00%
CTL 15h	30%	
NGF 15h	43%	13.00%
CTL 24h	35%	
NGF 24h	41%	6.00%

Table 2. Edu quantification with image J of limbal cultures cells selected after different time point of treatment with NGF. The table indicates an increase of cell proliferation after 15h of treatment.

Moreover Cytofluorimetric analysis also demonstrated that high affinity receptor TrkA became more expressed after 15 hours.

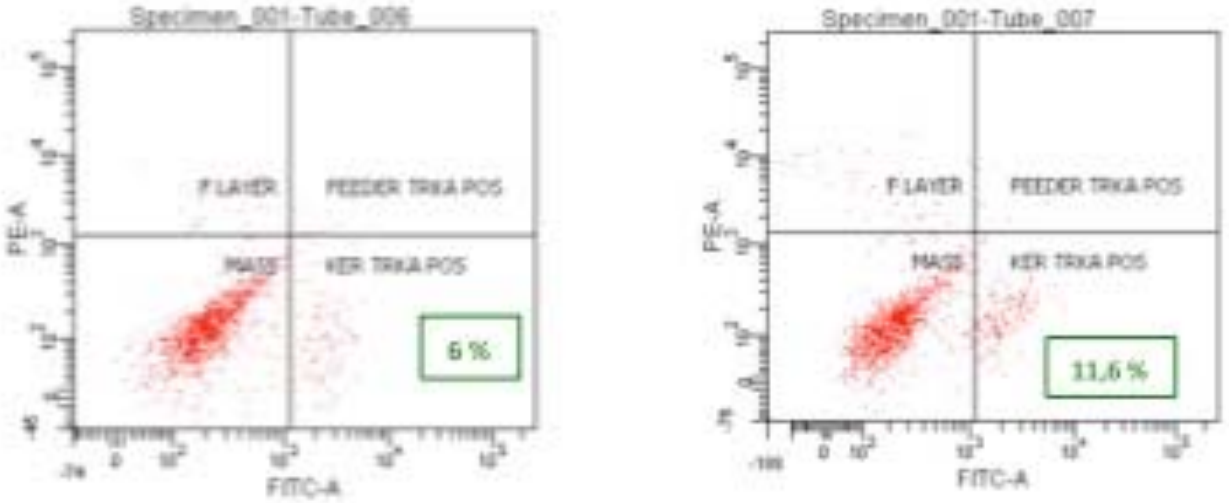


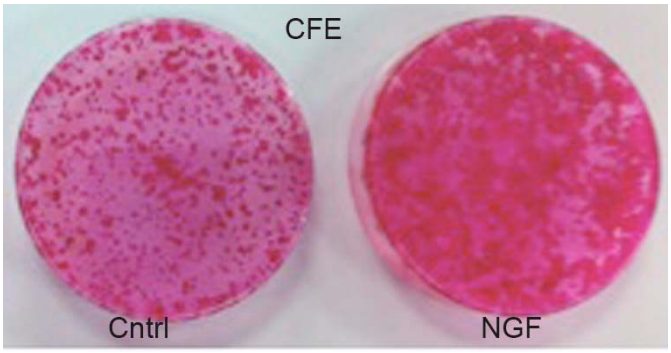
Fig 63. Cytofluorimetric analysis of TrkA receptor expression 15h of treatment with NGF.

In addition we quantified the percentage of p63-bright cells at each time point comparing treated and untreated samples. The data indicated that the percentage of serum (from 10% to 1%) doesn't influence the value of p63 bright. p63-bright cells increased after 15 hours with moderate decrease at 24 hours (Table 3).

NGF TIME POINT	DIAMETER	DENS MEAN	DENS MAX	p63 bright (%)	p63 bright increase fold
CTR fbs10%	17,28	962,67	2373,00	3,65	
CTR fbs1% 3 h	19,46	739,38	2230,00	3,50	
NGF fbs1% 3 h	19,12	886,19	2870,00	4,10	**1,17**
CTR fbs1% 6 h	18,02	541,15	925,00	3,65	
NGF fbs1% 6 h	18,66	803,85	1885,00	3,40	**0,93**
CTR fbs1% 15 h	18,19	1004,14	1990,00	4,15	
NGF fbs1% 15 h	16,89	895,58	2280,00	9,35	**2,25**
CTR fbs1% 24 h	18,85	763,80	1821,55	3,45	
NGF fbs1% 24h	17,75	1096,20	3021,00	9,20	**2,67**

Table 3. Quantitative immunodetection of p63 bright cells was performed on different time point after NGF treatment.

Three strains of limbal-corneal cultures were treated with NGF and the analysis of the colony forming efficiency showed an increase of stem cell clonogenicity.



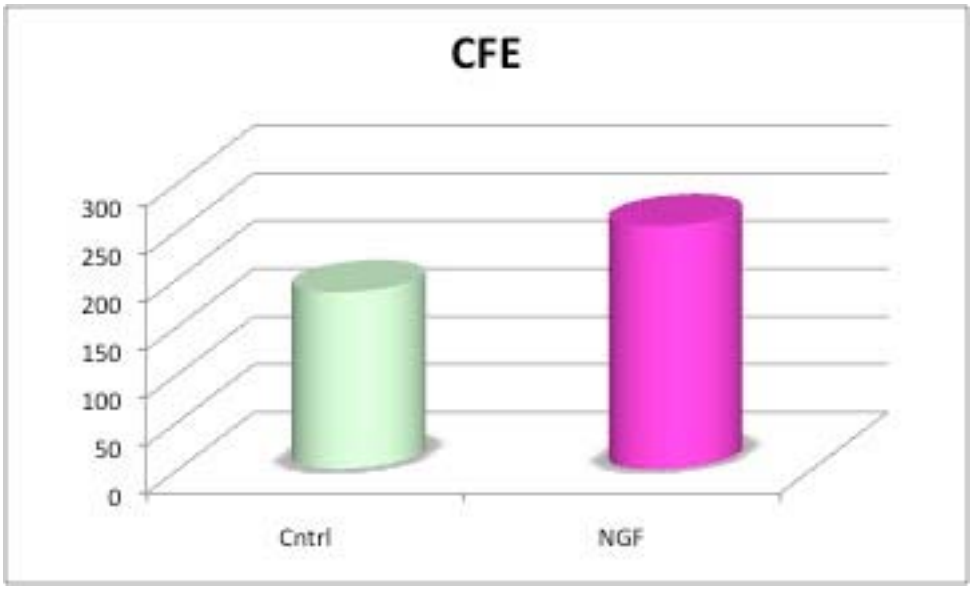


Fig 64. Colony Forming Efficiency (CFE) assay was performed on Limbal cultures comparing treated and untreated samples. The indicator dish and the graphic are representative of all three strains.

In limbal cultures NGF increases protein and mRNA level of Bmi1 (Fig 65A and B) while the levels of C/EBPδ (Fig 65C) remain constant. This suggests

the maintenance of the self renewal and the preservation of the proliferative potential.

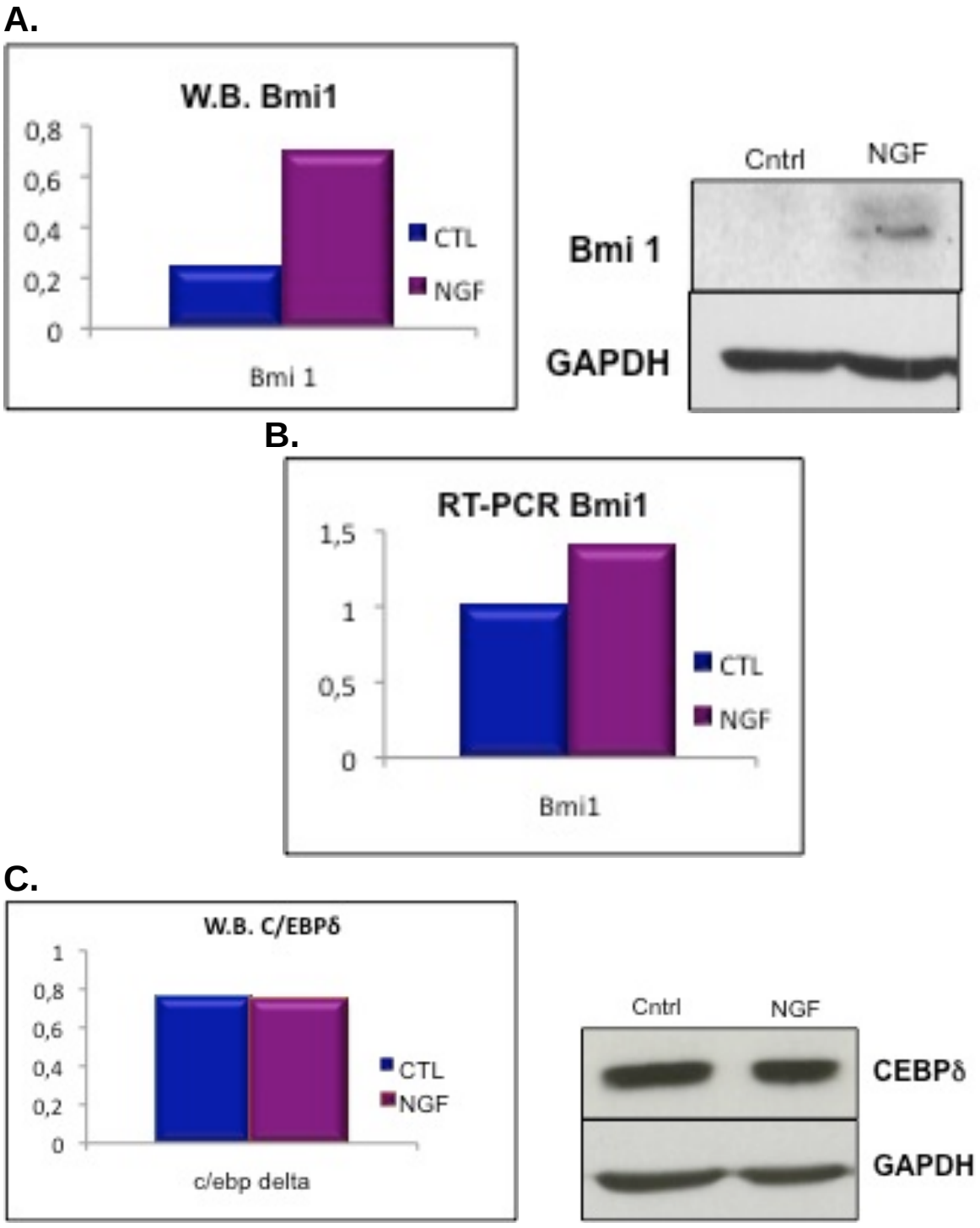
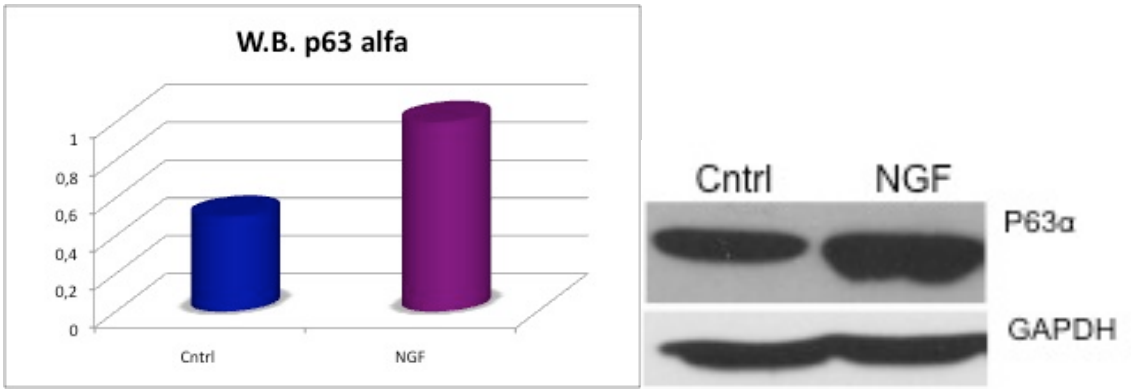


Fig 65. Western blotting analysis and RT-PCR of Bmi1 and C/EBPδ after NGF treatment. Protein samples were normalized on the expression level of GAPDH. mRNa samples were normalized on the levels of GAPDH

14-3-3σ and p75 protein levels are unchanged after NGF treatment. Conversely protein level of p63α increases and this data correlates with the increase number of Edu and TrkA positive cells.

A.



B.

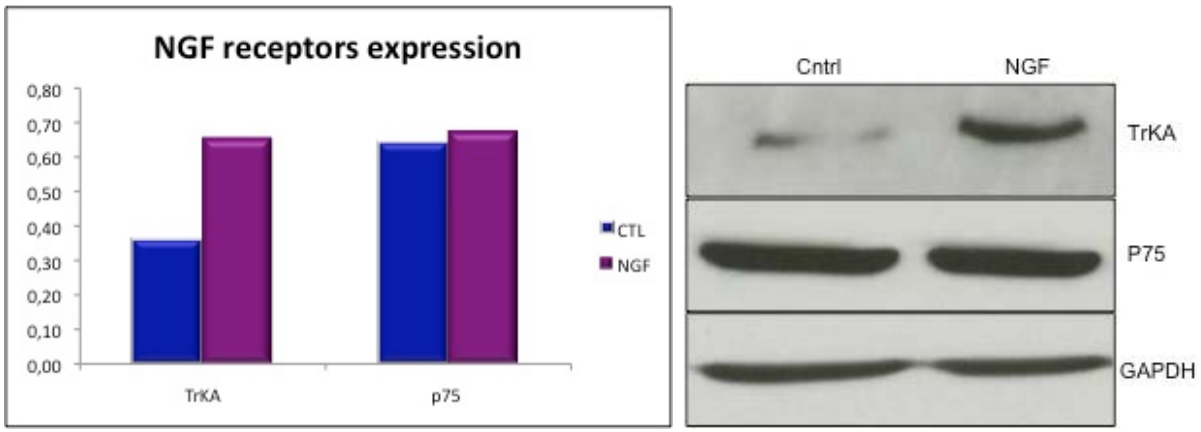


Fig 66. Western Blot analysis of p63α and TrkA proteins after somministration of NGF. Protein samples were normalized on the protein levels of GAPDH.

NGF treatment in three clonal types

In order to investigate whether NGF preserves limbal epithelial cell stemness, we performed clonal analysis from primary human limbal culture and we isolated 7 holoclones, 16 meroclones (early commitment progenitor), 3 paraclones (transient amplifying cells)

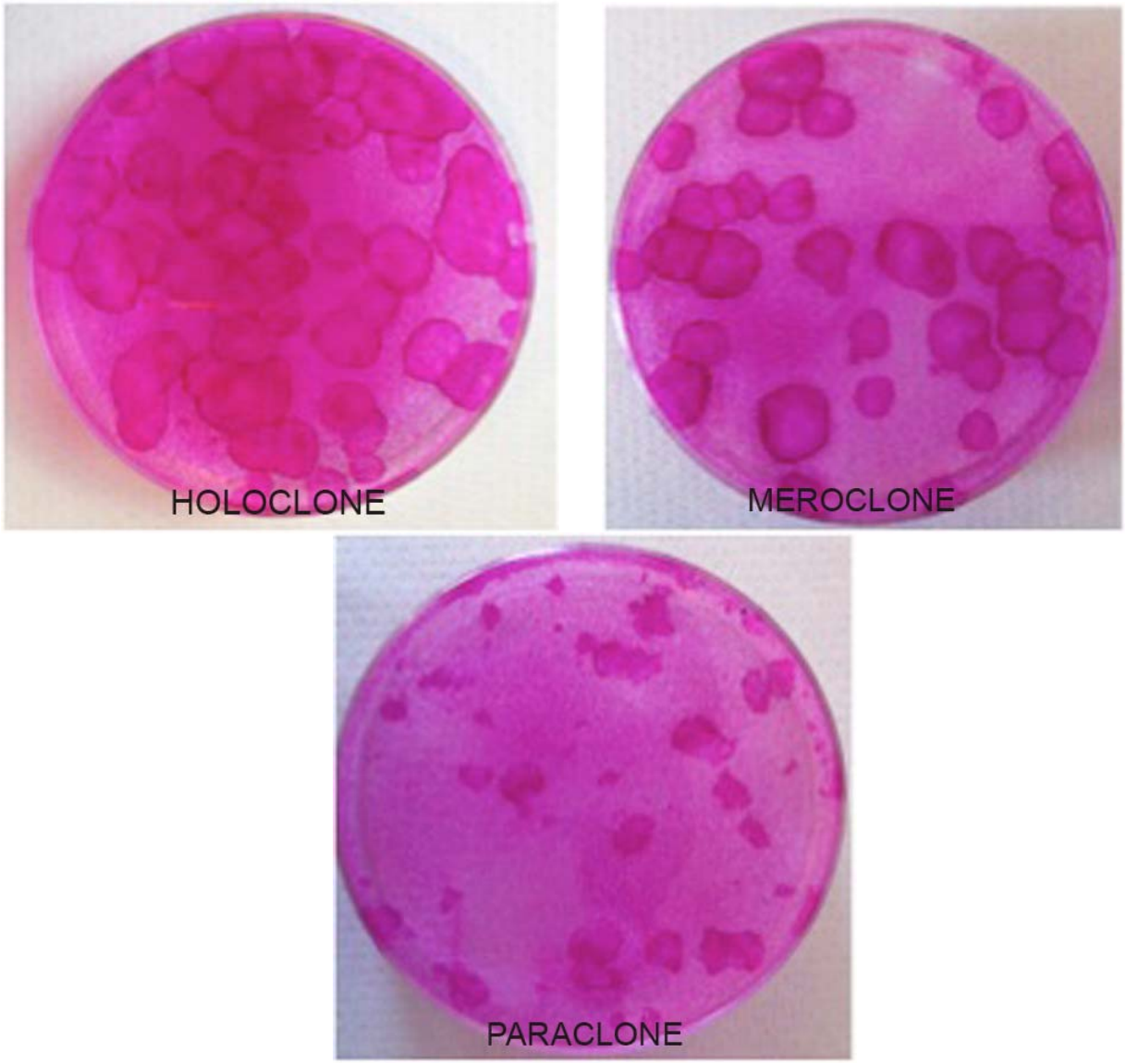
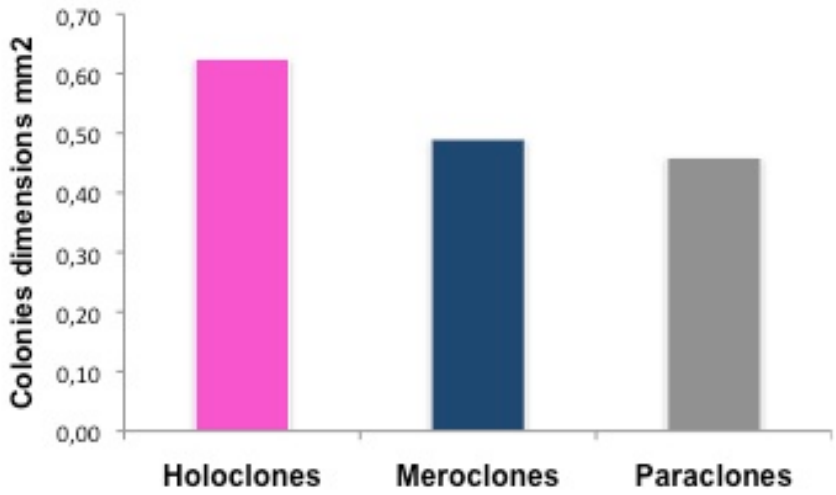


Fig 67. Indicator dishes for CFE analysis. Colonies scored at 12 days produced in indicator dishes by different clonal types of Human Limbal strain.

A.



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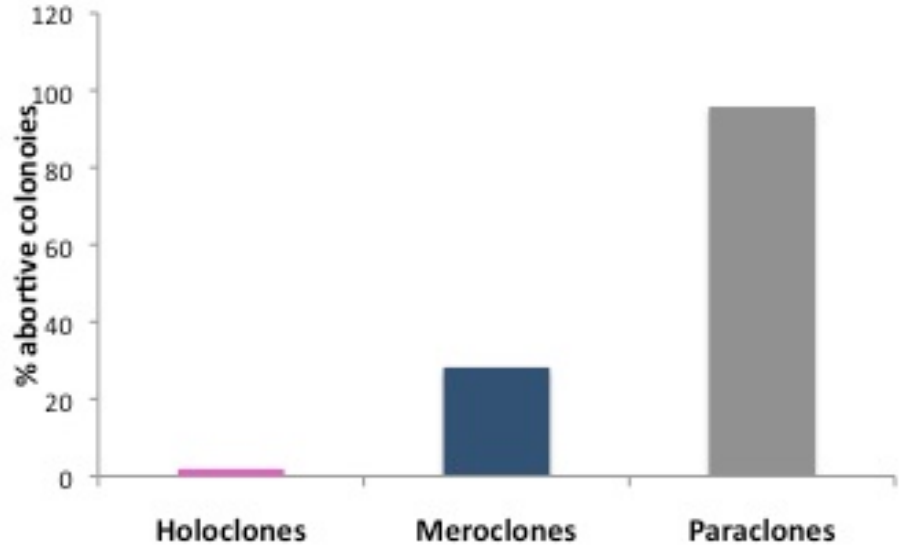


C.

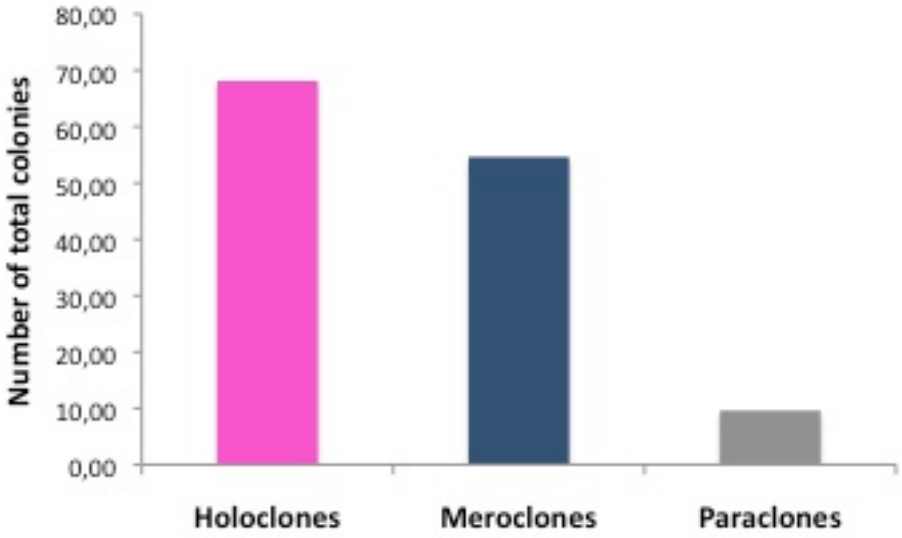
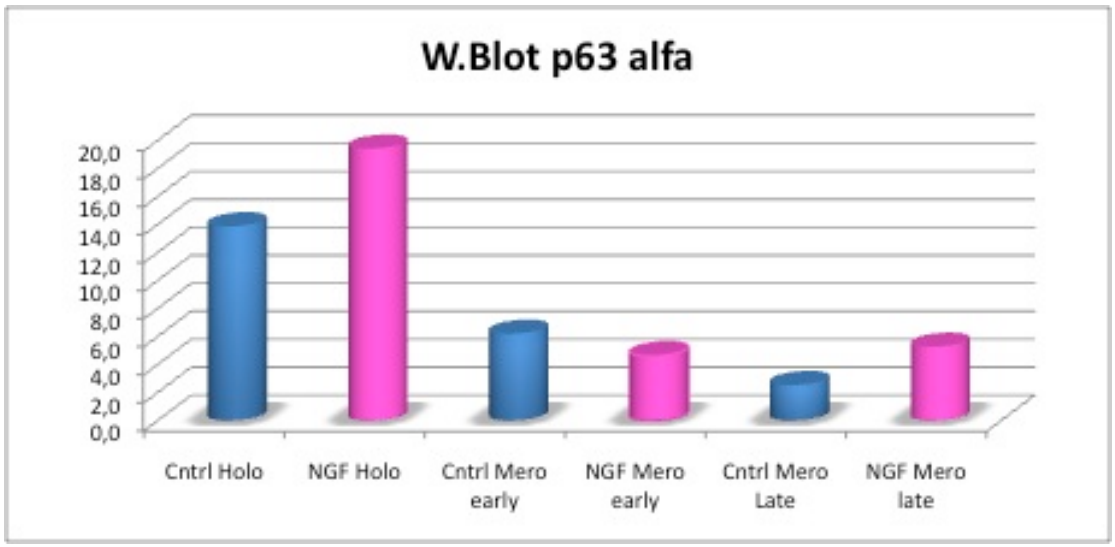


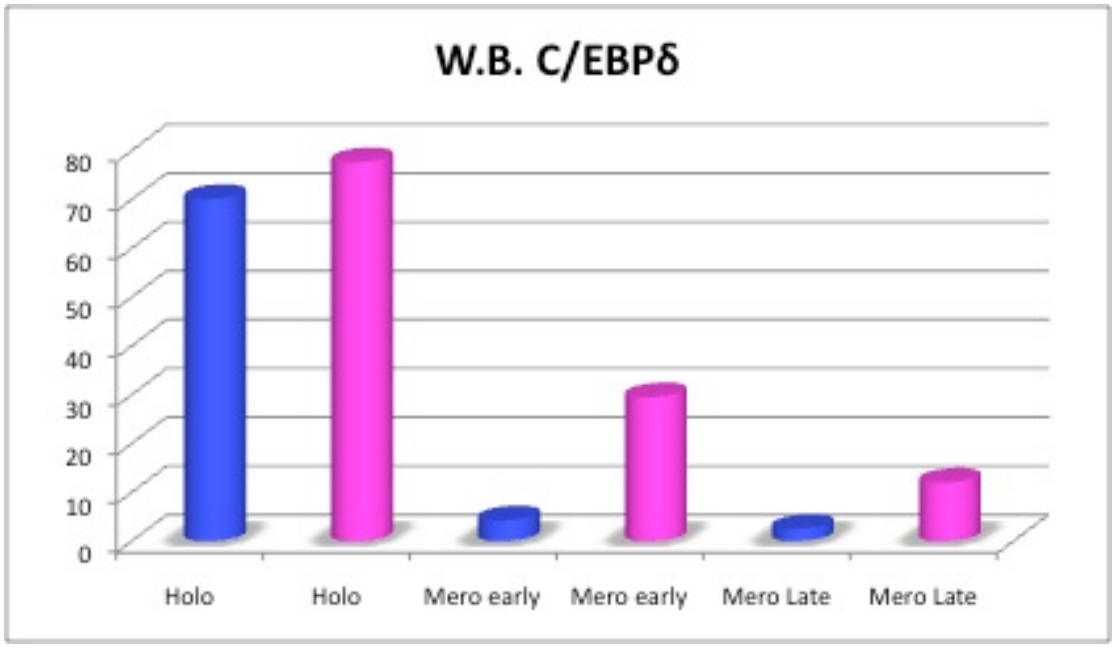
Fig 68. Graphics of the general characteristics of the three types of clones **A.** Mean of the colonies dimension in mm^2 **B.** Percentage of abortive colonies in indicator dish after 12days **C.** Mean of number of total colonies find for each clonal type.

NGF addition was associated with an increase of ΔNp63α and C/EBPδ protein level in Holoclones. Moreover we observed an increase of ΔNp63α, Bmi1 and C/EBPδ in NGF treated late Meroclones.

A.


W.Blot p63 alfa
20,0
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
Cntrl Holo
NGF Holo
Cntrl Mero early
NGF Mero early
Cntrl Mero Late
NGF Mero late

B.


W.B. C/EBPδ
80
70
60
50
40
30
20
10
0
Holo
Holo
Mero early
Mero early
Mero Late
Mero Late

C.

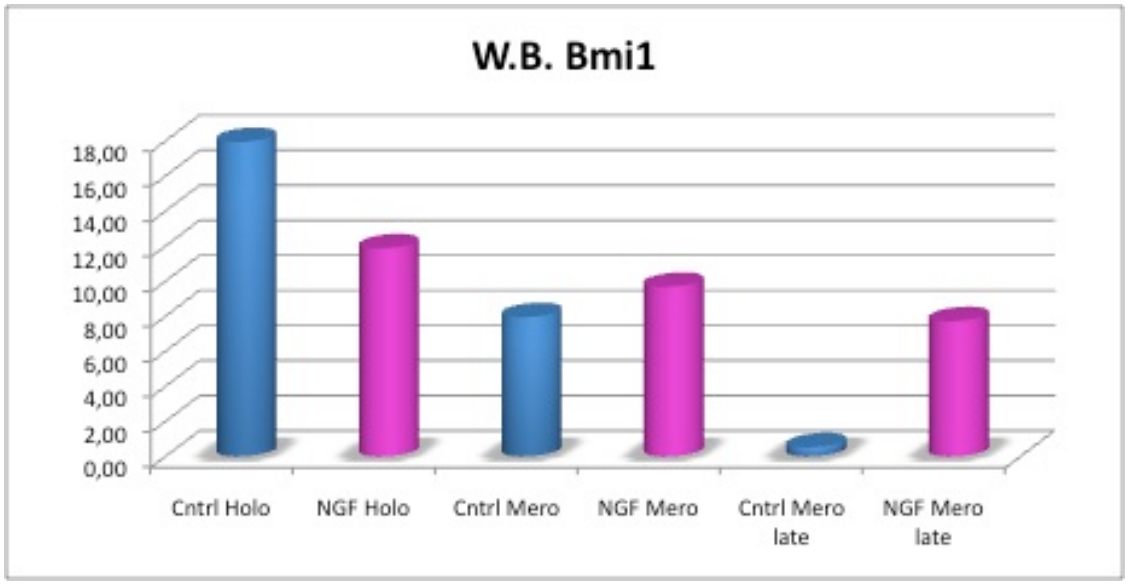

W.B. Bmi1
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
Cntrl Holo
NGF Holo
Cntrl Mero
NGF Mero
Cntrl Mero late
NGF Mero late

Fig 69. Western Blot analysis of three types of clones. The blue column represents the untreated cells and the pink column represent the treated cells with NGF. **A.** Level of p63α protein after treatment with NGF **B.** and **C**. Protein levels of C/EBPδ and Bmi1. Protein samples were normalized on the protein levels of GAPDH.

In addition we assessed the expression of p75, TrkA and endogenous NGFβ in Holoclone, after NGF treatment as showed in Figure 70, we demonstrated that those proteins are highly expressed in Holoclones confirming that these are stemness marker.

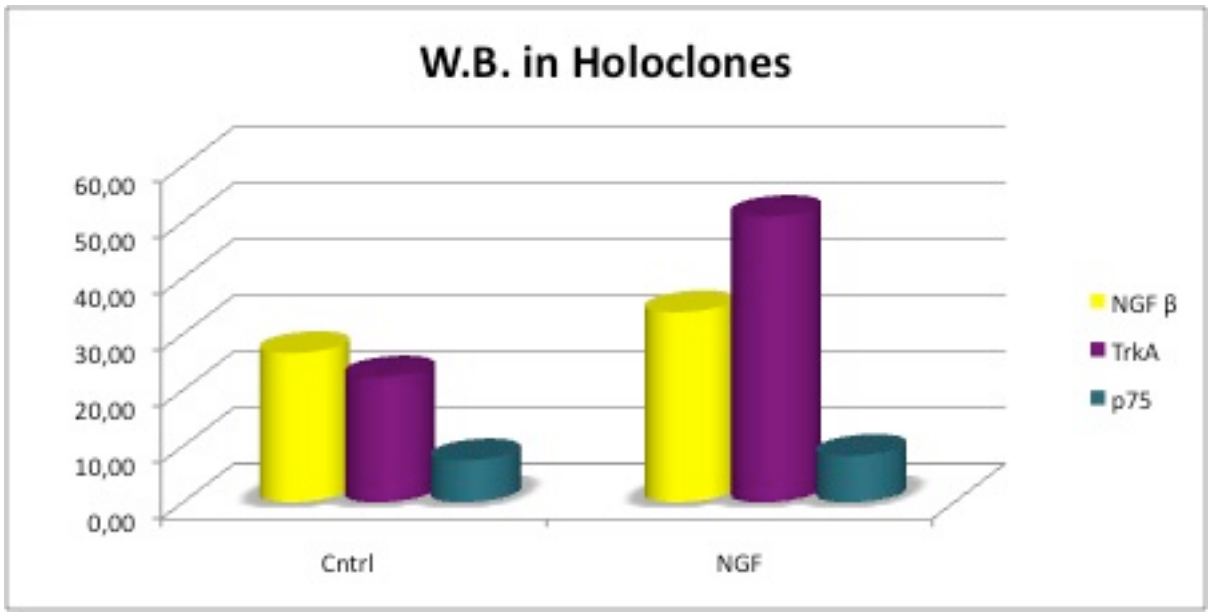


Fig 70. Western Blot analysis in Holoclones. Protein level expression NGFβ, TrkA and p75 in treated and untreated limbal cultures stem cells. Protein samples were normalized on the protein levels of GAPDH.

Since endogenous NGF is produced by the studied culture system, in order to evaluate the effect of NGF, we select an alternative approach. We inhibited TrkA and p75 receptors with specific small molecule inhibitor and specific antibody respectively. Unfortunately, we didn't observed any significant changes in the clonogenic ability of the limbal cells neither in the protein expression of the proliferation and differentiation markers (data not show). For this reason we used specific interference (siRNAs) for TrkA and p75. We used the cell lines HCLE for the validation of the functionality of these

siRNAs. This approach was repeated in human normal primary cultures but so far we haven’t reproduced the same efficiency yet. This discrepancy may be related to different characteristic of the cells or by different cell culture conditions. This approach is still in progress for the optimization of the system.

DISCUSSION

The human ocular surface is covered with the highly specialized conjunctival and limbal-corneal epithelia, These epithelia are continuous to the eyelid, lachrymal glands, meibomian glands and nasal lacrimal dotum. The cornea is a transparent avascular connective tissue which is formed by three different layers of cells (epithelium, stroma, endothelium) and two interfaces (bowman's layer, descement's membrane). Corneal epithelium is formed by superficial cells, wing cells and basal cells. This epithelium is the most richly innervated site of the body.

Conjunctival epithelium is highly vascularized and populated by goblet cells. These cells are monocellular glands generated from conjunctival stem cells. Conjunctival goblet cells synthesize and secrete mucin-5AC essential for ocular surface integrity maintenance and for the eye protection from the external environment.

Normally, squamous epithelia are constantly renewed during an organism life. The frequency of this regeneration changes from tissue to tissue. For example human skin is totally renewed in about a month while corneal epithelia takes from 9 to 12 months to undergo the whole process. This regeneration is possible thanks to the stem cells, the only one, which proliferate in adult tissue. Stem cells have the ability to self-renew and to generate progeny and transient amplifying cells. The corneal stem cells reside in the Limbus, in structures known as palisades of Vogt. Corneal stem cells are able to form holoclones in vitro (Pellegrini et al; 1999). Conjunctival stem cells are distributed in the basal conjunctival epithelium (Fornix, bulbar conjunctiva,). They have the capacity to generate conjunctival keratinocytes and goblet cells, so basically we're talking of bipotent stem cells (wei et al., 1997). Goblet cells generates from a stem cell-derived transient amplifying cells at a particular time points of their life in vitro. The first generation peak is originated by young progenitors after 45-50 cells doublings. More relevant is the second peak originated by an old transient amplifying cell just ten or

twenty cell doublings before senescence. This explains the suprabasal localization of goblet cells in vivo, the low number of goblets in growth colonies of conjunctival cells (200-500 goblets/cm^2) and the relevant increase of their number when the colonies create a stratified epithelium (less then 5.000 goblet cells/cm^2) (Pellegrini et al; 1999).

The identification of the ocular surface epithelial stem cells is based on the evolution of the expression of ΔNp63α. ΔNp63α supports the proliferative potential of stem cells of many stratified epithelia including those of the ocular surface. An other stemness marker is c/EBPδ which maintains mitotic quiescence of limbal stem cells, promotes their self-renewal and preserves their proliferative potential inducing the expression of ΔNp63α. c/EBPδ is co-expressed in limbal cells with an other stemness marker, Bmi1, which maintains the self-renewal of stem cells and regulates the cell cycle by repressing the expression of differentiation and cell death genes. (Di Iorio et al 2007).

Other proteins have been proposed as stemness markers, such as TGFB-RII, NOTCH1, ABCG2 (ATP binding cassette transporter protein), TrkA and p75. All these markers in parallel with the absence of differentiation markers (K3/K12, K7, involucrin, K19, connexin 43) can be used for the identification of limbal stem cells. We demonstrated that ΔNp63α and p75 are also expressed by basal cells of the conjunctiva. In addition we showed that TrkA has a diffuse expression pattern and keratin 7 is expressed only in apical layer of bulbar conjunctival epithelium section.

The integrity and the functionality of corneal epithelial, is essential for the view. The homeostasis of the ocular surface is dependent on a precise balance between cell proliferation, differentiation, activation and survival of epithelial stromal cells and these process are directed by a complex network of growth factors, cytokines, mucins and tears.

There are many diseases (genetic, autoimmune, traumatic...), which can

damage the eye surface with the consequent limbal and conjunctival stem cells deficiency. This leads to cornea opacification, visual loss and the decrease of the epithelial and goblet cells of the conjunctiva. Corneal innervation appears to be NGF dependent. Experimental studies in vivo showed that NGF topical administration in patients affected by neurotrophic corneal ulcers induces persistent corneal recovery and improvement of corneal sensitivity. Sjogren's syndrome which is an a severe form of dry eye syndrome and atopic keratoconjunctivitis are other ocular surface diseases which can be treated with NGF. These diseases are characterized by goblet cells reduction and by an alteration and/or disappearance of mucins with consequent instability of the tear film and ocular surface damage.

In healthy subjects the tear film consists also by the secreted and membrane mucins produced by conjunctival and corneal epithelial cells.

Currently there are no specific therapies for dry eye syndromes. The only treatments include artificial tear replaced or lubricants, corticosteroids, cyclosporines and tetracyclines. These therapies, however, are inconclusive. Studies on animal models suffering from dry eye syndrome and treated with NGF showed a higher production of the tear film with an increase of goblet cells suggesting a direct involvement of this neurotrophin in homeostasis of the tear film, goblet cell formation and reparative processes.

The NGF is a soluble protein discovered in 1950 by Rita Levi Montalcini. Its clinical effect is though to be mediated by a direct activity on the ocular epithelia since TrkA and p75 receptors are expressed on the both ocular epithelia. TrkA is the high affinity receptor and p75 the low affinity receptor. Trk receptors are responsible for the transduction of signals for cell growth and survival mediated by neurotrophins. The receptor p75 can act as a Trk co-receptor, enhancing the activity of the Trk, or can independently activate the signal transduction cascades that culminate the induction of apoptosis or survival.

In order to investigate the potential role of NGF on proliferation, clonogenicity, and/or differentiation in normal conjunctival epithelium we focused our studies on cultivated primary proliferating conjunctival cells derived from human bulbar region.

Untreated and treated conjunctival strains (four strains) with 100 ng/ml NGF expresses the two receptors, TrkA and p75. Cytofluorimetric and western blot analysis showed that the expression of TrkA is little modified after exogenous stimulation with NGF. mRNA and protein levels of p75 are increased after treatment; this suggests that the concentration of NGF does not saturates the receptors but it can be optimal for observe modulations both positive and negatives.

To evaluated the clonogenic ability of NGF treatment we performed Colony-Forming Efficiency (CFE) assays; we showed an increase number of the clonogenic cells in samples treated with NGF comparing to the untreated control. This suggests that this growth factor stimulates the clonogenicity of these epithelial cells.

The proliferative effect of NGF was evaluated by the investigation of Ki67 expression levels. We found a moderate increase after treatment of cells expressing this nuclear antigen. In addition was observed a variable quantitative increase in the expression levels of other proliferative markers such as p63tot and the truncated isoform, ΔNp63α, after treatment with NGF. Bi-parametric automated immunofluorescence analysis in cytospin preparations has highlighted an increase in the percentage of stem cells in cultures, after treatment with NGF, as indicated by the intersection analysis between the intensity expression of ΔNp63α and the cells dimensions.

mRNA and protein levels of C/EBPδ and Bmi1 remain constant or increase moderately after NGF treatment. This suggests that the growth factor does not modify the number of stem cells but rather stimulates asymmetric cell divisions.

In order to evaluated the effect of NGF on differentiation of this cultured epithelial cells we investigated mRNA and protein levels of epithelial differentiation markers such as 14-3-3-σ and K19. We observed an increase of these markers only in the strain, which contained more abortive colonies assed by CFE assays.

The most intriguing data is that NGF can induce the differentiation of conjunctival epithelial cells into goblet cells as suggesting by Lambiase et al.,2009. Our results showed an increase number of goblet cells in NGF treated cultures compared to the untreated samples. We identified the presence of goblet cells and we quantified them by immunofluorescence and alcian/PAS staining respectively.

Furthermore we demonstrated that NGF not only induce an increase of goblet cells but also stimulate the synthesis of the specific "gel-forming" mucin 5AC, essential for the stability of the tear film.

To further investigate the effects of NGF on gene expression profile in conjunctival cultures we performed microarray analysis at high quality treated and untreated RNA samples. The results of principal components analysis (PCA) showed the variation between the strains and the absence of a significant variation between the untreated and NGF treated samples . After this observation from the arrays analysis we hypothesize that could be a technical bias for the poorly represented goblet cells population. Therefore we try to enrich the analyzed population with goblet cells. For this propose we used the elutriation method, which allows to separate the different cell fraction based on the dimension and cell density. We choose this technique since goblet cells are different from keratinocytes in dimension, cytoplasmic/nuclear ration and morphology. We showed that small, undifferentiated cells can be separated from large terminal differentiated cells. In addition alcian-P.A.S. staining indicates that a specific cell fraction is highly enriched in mature goblet cells.

Moreover we evaluated goblet cells generation by alcian-PAS at three different growth stage after NGF treatment: sub-confluent, confluent and over-confluent. The highest goblet cell production was observed after NGF treatment at over-confluent stage; this suggests that NGF may accelerate the "cell doubling clock" which leads to goblet cells differentiation.

We wondered if 1-2 mm of bulbar conjunctival biopsy is able to generate this epithelium on supporting fibrin scaffold containing feeder-layer (fibroblast). Moreover we assessed if 100ng/ml of NGF treatment in organotypic culture is sufficient to induce the conjunctival cells to differentiate into goblet cells and to stimulate the production of mucin5AC.

We evaluated the epithelial layer organization and the expression of specific markers of epithelium differentiation through immunofluorescence analysis. Immunohistochemical staining revealed goblet cells formation in the suprabasal layer of the organotypic epithelium. Supernatant analysis by ELISA of the conjunctival organotypic culture has allowed us to confirm the positive effect of NGF on the synthesis / release of MUC5AC.

In addition we observed through western blot analysis after NGF treatment a moderate increase of stemness markers like C/EBPδ and ΔNp63α, Bmi1 protein expression remain constant like the level of K19. Both TrKa and p75 receptors are expressed suggesting their implication in the maintenance of epithelium homeostasis.

In all experiment we used as positive control for NGF function PC12 cell line (rat pheochromocytoma). In parallel, we treated with 100ng/ml NGF also the corneal human cell line (HCLE, gift from Gipson-expressing specific receptors TrkA and p75).

The involvement of NGF in inflammation and corneal healing was demonstrated by Lambiase et al. with in vivo studies (Lambiase et ; 2007). The research group demonstrated a strong expression of TrkA receptor in limbal/corneal region and the expression of p75 in patches of the basal layer

of limbus. This suggests that this receptor is expressed by the stem cells of the limbus and that NGF may play a role in the homeostasis of the resident cells of this epithelium.

For this reason, we focused our attention on the NGF effects on limbal corneal epithelium**.**

In order to investigate whether NGF modulates limbal cells proliferation and when this effect is the highest, we treated with 100ng/ml limbal cells and collected them at different time point (6h, 15h and 24h). The proliferation of cells at each time points was evaluated by Edu (5-ethynyl-2-deoxyuridine) marker, an analog of thymidine incorporated into DNA during active DNA synthesis. We measured (by image j) positive cells which have incorporated the edu;we observed that the highest peak of proliferation was after 15h. Cytofluorimetric analysis demonstrated that also the high affinity receptor TrkA became more expressed after 15 hours. In addition we quantified with immunodection the percentage of p63-bright cells in each point, comparing treated and untreated cells. The data indicated that the percentage of serum (from 10% to 1%) not influence the number of p63 bright cells which is increased after 15 hours with moderate decrease at 24 hours.

Three strains of limbal-corneal cultures were treated with NGF and the analysis of the colony forming efficiency showed an increase of stem cell clonogenicity.

Our results showed that in limbal cultures NGF treatment increases Bmi1 mRNA and protein levels but the level of C/EBPδ remain constant. These results indicate the preservation of self-renewal and proliferative potential after the treatment. 14-3-3σ and p75 protein level are stable after treatment conversely to the protein level of p63α which increases. This data correlate with the increase number of Edu and TrkA positive cells .

To assess whether NGF modulates the limbal epithelial stemness, we performed clonal analysis. We obtained 7 holoclones (stem cells), 16

meroclones (early commitment progenitor) and 3 paraclones (transient amplifying cells) from primary human limbal culture.

NGF addition to the three type of clones was associated with the increase of ΔNp63α and Bmi1 protein level and therefore with the maintenance of the stem cells in an early stage. This suggests that the shifting of the stem cells from their homeostatic state of self-renewal to terminal differentiation is slow down comparing to untreated cells. This dependence of this growth factor by limbal cells in vitro preserve their stemness.

To investigate the NGF effect on limbal and conjunctival epithelial cells, we used an alternative approach. It consist in the inhibition of the receptors TrkA and p75 with specific small molecule inhibitor and specific antibody. We didn't observed any significant changes in the clonogenic ability of limbal cells neither in the expression levels of proliferation and differentiation markers. We improved this approach by using specific interference (siRNAs) for the receptors TrkA and p75. We used the cell lines HCLE for the validation of the functionality of these siRNAs. This approach was repeated in Human normal primary cultures but so far it hasn't reproduced the same efficiency. This discrepancy may be related to different characteristic of the cells or by different cell culture conditions. This work is still in progress for the optimization of the system.

All together these data highlight the role of NGF in the modulation of stemness, proliferation, and differentiation of conjunctival and limbal epithelial stem cells.

The knowledge of the NGF molecular mechanism and its effect on ocular epithelial cells supports the clinical application as a pharmacological agent for the treatment of chronic inflammatory or autoimmune diseases of the ocular surface.

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A Rita Levi Montalcini

"La Vita non è altro che un elettrone che cerca un posto in cui fermarsi"