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# Limbal Stem Cell Markers

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## Abstract

*Limbal stem cell deficiency has an important position among corneal blindness, it is the second most common cause of blindness in the worldwide. Hyperemia in the eye, watering of the eyes, chronic pain attacks, blepharospasm, low vision, conjunctivalization, neovascularization, and fibrovascular pannus are observed in patients and these lowering their quality of life significantly. Keratolimbal allografts, conjunctivolimbal allografts and eccentric corneal grafts were used in the treatment of these pathologies. However, as a result of the recent studies, genes and markers that belong to stem cells such as p63 gene, ABCG2, ABCB5, Vimentin, K19 and connexin were defined and thus limbal stem cells were successfully reproduced. Today, the treatment of these diseases is possible by reproducing the stem cells in a small limbal biopsy using various methods. This approach is still current and still developing. In this study we aimed to review limbal stem cell's genes, markers and current treatment modalities.*

**Keywords:** ABCG2, ABCB5, ex vivo stem cell reproduction, connexin, K19, limbal stem cell deficiency, gene p63, vimentin

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## Introduction

Cornea is an optically transparent avascular tissue that reflects the light that enters the eye to the retina. Thus, sustaining the corneal transparency is important for the eye vision [1]. Every disease that interferes with corneal integrity threatens the vision and according to the World Health Organization (WHO), corneal diseases are the second prevalent cause of blindness after cataract [2]. Limbal stem cells provide the sustainability of corneal transparency and the integrity of corneal epithelium.

Limbal stem cells are defined as undifferentiated cells with regeneration capacity and could transform into different cell types. Almost all organs arise from stem cells in embryonic life. The role of stem cells in eyes is significant as in several other tissues in human body. Stem cells that provide surface homeostasis are located in the corneoscleral junction called limbus. These cells do not only provide sustenance for corneal epithelial integrity, but they are responsible from the lifetime restoration and renewal of the epithelial tissue. To sustain the integrity of the corneal epithelial tissue or as a response to a developing damage, these cells initially transform to transient amplifying cells and then to cornea epithelium cells [1,3,4].

Limbal stem cells are characteristically slow-proliferating unique cells with a high proliferative potential, displaying an asymmetrical division pattern and having their own special micro-frames. Each change that occurs in micro-frames of these cells affect their division capacities causing limbal stem cell deficiency symptoms and findings such as low vision, photopia, hydration, redness, irregularities in corneal epithelial surface, reduction in corneal transparency, and in serious cases, fibro-vascular panus conjunctivalization of corneal surface, corneal neovascularization, and persistent corneal epithelial defects [3,5-7]. The factors causing this pathology are considered in two different categories. In the first category (secondary), total destruction of limbal stem cells is observed. Chemical and thermal injuries, Stevens Johnson Syndrome, severe microbial infections with multiple surgical intervention and cryotherapy procedures in limbal region, iatrogenic reasons such as long-term contact lens use are among the most common cases that cause this pathology. In the second category (primary) direct response of the limbal stem cells is not observed, but limbal stroma would not support stem cells due to the disease and thus, stem cells cease to exist due to the lack of this support. This pathology is caused by hereditary factors such as aniridia and ectodermal

dysplasia, and inflammatory keratitis, neurotrophic and ischemic keratitis, pterygium and pseudopterygium [4,8-10].

In the treatment of these diseases, tissues obtained from limbal stem cells and other stem cell resources are used [11-13]. Especially autografts obtained from limbal stem cells using diverse methods provide significant results in the long-term, without the risk of rejection and without the requirement of immunosuppressive treatment [1,5,14]. İn parallel to the molecular genetic developments, several markers were defined both on the corneal surface epithelium and specific to the limbal epithelial stem cells. Some of these markers provide the definition of the cells, while others function in cell differentiation. The cells obtained with the help of these markers are reproduced in various culture media and prepared as tissues ready for transplantation. Some of these markers are discussed below:

**P63**

P63 gene belongs to P53 gene family. Although p53 is a tumor suppressor gene, p63 protein functions as a transcription factor. P63 gene plays a key role especially in the development, differentiation, morphology and connections between the epithelial cells. In p63 gene mutations serious structural anomalies occur especially in skin, limbus and olfactory region [15]. The total of p63 gene is denominated as Tap63 and the N-terminal part is called ΔNp63; transcript products of the N-terminal part have α, β and γ isoforms [16]. In a study by Chen et al., it was determined that p63 gene expression was very strongly expressed in epithelial, was expressed very poorly in corneal basal epithelium and furthermore no gene expression was traced in corneal suprabasal epithelium [17]. P63 gene products are accepted as a marker for limbal epithelial stem cells (LEKH); especially ΔNp63α isoform is accepted as a real marker and functions in LEKH activation [18,19].

**ABCG2**

Also known as breast cancer resistance protein 1 (BCRP1), it is the ATP-binding cassette subfamily G, member 2 (ABCG2). It is a marker defined for hematopoietic stem cells and was proposed as a universal stem cell marker [20]. In certain studies ABCG2 protein was shown in the membrane and cytoplasm of limbal basal epithelium using immune-histochemical staining, however it was not shown in supra basal and corneal basal epithelium [17,21]. In a

different study, when limbal epithelial cells were observed using flow cytometer, ABCG2 positive was determined in approximately 2.5-3% of the cells; this rate equals to the assumed LEKH ratio [21]. ABCG2 positive limbal epithelial cells display a faster colony formation than negative cells in culture media, which is in compatible with the stem cell characteristics.

**ABCB5**

ATP-binding cassette subfamily B, member 5 (ABCB5) gene is previously known as a gene highly expressed in skin and a marker defined for melanoma cancer stem cells [22]. Recent studies determined that ABCB5 protein functions in the development and repair of corneal epithelium, and it was stressed that it could be used as a marker for limbal stem cells. In a study by Xander et al., it was suggested that ABCB5 gene could both function in formation and repair of cornea epithelium and as a marker for limbal stem cells. Furthermore, the same study determined ABCB5 gene product in corneal epithelium of rats and found that it was expressed with p63α protein, which was previously defined as a limbal stem cell marker [23,24].

**Vimentin and Keratin 19 (K19)**

Vimentin and K19 are two components of middle filament localized in limbal basal cells. Vimentin and K19 are two proteins vital in the formation and adhesion of the cytoskeleton. It was observed that vimentin and K19 are expressed together in limbus epithelium cell clusters and K3 expression was negative, furthermore these two proteins were morphologically shown in limbus using electron microscope [25,26].

**Connexin**

It is a protein functioning in cellular communications. There are two types of connexin isolated from the corneal epithelium; these are connexin 43 and connexin 50. Connexin 43 is not expressed from limbus basal epithelium, while it is expressed from corneal basal epithelium. On the other hands connexin 50 is expressed from both limbus and cornea supra basal epithelia, however it is not expressed from basal cell epithelium [27,28].

Several markers are defined for both the definition and the differentiation of limbal stem cells (Tables 1 and 2).

**Table 1.** Markers for limbal and corneal epithelia related to stem cells [17].

| **Markers** | **LEB** | **LESB** | **CEB** | **CESB** |
|---|---|---|---|---|
| **İntegrin α9** | +++ | +/- | - | - |
| **P63** | +++ | +/- | +/- | - |
| **ABCG2** | +++ | +/- | - | - |
| **NGF receptor (trkA)** | +++ | ++ | +++ | ++ |
| **α-Enolase** | +++ | + | ++ | + |
| **$Na^+/K^+$ATPase** | +++ | + | ++ | + |
| **Carbonicanhydrase** | +++ | + | ++ | + |
| **Cytochromeoxidase** | +++ | + | ++ | + |
| **Keratin 19** | +++ | + | +++ | +++ |
| **Vimentin** | +++ | + | +++ | +++ |

**LEB:** limbal epitelhium basal, **LESB:** limbal epitelhium suprabasal, **CEB:** corneal epitelhium basal, **CESB:** corneal epitelhium suprabasal, **-:** no expression, **+/-:** very weak expression, **+:** weak expression, **++:** moderate expression, **+++:** strong expression.

**Table 2.** Markers for limbal and corneal epithelia related to the differentiation of stem cells [17].

| **Markers** | **LEB** | **LESB** | **CEB** | **CESB** |
|---|---|---|---|---|
| **K3/K12** | - | +++ | +++ | +++ |
| **Konneksin 43** | - | +++ | + | +++ |
| **Konneksin 50** | - | +++ | - | +++ |
| **İnvolokrin** | - | +++ | + | +++ |
| **Nestin** | - | +++ | + | +++ |
| **NGF reseptör ($p75^{NTR}$)** | - | +++ | +++ | +++ |

**NGF:** nerve growth factor, **LEB:** limbal epitelhium basal, **LESB:** limbal epitelhium suprabasal, **CEB:** corneal epitelhium basal, **CESB:** corneal epitelhium suprabasal **-:** no expression, **+:** weak expression, **+++:** strong expression.

**Diagnosis of limbal stem cell deficiency**

In addition to the clinical symptoms and findings which mentioned above certain tests are significant in the diagnosis of limbal stem cell deficiency. In fluorescein staining, conjunctiva epithelium displays an abnormal staining pattern since it is more permeable than the corneal epithelium. Furthermore, conjunctivalized corneal surface displays spotted late staining pattern and adjacent corneal epithelium is thinner than normal corneal epithelium, thus it is more sensitive to recurrent corneal erosions and to transition of veins. This could cause recurrent epithelial damages, infections and finally blindness [10,29]. Limbal stem cell deficiency diagnosis could be best confirmed histologically. In normal cornea, goblet cells are not found in epithelial tissue, goblet cells are a characteristic of conjunctival epithelium. Thus, the indication of goblet cells on cornea surface with impression cytology confirms the limbal stem cell deficiency diagnosis. Furthermore, the lack of differentiation to corneal type with immune-histochemical staining, which is a more sensitive method, and the indication of the mucin containing goblet cells confirm the diagnosis [1,10,30,31].

Limbal stem cell deficiency could be observed as partial or total depending on the damage severity limbal region. In partial limbal stem cell deficiency, there is a line between corneal and conjunctival phenotype cells and when stained with fluorescein, the stain is accumulated in the conjunctival side [10,32,33].

Diagnosis of the limbal stem cell deficiency is very important. Because, these patients would not benefit from corneal transplantation without the diagnosis and treatment of the stem cell deficiency [10].

**Treatment of limbal stem cell deficiency**

In the treatment of limbal stem cell deficiency several surgical methods have been used for many years. These are keratolimbal lamellar allograft, conjunctival limbal graft, and conjunctival limbal graft from a living relative. In addition, eccentric penetrated keratoplasties were also used [34-37]. These methods are often used in single-side deficiencies or in patients with partial deficiency by transplanting limbal autograft from the healthy eye to the one with the deficiency. However in this method, there is the risk of the eye that the graft was removed developing limbal stem cell deficiency. In patients with severe bilateral deficiency allografts

could be used. These allografts could be taken from a corpse or a living relative. However, the most significant risk with the allografts is rejection.

Thus, recently Pellegrini et al. [14] were able to engineer 1-2 mm2 healthy autologous graft epithelial tissue in their study. This method causes very low morbidity in donor area and provides autologous treatment for the patient. However, cases with bilateral affection such as Stevens Johnson syndrome or aniridia could not be treated with this method. For these cases, the sample could be obtained from a living relative or a corpse [29]. Today, there are two common methods used for the reproduction of the cells. Reproduction of the tissue transferred onto human amniotic membrane in cell culture media and reproduction of limbal stem cells with 3-dimansional tissue culture.

**Amniotic Membrane Limbal Stem Cell Culture**

Amniotic membrane is a part of mammal placenta and formed by single-layer epithelium enveloping the amnion fluid. Human amniotic membrane that is seronegative for contagious diseases, removed from volunteering Caesar-section cases is transferred to the laboratory in sterile phosphate-buffered saline solution (PBS). After being divided into 4 or 9 $cm^2$ sized pieces under sterile laboratory conditions, amniotic membrane could be stored for 2 years in -80$^{\circ}$C. Tissues obtained from the limbal region of the healthy eye, after being treated with various chemicals, are transferred onto amniotic membrane. It is cultured for approximately 2 weeks in 5% CO2 incubator in 37oC, and after the tissue reaches the required dimensions, it is transplanted using various surgical methods. Usually the tissue is obtained from the limbus of the healthy eye, however if both eyes are damaged, it could be obtained from another person with HLA tissue compatibility and transplanted after reproduction. The most significant disadvantage of amniotic membrane transplantation is the risk of HIV, hepatitis and other viral infections [38-40].

**Three-dimensional Cell Culture**

In primary tissue cell cultures or cell-line cell cultures, usually two-dimensional (2D) cell cultures are used. It is technically less expensive and easy to reproduce the cells in two-dimensional cell cultures. Cells reproduce in colonies in two-dimensional cell cultures and could not form tissue integrity, which in turn decreases or destroys the success rate of tissue

transplantation in cases like limbal stem cell deficiency. Recently, 3-dimensional (3D) tissue cultures, formed by gel or murine layers were developed. The most significant advantage of three-dimensional tissue cultures is the fact that the cells develop as a whole, in the form of tissue. In certain times, even in three-dimensional cell culture media tissue integrity could not be obtained. For the tissue to develop as a single layer epithelium and in integrity, tissue cultures called three-dimensional sandwich method were developed. One of the most important advantages of 3D cell culture is the fact that while 2x2 mm tissue is required for amniotic membrane cell culture, for 3D cell culture, 3x104 cell/$cm^2$ is sufficient. Another advantage is its safety with respect to infections [41-43].

**Transplantation of limbal epithelial stem cells**

Limbal epithelial stem cell transplantation is similar in all procedures; after 360 degrees conjunctival peritomy, conjunctival tissue on fibro vascular pannus and corneal surface is dissected from cornea and limbus. Later on the graft obtained is placed on corneal surface and limbus. To prevent disintegration during the removal of cells from the medium, sodium hyaluronic acid is used. When amniotic membrane should be used, graft is placed on the surface so that the cells would be facing the tear side. Later on graft is sutured with 10/0 vicryl or nylon suture. If epithelial tissue is transplanted without a vehicle, basal side of the cells should face the surface, however then suture is not required. In partial limbal stem cell deficiency, this procedure should be applied to the region of deficiency [12,44,45].

**Conclusion**

Limbal stem cell deficiency is a disease group that affects quality of life seriously and results in reduction of vision. Various treatments have been used in treatment of this disease over the years until today. Parallel to the developments in molecular genetics stem cells in the eye were indicated and ex vivo engineering of these cells became possible. These cells are utilized in the treatment of this patient group after reproduction as auto or allografts. The developments in the field still continue.

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