Regeneration Biology
Rajesh Ramachandran
Department of Biological Sciences
IISER Mohali
Week: 12
Lecture: 59

W12L59_Limbal stem cells and artificial cornea

Hello, everyone. Welcome back to another class on regenerative biology. And in today's class, we will learn about limbal stem cells and the artificial cornea. So we have discussed limbal cells very casually in one of the earlier classes. In general, limbal cells are the transition zone between the cornea and the conjunctiva. So that is the transition zone.

The limbal stem cells play a major role in maintaining the transparency and functionality of the cornea. So let us see that the cornea of the eye is a transparent structure present at the front part of the eye. And it is our window to the whole world. You are seeing the world through the cornea, although you can tell it is I who see the world through the eye.

When you donate eyes from cadavers, when you collect them, it's mainly the cornea because the cornea can be transplanted without too much immunological complication from any donor, and a recipient can have it for several years. So the maintenance of corneal tissue transparency is very important for vision. So the integrity and functionality of the outermost corneal layer, which is the epithelium, can play a key role in the refraction of light onto the retina at the back of the eye. The thickness of the cornea can vary; for example, people who watch a lot of gadgets may have thicker corneas, which can cause them to act like a lens. Which is the cause of your, you know, the shortsightedness or the myopia that the image will be formed in front of the retina, not on the retina.

So that is why doctors will give you a concave lens, which will deflect the light so that the image will be clear. Form because it looks like you have a lens in your eye, and in front of that, there will be one more lens. The thickness of the cornea is an important factor in deciding your eye power. These days, LASIK surgeries have come up where you can reduce the thickness of the cornea, and you can even get rid of your glasses. But the thing is, you need to have a good power of eight or above; then the success rate is higher.

If you have a power of two, three, or four, it may be very difficult to get it done through LASIK. I don't know; maybe technology has improved now. So the integrity and

functionality of the outermost layer of the cornea, the epithelium, are very, very important in projecting the image to the back of the eye. Similar to other epithelia, the epithelium of the cornea is maintained by stem cells. Exploring the properties of these stem cells, along with the clinical consequences of stem cell failure and the potential for stem cell therapy in ocular surface regeneration, is a strong field of regenerative medicine because there are numerous scenarios, not just those where you have to be exposed to an acid or some chemical to damage your cornea.

It can also be a mechanical injury. Sometimes what happens is that these limbal stem cells are not doing their job adequately, which can cause the cornea to lose its transparency due to the presence of vasculature, etc. So there are multiple scenarios in which the cornea can lose its perfect transparency or optimal functioning. So limbal stem cells and the development of artificial corneas, if you look further, you can see that limbal cells or limbal stem cells and the development of artificial corneas are very much connected. They are related through their roles in corneal repair and regeneration.

So your cornea is a dynamic structure in the sense that it constantly gets repaired. It is constantly rejuvenated. That is why the thickness is also varying. So it's not that your cornea is a dead structure. Just that it doesn't have blood vessels.

Limbal stem cells, short form known as LSCs, are crucial for maintaining a healthy corneal epithelium, and their deficiency can lead to significant vision loss in multiple ways. It can be as simple as peeling off the epithelial cells because they're not getting replenished. There can be many such troubles that can come due to the lack of an adequate amount of LSCs. So research on LSCs is pushing the development of artificial corneas, which aim to provide a functional substitute for damaged corneas and often uses LSCs or their derived cells. Although we call it an artificial cornea, it simply means that it is not made of plastic, rubber, or something similar.

It is made only of cells. But you collect these cells and tweak them in vitro. and expand in number and see whether I can fix the damaged cornea. Just kind of take the limbal cells and you culture them and then repopulate back. Limbal stem cells reside in the limbus at the border between the cornea and the sclera.

That is, as I told you, in your eye, if you look through the mirror, you see the black and the white. So the black portion is called the pupil, which is covered by the cornea. Adjacent to that, transitioning into the white, that middle portion, we have the limbal cells. They are responsible for regenerating and maintaining the corneal epithelium, which is the outermost layer of the cornea. Damage to the limbus or loss of LSCs is due to various reasons.

LSCs can be low in number, and their life expectancy has reduced, etc. So the loss of LSCs can lead to limbal stem cell deficiency. It is known as LSCD, Limbal Stem Cell Deficiency, resulting in corneal conjunctivization, which basically means the conjunctiva is growing into the cornea, making it opaque and causing neovascularization and vision loss; if the cornea becomes opaque, it is like closing your eyes—you don't see anything, it's nothing but cutting off the light. In the same way, if your cornea becomes opaque because of the movement of the conjunctiva into the cornea. And naturally, the blood vasculature will come, and your vision will be lost.

LSCs are being explored for their use in various corneal repair and regeneration strategies, including the transplantation of LSCs or cells derived from them. Both ways can be approached. So how do we develop artificial corneas? The development of artificial corneas is driven by the need to treat LSADs, that is, corneal deficiency and limbal stem cell deficiency, because of which the corneal structural deficiencies have to be met. And the other corneal disorders can also occur when natural regeneration becomes impossible. That is why you have trouble with the cornea's transparency or its translucency.

So researchers are exploring different types of approaches, materials, and methods, including bioengineered scaffolds and cell-based therapies using limbal stem cells and their derived cells. You can have the limbal cells, culture them, expand their number, and then try to put them back. So some approaches focus on creating a corneal substitute that can be implanted to restore vision and maintain corneal clarity. So this is the approach that is usually done. LSEs and their derived cells are being investigated for their potential to repopulate and maintain the artificial corneal surface, mimicking the natural corneal epithelium.

Slight changes in the corneal dynamics, etc. Can be adjusted using the correction glasses. Not everyone who has a thickened cornea has to get an artificial cornea. They can simply do a corrective reading; glasses can be fixed. But there are some rare cases where the cornea, although it is thick, is not transparent.

It is not a biologically usable cornea. That is the situation in which cornea fixing is needed. In summary, limbal stem cells are essential for corneal health and regeneration. And their study of the working or the exploration of the limbal stem cell is crucial for advancing the development of artificial corneas. Artificial cornea development aims to provide a functional substitute for the damaged cornea, often by using the limbal stem cells themselves or their derived cells to create a self-renewing and transparent corneal surface.

When you expand the number of these limbal stem cells and repopulate them back into the same eye, it has to be a self-sustaining process; you cannot keep... Supplying limbal stem cells every now and then means one has to find out the circumstances through which the limbal stem cell population is declining, or even if the limbal stem cell population is present, it is not repopulating the corneal epithelium; one has to figure this out. Otherwise, it will be like giving parcel food every day, so one has to find out the root cause so that these newly introduced cells are doing the job that needs to be done in an ideal scenario.

So, if you see the human cornea in cross-section, as you can see in this picture, at the outer surface of the cornea, there is an epithelial cell layer that sits on the basement membrane above the Bowman's layer. We'll come to this structure. And the middle stromal layer, which is sparsely populated with keratinocytes, is surrounded by dense connective tissue. Remember, it is transparent. The final layer consists of a single sheet of endothelial cells which sits on the basement membrane.

So you can see here that there is an epithelial layer, a basement layer, stromal cells, and a Desmond layer, and then you have got the endothelial cell layer, and each of them is marked here. Desmond's membrane is there, the basal cell is there, the wing cell is there, the squamous cell is there, and remember the majority of this portion is transparent, as you can see in the structure, and that is essential for the light to pass through effectively. So, homeostasis in the corneal epithelium, corneal integrity, and therefore function depend upon the self-renewing properties of the corneal epithelium. We have mentioned this very clearly. The prevailing hypothesis is that the renewal of this corneal epithelium relies on a small population of putative stem cells located in the basal region of the limbus.

Limbus is a structure, as I told you, between the cornea and the sclera. And in the basal region of the limbus, there is a small population of cells that we call limbal stem cells. So these putative stem cells are primitive in nature, and they can divide symmetrically to self-renew and asymmetrically to produce daughter cells. Cells that are transient amplifying cells, also known as TAGs, migrate centripetally to populate the basal layer of the corneal epithelium. So limbal cells have to divide in two different ways.

One is a symmetric way to make a copy of itself, and the other is an asymmetric way in which one cell will keep a copy of itself. The other one will become part of the cornea that is called transient amplifying cells. These are daughter cells. So the TAC, known in short form as TAC, transient amplifying cells, divides and migrates superficially, progressively becoming more differentiated, eventually becoming post-mitotic and

terminally differentiated. Post-mitotic cells mean they are terminally differentiated.

It has no plans of entering into the cell cycle. And many differentiated tissues have terminally differentiated cells. And this is how the establishment or dynamics of the cornea is done. Let us quickly see the human limbus. So the limbal epithelial stem cells reside in the basal layer of the epithelium and are marked as EP, as you can see here, which undulates at the limbus.

You can see that this portion is called the limbus, and the conjunctiva is present in the scleral layer, while the cornea includes the peripheral cornea and central cornea, along with the limbus and conjunctiva. And this is the area we are talking about. Daughter transient amplifying cells, known as TACs, divide and migrate towards the central cornea, as mentioned in RO. As you can see, the movement here, which is migrating from here, has been given. To replenish the epithelium that rests in the Bowman's layer (BL), as marked here, and the stroma layer (ST) you can see here, the stroma layer of the limbal epithelium cell niche is populated with fibroblasts and melanocytes and has a good blood supply.

The stromal layer has the blood supply because it gets the nutrition. Because of this stromal layer, the corneal epithelial cells are getting the nutrition. Otherwise, there are no blood cells. So how will corneal cells get oxygen and glucose? So they also have a metabolism. So thanks to this underlying stromal layer.

So this is the structure. It's a very simple structure that is easy to understand. So this is the black region of your eyes and this is the white region. So, limbal epithelial stem cells throughout life, our self-renewing tissue relies upon populations of stem cells and progenitors to replenish themselves following normal wear and tear or even after an injury. So the corneal epithelium on the front surface of the eye is no exception. It also follows the same rule that dead squamous cells are constantly sloughed from the corneal epithelium during blinking.

You're closing your eyes; you're creating a frictional force. Especially, some people have a condition called dry eye, for which they use lubricants. If a dry eye situation is present, there will be more wear and tear because wear and tear is less when you have enough tears in your eye. So, no matter whether tears are there or not, when you blink, you are damaging the corneal epithelium. At the corneal scleral junction, an area known as the limbus, there is a population of limbal epithelial stem cells known as LESC, slightly different from LSE.

LESC, limbal epithelial stem cells. Limbal epithelial stem cells share common features

with other adult somatic stem cells, including their small size, high nuclear to cytoplasmic ratio, etc. They also lack the expression of differentiation markers, such as cytokeratins 3 and 12, because they are stem cells. This LESC is also stem cell. So they don't have any differentiation markers, but they have the potential to give rise to corneal epithelial cells. So this is an illustration of stem cell control by the niche.

It is thought that the LESC limbal epithelial stem cells undergo asymmetric cell division, producing a stem cell that remains in the stem cell niche to repopulate the existing stem cell pool and a daughter cell that is transiently amplifying, which migrates to the corneal layer amplifying cell known as the early transient amplifying cell, also known as ETAC. So this more differentiated ETAC is removed from the stem cell niche, and it can divide further. It will continue to divide, producing transient amplifying cells known as TAC. ETAC becomes TAC, eventually producing the terminally differentiated cells known as DCs. So the double arrows, as you can see in this picture, represent the self-renewing capability of the stem cell.

That is like, you know, a recycling kind of arrow. The supporting niche cells, which are shown in blue, surround the stem cells, which are light green and dark green. DC cells are marked here; differentiated cells and the tags are present. ETACs are there, and the blue-colored cells are the supporting niche cells. So that's how they keep migrating. So to conclude this portion, LESCs are important for vision, and efforts to specifically and prospectively identify these elusive cells are proving to be very difficult because you do not have any bona fide or classified marker, hence one has to do a hit-and-trial method.

However, despite this, mixed populations of epithelial cells isolated from the limbal region can potentially restore the ocular surface and improve vision in patients with LESC function failure. If you have any problem with the limbal epithelium, you can collect the cells from the limbus and culture them even if you don't clearly know what LES is. So the mode of clinical efficacy, that is treatment failure, may become apparent once a more thorough understanding of the normal LESC regulation, that is Limbal Epithelial Stem Cell regulation, and the role of the niche is gained. As of now, we do not have any bona fide markers available for them. The current approach is just like, you know, in some treatments, if you don't know the functional alkaloid, you take the whole tree, the whole plant, grind it, and consume it, assuming that the alkaloid is there; it's not a pure form.

Something like that researchers do now: limbal epithelial stem cells in corneal surface reconstruction. How does it contribute? The integrity of the corneal surface is essential for ocular function. Injuries or congenital diseases could significantly disrupt the homeostasis of the ocular surface, especially the microenvironment of limbal epithelial

stem cells. This will eventually cause corneal regeneration dysfunction and a decrease in limbal epithelial stem cell numbers. So the loss of LESC for different reasons is named limbal stem cell deficiency or LSCD.

Usually, that is a medical term. One of the leading causes of vision loss worldwide is. To restore the corneal surface, LESC transplantation in the form of tissue or cell culture is currently a viable alternative for treating LSCD. So are limbal epithelial stem cells and their niche. We need to understand what the niche is and what the importance of the niche is. Eliases are a group of somatic cells that constantly asymmetrically divide, differentiate, and centripetally migrate towards the corneal center to replenish the worn-out corneal epithelial cells.

These corneal epithelial cells are known as the cornea's outermost layer, and they maintain the avascular and dehydrated features for corneal transparency and integrity so that clear vision can be obtained or maintained. LESCs are situated at the limbus, specifically at a transition zone of the cornea and conjunctiva, and they move in a systematic manner; it is known as Vogt's palisades, which is a Latin term, and in short form, it is called PV. The area is composed of radial fibrovascular ridges, and it is also accepted as the area for the limbal stem cell niche, or LSCN; in short, it is called the limbal stem cell niche. So, the limbal basement membrane expresses a series of ECM proteins, including laminin and collagen family proteins such as laminin 1, laminin 5, and type 4 collagen. Recently, many independent researchers in their studies have shown that corneal regeneration could happen in the central corneas without involvement of the limbus.

These phenomena indicate that the central corneas could be the second niche of the aliases themselves. Several studies from animal models done in rodents show that corneal homeostasis can be maintained after artificial limbal destruction. You can damage it in the model organisms. This indicates the existence of LESC in the central part of the cornea itself. That means even if the limbus is damaged, sometimes the cornea is not affected very badly because it has the stem cell niche in the cornea itself.

So researchers suspect that the corneal epithelial regeneration in the central cornea could be due to the contribution of the transiently amplifying cell TAC. Migrated from the limbus sometime ago, which is making a copy of itself and also maintaining itself. So limbal stem cell deficiency, if you look closely, is defined as the irreversible impairment and dysfunction of LESC, limbal epithelial stem cells, and their essential supporting structure, the niche cells. In the limbus. So, without LESCs and LSCN, the cornea loses its ability to regenerate towards the epithelium.

Besides this, neovascularization and conjunctivalization in the cornea can also be recognized as features of LSCD by simple microscopy at a pathological angle. But microscopy means you can put it near the eye and then visualize it, which doctors will do. The diagnostic modality of LSCDs is currently poor and relies only on clinical observation. Impression cytology, which involves transferring, blotting, and immunostaining superficial ocular surface cells, is currently the best option for LSCD identification. Limbal stem cell deficiency, if you look closely, can occur due to various reasons with different pathogenicity.

A is a healthy eye. B is an LSCD eye caused by a chemical burn. As you can see here, how the vasculature has come. And C, LSCD eye caused by Stevens-Johnson syndrome, which we will not go into the details of. And D, LSCD eye is caused by the herpes simplex virus. In all cases, it has become opaque or less transparent. So the clinical symptoms of LSCD, which is a deficiency of limbal epithelial stem cells observed in the patients, include chronic ocular surface discomfort, vision loss, and photophobia.

That means an aversion to light. This causes the LSAD to vary from individual to individual. and it can be categorized broadly into two groups. The extrinsic causes include chemical burns, radiation, infections such as herpes simplex virus, keratitis, et cetera. The intrinsic causes are also there, such as Stephen Johnson syndrome, aniridia, and chronic limbitis, et cetera.

Cell transplantation therapy is one approach. The treatment of LSCD has gradually matured with recent progress in surgical techniques and regenerative medicine. To restore LESs and LSN as an ultimate aim of LSCD treatment, regenerative medicine is defined by using somatic cells to regenerate biological substitutes and improve tissue functions. This is the approach you take: the cells have a scaffold that allows them to grow, and you make it. And it has become the most promising strategy for LSCD management in the past 30 years. So, cultivated limbal epithelial stem cell transplantation is known as CLET.

So regenerative cell therapy using somatic stem cells has gained a great deal of attention for the treatment of multiple diseases in recent years. We know that. And a scientist, Pellegrini et al., first applied this CLIT for the ocular disease. In this study, the autologous human corneal epithelium was co-cultured with the mouse fibroblast feeder layer and then transplanted into two unilateral LSD patients.

This is one approach. And in 2001, Coley et al. established a method using human amniotic membrane as a substrate to facilitate cell expansion. This method uses feeder-free culture; no feeder layer is needed. With no animal products. And this new technique successfully targeted and treated around eight patients.

So Mariappan et al. also published a standard protocol for the expansion of limbal stem cell epithelial cells in vitro at the same time, which is also a feeder-free technique using HAM, that is, human amniotic membrane, as the culture substrate. So now CLET is one of the most mature and widely used stem cell therapies that have been reported. During the general procedure for the CLET technique, a small limbal biopsy is performed to harvest healthy LESCs from the donor eye first. That is the first step.

Then the harvested LESCs are cultured and expanded in number in ex vivo. And the amount of in vitro cells required for transplantation varies from patient to patient, and it carries substances such as amniotic membranes or other substrates that will be simultaneously applied to support the transplanted cells because they need some transient support to start with. Depending on the diverse LESC sources, this technique has been widely used in unilateral, partial, or total LSCD scenarios. So cell transplantation therapy is also used for other approaches. Although the clade performs well in LSCD treatment, the limitation of its source is very obvious.

Every time you don't get enough cells to overcome the shortage of LESCs. Several recent studies have investigated alternative sources of transplantation solutions. So the cultivated oral mucosal epithelial cells, known as the COMET technique, use epithelial cells lining the oral mucosa and were first developed as a promising alternative to CLET. So, in 2004, Nishida et al. First, we applied the transplantation of the cultured autologous oral mucosal cells to four bilateral LSCD patients.

Bilateral means both sides. And all four patients showed obvious visual improvement. However, the long-term effect of the COMET shows a disadvantage, as neovascularization in the cornea was observed in all patients after transplantation, which severely affects visual acuity. Inactivated mouse fibroblasts were used as the culture substitute in this study, which also limits the future usage of the COMET. Hair follicle-derived stem cells are used.

Human skin epithelial stem cells are utilized. PAX6 and Wnt7 have also been identified as key molecules only for corneal epithelium differentiation, but not for dermal keratinitis. So, to conclude, LESC's research has progressed greatly in recent years, but the development of many improvements is still urgently needed. Significant progress in tissue transplantation therapies has also been achieved in the past three decades. In the future, what we may find is that a xenofree culture can be brought into existence, and new stem cell research and LSCD treatment must be developed; more effort should be put into this blooming research area to achieve a more efficient therapeutic approach.

Conquering the troubles of cornea fixation, we will study more about regenerative biology in the next class. Thank you.