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Review Article

Limbal Stem Cells: A Review

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Abstract

The two distinct types of epithelial cells are present in conjunctiva and cornea. Although getting continuous anatomically, the two distinct cell population get comprise by their epithelia. The limbus locate the corneal stem cells. The stembess of stem cells is important in maintaining the microenvironment of the limbus and conjunctival epithelial cells act as a barrier preventing to migrate onto the surface of cornea. The limbal damage results in limbal stem cells deficiency to varying degrees, including conjunctiva location of cornea as it's characteristic clinical features. Two approaches get comprise as regenerative management of corneal conjunctival satin utilising stem cells, one as limbal onto and the other as allograft by the use of existing stem cells and regeneration and inducting of ocular tissues from embryonic stem cells. Herein we review stem cells and limbal stem cells along with different types of epithelial cells in the cornea, different stages of markers of corneal epithelial cells as well as current approach towards corneal epithelial generation.

Keywords: Conjunctiva; Cornea; Epithelial Cells

Introduction

Animal kingdom represent several examples of the ability to regenerating tissues following injury, as remarkable capacity to regenerate while limbs, is one of a kind of best illustrations. As regenerating the whole organ is not performed by humans, however during normal wear and tear we are able to replace certain tissues such as, our hair and epidermis. Whereas, trauma is followed by our ability to initiate a wound healing response and if the initial injury is not too severe, parts of our liver can even get replace, small intestines and blood cells. Replacing these tissues relate our ability largely to a small population of stem cells having the capacity to self renew and lifelong differentiation along with the specialised molecular pathways. The key to the maintenance of tissues integrity are stem cells throughout the body, including the eye and cornea.

The most specialised body surface is the corneal surface consisting of a constant state of cell renewal and regeneration. The cell proliferation and degeneration continuously replace the outermost layer of the corneal epithelium. The cornea comprises 3 major cell types: epithelial cells, corneal fibroblast and endothelial cells.

The epithelium of the cornea is non – keratinized, stratified and squamous and of ectodermal origin. The ocular surface gets cover together with the peripheral limbal epithelium and conjunctival epithelium. Serving as a protective barrier against fluid and penetration of pathogens are the two main functions of corneal epithelium.

The major role in all main functions of the mature cornea are performed by corneal stroma i.e., Protection, Transmittence, and refraction of light.

The highly organised extracellular matrix are related to all functions which is formed primarily by aligned bundles of collagen termed lamellae stacked in an orthogonal pattern.

The relatively dehydrated state of stroma is maintained by the function of corneal endothelium. Presence of a barrier formed by focal tight junction prevent fluid flow along with pumping action provided by Na+ and K+, ATPase and Mg+ has the former function depend on it.

Corneal organogenesis in humans

The derivation from the ectoderm overlying the crystalline lens initiates the development of cornea as a tissue. The derivation initiate as early as five weeks in the human embryo. A two cell layer thick epithelium primitively is apparent first at about five weeks which is contiguous with the surface ectoderm.

The epithelial stratifies to 3 to 4 cell layer during next one to two weeks, the lens completes its formation and the ectoderm detaches and the eyelid form and fuse.

Immediately after the separation of the lens from corneal epithelium, the waves of neural crest cells migrate into the space between the lens and epithelium. The corneal endothelium and the stromal keratocytes changes to these form of cells. The eyelid opening marks the time of continuous corneal development, which associates with the major development changes.

Approximately 20% of epithelial and stromal cells and 12% of endothelial cells actively progresses through the cell cycle during birth. The actively proliferating cells in both the stroma and endothelium has decreased to nearly zero by the time eyelid get open. Proliferating at a low level constant throughout life. The proliferating level increases noticeably in the corneal epithelium and remains at peak after eyelid opening with actively proliferating 75% basal cells. The stratification of the epithelial get correlate well with the burst of proliferation.

Stem cells in the eye

Discussed below are the several unique inherent properties enabling stem cells which helps maintaining tissue integrity as an important task to get accomplish throughout the body.

- **Error free proliferation:** The essentiality of error free mitosis is utmost since genetic error at the level of stem cell gets continuously and permanently pass to the whole clone of cell, resulting in cellular dysfunction and abnormal differentiation. To minimise any sort of error produce during stem cell mitosis, develops several protective mechanism. During the state of steady growth, firstly the stem cells are relatively quiscent. They leave the task of active DNA synthesis and cell member amplifies to transient amplifying cells (TACs). So, as all cells except stem cells has a limited life span an error mode at the TAC level gets self limited. Stem cells have a longer cell cycle time in Cellular Kinetic Terms. (180 Vs 90 hrs) and a shorter S – phase duration (2-3 Vs 9-21 hrs) as compared to TACs of skin epidermal case. Secondly, it is demonstrated by Portent., *et al*. that asymmetrical DNA segregation occur during Stem cell mitosis, which suggest to retain stem cells their original genetic message, during mitosis passing never copies onto TACs only.
- **Poor differentiation:** The further differentiation get excluded as a necessary property from the concept of "stemness". Therefore, it's been long recognised that the stem cell cytoplasm appears to be primitive and contains fever, if any differentiation products. The process of differentiation means removal of cells from the stem cell population if differentiation is envisioned as reprogramming of the genome. Therefore, a stem cell gets separated from stem cell population if it respond to a differentiating stimulus. A differentiating event getting induce can be explain by two possible mechanisms. First a full mitotic cell cycle needed to asymmetric cell division into two different cells. One remains a stem cell (self renewal) while the other one is destined for cellular differentiation. Second, a complete cell cycle may not occur; instead, stem cell at the Go stage gets affected by differentiating stimuli in which most of the stem cells are going through steady state growth. The cells which get affected, get removed from stem cell population by virtue of the change induce by differentiation process and does not enter the stem cell mitotic cell cycle.
- **Long lifespan:** The lifespan of a stem cell gets equivalent to the organism in which they reside.
- **Long cell cycle time or slow cycling:** The low mitosis activity gets indicated by this property. Although, high proliferation potential is governed by stem cell under steady state conditions, the extremely low rate of proliferation get exhibited.
- **Symmetric and asymmetric division:** In terms of stem cell division it can be either symmetric or asymmetric in terms of daughter cell fate. A daughter cell remain similar to its parent when cell division is obligatorily asymmetric and replenish the

pool by serving it, while the other daughter cell is destined to divide and characteristics the specific tissue by differentiating with acquisition of its features. Such a cell is called TAC and is less primitive than its parent cell. While, on the other hand similar daughter cells are induce by the asymmetry in division, so that they behave differently.

Finally each division of the stem cell may be symmetric but half of the time are self – renewing. Origin of the corneal epithelium is explained by the help of two opposing theories. One states the conjunctival transducers Titian that is derived from the adjacent conjunctiva, while the other depicts about the origin of corneal epithelium is corneal stem cells in the limbal basal epithelium. In the first theory, denuded cornea gets ingrowth into itself the conjunctival epithelial following large epithelium wounds differentiation into corneal epithelium the process describes the term as 'Conjunctival Trans differentiation'.

However several evidences are present experimentally against the above mentioned assumption; Wei., *et al*. demonstrated in culture media that identical keratins are synthesise by limbal and corneal cells, including greater amounts of K3 and K12 markers of corneal type differentiation. By contrast another keratin pattern are produced by the conjunctival epithelium with large amount of simple epithelium keratins but only minimal K3 - K12 keratin, indeed several other biochemical properties along with glycogen content of conjunctiva derived corneal epithelial remain abnormally long after completing Trans differentiation. Responding to Corneal vascularization forming goblet cells can be performed by conjunctival derived corneal epithelium and by expressing immunoglobulin which is a secretory component, two markers of normal conjunctival epithelium. Although normal appearance on light microscopic studies by conjunctival derived corneal epithelium, while the electron microscopic studies showed that the corneal epithelium has narrower intercellular space than the epithelium. It was also found that in humans the persistent epithelium defects get itself frequently associated with the conjunctival derived corneal epithelium along with recurrent erosions, stromal vascularization, necrosis and slow healing rates. Taken together two separate sets of cell lineage are essentially different, one of which are limbal corneal epithelium and the other is conjunctiva epithelium. These data also indicates that true conversion of a differentiated corneal phenotype does not get represented by a conjunctival Trans differentiation' but rather describes an environmental modulation of the conjunctival epithelium.

Corneal stem cells localization

The localisation of limbal stem cells has been based due to the lack of a stem cell marker, on clinical and laboratory evidence supporting the location of corneal epithelium stem cells at the limbal region. The basal layer of limbal epithelium gets identify by six sources of evidences as the one harbouring stem cells for the corneal epithelium.

- **Pigment migration studies:** For the very first time in 1971, the papillary structures was proposed (Palisades of vogt) as generative organs for corneal epithelium cells are at the basal layer of the limbal epithelium. They demonstrated limbal epithelial's basal layer of guinea pig eyes is pigmented, the cornea get healed with pigmented epithelium when the normally non – pigmented corneal epithelium was removed.
- **Radio – labeeled thymidine studies:** The basal cells of the limbal epithelium are normally slow cycling in nature were demonstrated in 1989 by Costarells and co workers, but it can be made to cycle much more rapidly when the central corneal epithelium get to damage.
- **Immunohistochemical studies:** Initial immunohistochemical data suggest that lowest level of differentiation among all corneal epithelial cells is shown by limbal basal cells. The desmosome associated 10 NM intermediate filament in almost all epithelial are form by the group of water insoluble cytoskeletal proteins called keratins. Perhaps the K3-K12 pair is the most important keratin type for ocular surface epithelia, which gets synthesised by some oral mucosal amounts in conjunctival epithelia.
- **High proliferative capacity of limbal epithelium in cultures:** Limbal basal epithelium cells have higher proliferative potential in cell cultures than central and peripheral corneal epithelial cells. The central corneal wounds get respond from Limbal basal cells and by undergoing greater proliferation from tumor promoting agents than central corneal epithelial cells, which terminate the differentiation initiated by proliferation.
- **Corneal conjunctivalization:** When the Limbal epithelium gets removed partially or completely, there occurs a spectrum of corneal abnormalities which gets characterised by conjunctival epithelium ingrowth (conjunctivalisation), vascularization and chronic inflammation which can explain the Limbal stem cell deficiency.

- **Corneal epithelial neoplasm:** The Limbal localisation of corneal epithelial stem cells could get into account for the relative preponderance of Limbal neoplasms and the corneal epithelial tumor scarcity.
- **Corneal regeneration:** Kinetics of maintenance of corneal epithelium mass by mathematical analysis confirms the corneal epithelium can maintain through centripetal migration of epithelium cells which originate from limbus without getting contribution from adjacent conjunctiva.

The mentioned sources of point of evidence to the 1.5-2mm wider area of basal layer in Limbal epithelium as the main region for corneal epithelium harbouring stem cells. The total tissue are a small sub – population for these cells and is estimated to make 0.5% to 10% comprising the whole cell population located in the Palisades of vogt.

The limbus as limbus stem cell niche

It's not well understood that what maintains the stembess of a stem cell. Characters which are inherent to stem cells additionally may plays a role influencing extrinsically from surrounding environment. Schofield suggested firstly that this very property of a stem cell get maintained in its microenvironment by its extrinsic factors. Upon division of a stem cell and replenish the stem cell by returning to niche while the pool, another daughter cell becomes a TAC, that eventually gets terminally differentiate. Limbal microenvironment differs from that of the most striking difference. Undulating network in the Palisades of vogt gets form from its blood vessels and the epithelium allow close proximity from this arrangement. Potentially it provides increased levels of nutrients and to the cells a blood borne cytokinesis at the limbus.

The cornea differs from that of the Limbal basement membrane in that with pegs of stroma it undulates extending upwardly interlinked with anchoring fibrils linked to the basement membrane with an adherent niche this could provide resident stem cells, shielding them against injury and performing movement within their microenvironment. Corneal basement membrane is composed of collagen type IV (3, alpha 4 and alpha 5 chains), collagen type VII. Laminin, fibronectin and heparin sulfate proteolysis. An additional laminin alpha2 and Beta 2 chains together with alpha 1 and alpha 2 chains of collagen type IV is possess by Limbal basement membrane. Composition differences of the basement membrane in Limbal and corneal epithelium might be at least responsible partially for different cellular phenotypes and proliferating behaviours of these distinct cell population. In future studies are required to clarify the role of stromal microenvironment in the regulation stem cell function.

Expression of several proteins at higher concentrations in the basal cells of Limbal epithelium is another difference between the limbus and the cornea is as compared to central corneal epithelium such as cytochrome oxidase, Na+/K+, ATPase and Carbonic anhydrase. The involvement of whether any of these proteins in maintenance and regulation of stem cells is to be seen in the distribution and concentration of various regulatory factors such as human Limbal and corneal epithelium contains retinoic acid as well as in the underlying stroma and fibroblast that affects stembess properties. Different expression of cytokinesis in the limbus and cornea are other important difference.

There have been three types of cytokinesis determined in limbus and cornea.

- Type I cytokines.
- Type II cytokines.
- Type III cytokines.

Types and functions of corneal epithelial cells:

- **Stem cells:** These are fewer in number but have high capacity for large proliferative potential and self renewal. There tendency is to remain quiscent in healthy tissues. When stimulated they divide and undergo differentiation to form TAC and the Limbal epithelium are located in the basal layer.
- **Transient amplifying cells (TAC):** The Limbal basal epithelium most likely consist not only of stem cells but TACs also which get locate in the basal layer of the limbus and peripheral corneal epithelium. They play an important role in wound healing process. Some characters of stem cells are display by TACs in the limbus and peripheral cornea such as long life slow cycling with low mitotic activity and less differentiation in the normal steady state. A shorter life span is shown by TACs in the cornea are getting cycles rapidly and can amplify effectively cellular mass through limited mitosis and get differentiate into corneal superbasal post mitotic cells and terminally differentiated cells (TDCs) at a critical point.

The exact mechanism although remains unknown, while the control of mitotic kinetics in TACs for Limbal stem cells is different from that for corneal cells have been indicated through various studies. The Limbal and corneal TACs provide advantages including;

- Stem cell division gets amplified and minimise the need for proliferating stem cells and conserving stem cell energy.
- Chance for introducing replicative DNA errors into the stem cell population got minimise.
- Much closer provide was of newer cells to the terminally differentiated functional cellular component for ex, The central cornea gets cover by the epithelium.

Terminally differentiated cells (TDCs) and post mitotic cells (PMCs)

The non dividing PMCs gets form by the division of TACs which gets migrate and differentiated towards the central cornea and as TDCs it specifically take on the final corneal cellular phenotype. They get shed continuously in a normal healthy tissues and get replaced because of their limited lifespan. The central cornea locate them in its superficial part and has many tight junctions.

Corneal and limbal markers

The location of the stem cells should not only be able to get pinpoint by the ideal stem cell marker, within the epithelium but it should also allow for isolation, enrichment and molecular characteristics of stem cells which are viable. To date there have been proposed several putative stem cell marker although there have been no identification of a single specific molecular marker. Our capacity to study the characteristics and behaviour got limited by this.

Titrated Thymidine get continuous administered for a prolonged period labels all dividing cells. Remain labelled for a long time are the slow cycling cells termed as label retaining cells and are believed to be stem cells. Capacity to remain highly proliferative *in vitro* are another characteristics of stem cells.

- Glycolytic enzyme alpha – enolase.
- Aldehyde dehydrogenase (ALDH) And P1. Transketolase (TKT)
- Lectin
- Gap junctions.
- P63
- Cytokeratins.
- PAX6
- Cadherins.
- Integrin.
- Transferring Receptors.

Factors regulating limbal stem cell proliferation

- Growth factor receptors (GFR)
- ABCG 2

Limbal stem cell deficiency

Deficient stromal microenvironment supporting the stem cells may cause LSCD such as aniridia, congenital erythrokeratodermia, neutrotrophic keratoplasty and chronic limbitis or more following commonly the external insults that destroy the Limbal stem cells, such as Thermal or Chemical injuries, ocular cicatricial Pemphigoid (OCP), Stevens – Johnson's Syndrome (SJS), Multiple surgeries or cryotherapy rs, contact lens wearers and severe microbial infections. The deficiency of Limbal stem cell can be sectoral or diffuse. The deficient areas is limited to conjunctivalisation of cornea in the latter cases. At the time of insult, Limbal deficiency may be subclinical, in some patients, this can get progress eventually to overt deficiency as the over time depletion may occur of stem cell population. Condition such as, aniridia, keratitis get associated with multiple endocrine deficiencies, neurotrophic keratopathy and Pterygium/pseudopterygium at foremost represent wilder forms of subtotal Limbal stem cells deficiency in which a poor TAC generation, amplification and gradual loss of stem cells occurs.

These kind of patients may experience severe photophobia, pain, reduced vision and even blindness. This seemingly diverse group of diseases represent some common pathogens features as depletion of stem cell population from the limbus, that results to ingrowths of conjunctival elements or in conjunctivalization onto the corneal surface. For several reasons these patients are poor candidates; 1. Not stem cells but only the corneal TACs get transplanted. (2). The risk of allograft rejection is increase by inflammation and pre existing corneal vascularization (3). Due to stem cell dysfunction these eyes tend to develop recurrent conjunctivalization.

The stem cell function may get restore as it is possible theoretically by expanding the population of stem cell modulating it through the microenvironment or using the appropriate growth factors by TACs mitosis.

Harvesting Limbal stem cells along with carrier which may be cornea or conjunctiva. Various methods have been employed preparing transplant sheets for it e.g., Corneal epithelium cell sheet alone, a corneal epithelial cells sheet getting culture on a polymer substrate, collagen or fibrin or an amniotic membrane as a biomaterial. Procedures use for the treatment of LSCD are discussed below.

Amniotic membrane transplantation

The innermost layer of placenta is the human amniotic membrane. Histologically the amnion is 0.02 mm to 0.05 mm in thickness, composing of three basic layers; the epithelial monolayer, the thick basement membrane and avascular hypocellularity stromal matrix. Currently the structural integrity, Transparency and elasticity has made it the most widely accepted tissue replacement for ocular surface reconstruction. The epithelial cellular migration is known to be promote by amniotic membrane.

Amniotic membrane transplantation (AMT) was firstly reported for reconstruction of corneal surface in a rabbit model of total Limbal deficiency. This technique was first use by Tsubota., *et al*. combining with the allograft Limbal Transplantation to construct it effectively the corneal surface in patients having severe dry eye caused by OSP and SJS. It has been earlier reported that alone AMT is satisfactorily to conserve the corneal surface with partial LSCD in eyes, suggesting AMT may help to expand the Limbal epithelial stem cells remaining *in Vivo*.

Amniotic membrane contains high levels of EGF, HGF, TGF (Tumor growth factor) and bEGF (Basic fibroblast growth factor) which potentially are involved in epithelial stromal interactions of human ocular surface involving modulation of proliferation and epithelialisation and differentiation of stromal fibroblast.

Therefore cytokinesis might be provided by amniotic epithelium, which plays a very crucial role in the microenvironment niche of Limbal progenitor cells. In addition amniotic membrane basement membrane contains type IV, V and VII collagen, Ln1, Ln5 and fibronectin that plays an important role in migration and corneal epithelial cells adhesion. The stromal matrix also suppresses the expression of certain inflammatory cytokinesis that originate from ocular surface epithelia, which includes interleukin 1 alpha, IL -2, IL -8, Interferon – alpha, tumor necrosis factor -beta, Basic fibroblast growth factor and platelet derived growth factor. Sequesters inflammatory cells infiltrating the ocular surface and the amniotic membrane attracts and contains various forms of protease inhibitors which explain some of its and anti – inflammatory properties.

High amounts of nerve growth factor is contained in high amounts by Amniotic membrane stroma, that plays a key role in epithelium integrity and survival of stem cell. When a layer of human amniotic membrane covers a rabbit cornea after excimer laser ablation the acute inflammatory reaction got markedly reduced provide evidence by apoptosis of polymorphonuclear neutrophils. This analysis also supported the Han patients with acute burns where amniotic membrane and exhibited apoptosis trapped the lymphocytes. When alkali burns get created in rabbit corneas it get use as a temporary patch by amniotic membrane transplantation which reduces acute and a severe inflammation evidence by a polymorphonuclear neutrophils to a smaller amount of infiltration. These explain how the anti – inflammatory properties help a non – inflamed stroma get created by AMT is essential for successful LSC Transplantation and survival. Other biological effects of amniotic membrane may explain how the preservation of the normal phenotype of human conjunctival and corneal epithelial cells in culture get facilitate and a stem cell expansion get provided by an ideal stromal niche.

Complications of AMT

The major complications are not entail by AMT, however surgery may be followed by minor events. A hematoma may not get form under the membrane in the immediate postoperative period. This blood absorb usually, but if get excessive may need drainage by a small incision in the graft. Occasionally, there persists a residual subepithelial membrane and the visual axis get opacified. The post – AMT microbial infections is lower to as much as 1.6%. This figure is quite lower than the 8% rate with the use of fresh amniotic membrane, whereas the most frequent isolates are the gram +ve organisms. Gabler., *et al*. reported a case of a sterile hypopyon after repeating AMT. Calcification occurs to about 12.8% cases and white

plaques of ciprofloxacin deposits might occur reducing postoperative complications is the key to meticulous selection of donors and recipients and maintaining higher standards of quality.

Limbal auto/allograft transplantation

The autologous or allogenic Limbal epithelium stem cells need to be transplanted in total LSCD. This techniques was introduced in 1989 by Kenyon and Tseng and subsequently for treating patients by many others with focal or unilateral LSCD in a different way of clinical settings. A healthy fellow eye can be harvested from donor tissue. (Conjunctival Limbal autograft) from a related donor which is living or from an eye of cavader (Kerarolimbal autograft).

Transplantation of large pieces of healthy Linus from the donor are involved in a limb grafting. Technique for each of the varieties has drawbacks in the case of allografts get and autografting from living related donorz. The amount of Limbal tissue that can be harvested is limited due to the risk of producing iatrogenic LSCD in an eye of donor.

Allografts from related to living or cadaver donors entail the risk of rejection of a tissue and their survival systemic immunosuppression aggressively which associates with significant morbidity and reduction in quality of life.

Ex – vivo limbal stem cell transplantation

With the increased knowledge in the near past on stem cell biology techniques have been developed of a Limbal tissue to expand small biopsies in culture for subsequent transplantation, by this some of the main hurdle get overcome entailed by whole Limbal tissue transplant. The culturing of stem cell conception was derived from using cultured corneal epithelium stem cells has being the most promising and exciting technique in Limbal Transplantation. It is possible culturing stem cells using tissue to a small amount thereby to minimise the damage of a donor and depletion of reserving it's stem cell using cultured human epidermal cells as grafting autologous in patients with burns and in plastic reconstruction energy.

Only with this technique the epithelial cells (Not Langerhans cells and blood vessels) get transplanted therefore theoretically reducing the rejection possibility.

There are in particular 3 main techniques for culturing Limbal epithelium. The first of it involves the co – culturing of Limbal epithelium with inactivated mitotically, 3T3 mouse fibroblasts, the second of it entails using human amniotic membrane as a substrate for Limbal epithelium cultivating and the third of it combines the above two mentioned methods.

The original method which states about culturing Limbal epithelium on amniotic membrane involves taking small biopsy of a limbus or explants and to culture it in the center of amniotic membrane.

The Limbal epithelial's expansion *ex – Vivo* grows out from the explant onto the amniotic membrane when the amniotic membrane get by *ex – Vivo* expansion of Limbal cells, it get transplanted to the eye with LSCD. The epithelialisation in promoted by amniotic membrane, reduces inflammation and scarring, which preserves and maintain existing Limbal stem cells and is serve as a natural substrate on which the growing and proliferation of Limbal stem cells may occur, which also enables cultural Limbal stem cells for easier handling. It is believed that the amniotic membrane may act as a barrier for immune cells, which diminishes by inhibiting IL – 1 beta and IL-8 expression from the immune response, and it may also produce anti - angiogenic proteins. Culturing limbal stem cells has been of used by fibrin substrate. The culturing Limbal stem cells has been of used by fibrin substrate. The culture system may get maintained for 14-28 days and it either get transferred to the recipient bed or subject to air lifting in order to promote the stratification or epithelium tight junctions.

Conclusion

The concept of Limbal stem cells has greatly improved our understanding of corneal epithelium proliferation, migration and regeneration. Now, this has also contributed directly to improves medical and surgical management of a wide range of ocular surface disorders. Though many questions remain unanswered.

Clinically the most important is the issue of Limbal allograft rejection and the long – term survival of Limbal transplants and that of improving immunosuppressive regimens. In terms of stem cell biology, the unanswered questions include; How does "stemness"

of stem cells maintained? Which factors regulate the Asymmetric division of stem cells? What are the external and internal modulators influencing stem cells? And what role does the microenvironment play in stem cell function and Regulation? [1-10].

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