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Review Article

Current Perspectives on Tissue Engineering for the Management of Limbal Stem Cell Deficiency

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Abstract

Limbal Stem Cell Deficiency (LSCD) encompasses a group of eye disorders characterized by abnormal maintenance of the limbal stem cells. This painful and potentially blinding condition poses a challenge in transplantation biology; whereby whole corneal transplantation would normally fail due to the depletion of the recipients' limbal stem cells. The most preferred technique of management is transplantation of *ex vivo* expanded limbal stem cells to the damaged eyes. This article discusses the therapy options for unilateral and bilateral cases of LSCD, the clinical outcomes, components of cells and substrates that are currently being investigated or have been utilized by this technique, and brings into focus the newer therapy of using a scaffold-free cell delivery system to treat LSCD.

Keywords: Limbal stem cells; Limbal stem cell deficiency; Tissue engineering; Cell sheet

Abbreviations

AM: Amniotic Membrane; BM: Bone Marrow; EGF: Epidermal Growth Factor; ECM: Extracellular Matrix; ESC: Embryonic Stem Cells; GMP: Good Manufacturing Practice; iPSC: induced Pluripotent Stem Cells; LEC: Limbal Epithelial Cells; LSC: Limbal Stem Cells; LSCD: LSC Deficiency; MSC: Mesenchymal Stem Cells; PIPAAm: Poly N-Isopropylacrylamide; 3D: Three Dimensional

Introduction

The corneal limbus forms the narrow transition zone between the corneal and conjunctival epithelia and is believed to harbour the cornea stem cells [1-3]. The limbal stroma is an area rich in blood vessels, contains melanocytes, Langerhans cells and abundant with nerve supply. Adult stem cells are now believed to reside in most tissue populations for regenerative purposes and tissue repair. Stem cells are protected from hostile external factors in a specialized microenvironment called the "stem cell niche". It is hypothesized that the stem cells for the cornea are deposited deep in the basal layer of the limbus [3-7].

More recent advances using the lineage tracing technique in K14+ve Confetti mice supported the evidence that mouse limbus significantly contributed to self renewal and regeneration of the mouse cornea [8,9]. Limbal cells also responded rapidly to major wounding compared to the wound healing potential of the long-term corneal clones which mainly responded to minor injury [9].

Limbal Stem Cell Deficiency (LSCD)

This is a painful and blinding condition of the eye caused by abnormal maintenance of the LSC [10]. It can be broadly categorized into unilateral or bilateral involvement, acute or chronic conditions. Among the causes are hereditary genetic disorders called aniridia, where there is developmental dysgenesis of the anterior chamber of the eye due to PAX6 gene mutation [11,12]. Acquired causes of LSCD include chemical and thermal injury, inflammatory conditions i.e Steven-Johnson syndrome, ocular cicatricial phemphigoid and chronic limbitis. Trauma, surgery and cryotherapy to the limbus, radiation and topical instillation or subconjunctival injection of toxic drugs are some iatrogenic causes.

In the majority of cases involving corneal blindness, whole corneal transplantation is the therapy of choice; but this is not the solution for LSCD. Failure of transplantation in this condition lies in the loss of host stem cells and thus, inadequate self renewing cells to replenish the epithelial surface of the grafts taken from the donor. Due to this, the management of LSCD shifts to transplantation of healthy limbal tissue to the damaged limbal areas. This follows the rationale that re-epithelisation will take place when there are residual healthy limbal cells in the diseased eye, or a sufficient number of limbal cells are replaced by transplanting whole pieces of healthy limbal tissues [13]. However, this surgical method will usually involve a large area of graft taken from a donor site, thus rendering it susceptible to secondary LSCD.

Current Perspective on Tissue Engineering for LSC Transplantation

Tissue engineering was first introduced as an interdisciplinary approach using cell biology and engineering to restore or enhance the biological functions of tissues and organs using substrates [14]. A landmark report in 1997 by Pellegrini revealed a successful transplantation of *ex vivo* expanded limbal epithelium grown on a fibrin carrier [15]. Another commonly used substrate is the amniotic membrane [16]. The outgrowths from the explants originated from a contra lateral healthy eye were allowed to proliferate to form a cell sheet before transplantation to the damaged eye. The advantage of using autologous *ex vivo* expansion of limbal epithelium is the small sized-biopsy taken from the healthy eye which will prevent secondary LSCD in the donor eye. The need for a long term immune suppression is usually eliminated [17]. These bioengineered tissue constructs comprising of a cellular component and a substrate counterpart allow the cells to grow and differentiate towards corneal epithelial

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lineage and proliferate to form an epithelial sheet to be transplanted to the ocular surface. The purpose of this is to maintain the corneal epithelial barrier function from external insults such as pathogens, allergens, desiccation or mechanical injury.

Clinical Trials for Limbal Stem Cells Transplantation

Translational research involving *ex vivo* expansion of LSC and transplantation to treat LSCD was among the first stem cell tools to reach the patients. Using keywords such "limbal stem cell deficiency" and "limbal stem cell insufficiency" in a search at the database for human clinical trials https://clinicaltrials.gov, we found only 10 registered clinical trials (one trial on the use of collagenase in a tissue culture protocol was disregarded). These clinical trials mainly covered the use of established protocols for *ex vivo* expanded LSC and oral mucosa. This implies that the growing numbers of clinical studies for the treatment of LSCD remains in the academic institutions and laboratories; needing further optimization of protocols before materializing into registered clinical trials. Furthermore, the long and complex pathways from preclinical trials to regulatory approval and consent, Good Manufacturing Practice (GMP)-compliant laboratory protocols and industrial partnership for funding of clinical trials and production of "accredited tissues" are among the obstacles from making these studies nearer to the clinic [18,19]. Till date, Holoclar is the only *ex-vivo* expanded autologous human corneal epithelial stem cell product authorized to be used as an advanced therapy recommended by The European Medicines Agency (EMA) for the treatment of LSCD in the European Union. However, Holoclar still awaits comprehensive data report before being adapted for general clinical practice.

The Outcome of *Ex Vivo* Expanded Limbal Epithelial Transplantation

Several investigators have investigated into LSC fate and how restoration of the damaged ocular surface takes place after LSC transplantation [20]. It is very unlikely that it is due to replacement of stem cell numbers alone. It was suggested that LSC transplantation has stimulated dormant LSC to renew and proliferate to the site of injury. In addition, LSC transplantation was also believed to attract circulating corneal progenitors or directly from Bone Marrow (BM) to repopulate the site of injured ocular surface by a chemotactic stimulus. Studying the LSC fate in different aetiologies of stem cell deficiency and the types of tissue transplantation would be a future direction to explain the process of cellular restoration. At present, there is no consensus on LSC fate in different types of transplantation [20-22] such as in penetrating keratoplasty, alone or in combination with limbal allograft transplantation, or in the case of *ex vivo* LSC transplantation.

A short term review of 28 clinical studies on cultivated corneal epithelial transplantation since 1997 to 2010 shows a success rate of 67% [23]. This would probably be due to the majority of tissues used in these studies being autologous in nature (84%). Another long term study on the outcome of cultured limbal epithelial transplantation using fibrin as a carrier gave 66% of full success, 19% for partial success and 15% of failure rate respectively [24]. In another review of clinical outcomes [25], despite the heterogeneity of the type of grafts, the biological carrier to transplant LSC, culture methods, and the clinical cause of the disease, the overall outcome of 17 studies was similar at 67% success rate. In a recent systematic review and meta-analysis of LSC transplantation using AM, an almost similar outcome was recorded. The success rate and vision improvement was at 67% and 62% respectively [26]. Surprisingly, there was no significant difference between autografts and allografts. A longer post transplantation period of observation is warranted to justify this conclusion.

It could not be emphasized enough that long term success of LSC transplantation depends on the quality of the grafts or the frequency of LSC on the grafts [27]. Rama et al, observed the presence of more than 3% of p63+ve stained cells in the holoclones were associated with 78% of success rate. The outcome could be improved by a more effective identification or isolation of LSC, or the use of stem cell enrichment methods such as the side population assay [28,29].

Similar to the allogeneic response occurring in solid organ transplantations, the issue with allogeneic tissue or cell transplants in LSC remains their immunogenicity due to major histocompatibility complex mismatch [30]. Major allogeneic responses include the "graft-versus-host" immunological reactions which need tolerance-inducing strategies [31,32].

In the case of total and bilateral LSCD, cultivated oral mucosa epithelial transplantation on AM has also been clinically applied with promising results [33,34]. This approach when reviewed for 15 treated eyes showed a success rate of 67% total re-epithelisation, without any major complications for a period of at least 34 months. A similar method, but in the absence of 3T3 feeders and animal serum has also been trialled in two patients with successful regeneration of corneal epithelium [35]. However, the phenotypic difference in the corneal and oral mucosa epithelia leads to new vascular formation and corneal opacity. A secondary penetrating keratoplasty may sometimes be performed to achieve a clear central cornea and improve visual acuity [36,37].

Alternative Sources of Cells

The lack of donor corneas in sufficient quantity and of sufficient quality to generate limbal epithelia for transplantation has motivated many clinicians and scientists to search for alternative sources of cells for cellular therapy. The option for replacement of adult limbal epithelial stem cells sourced from outside the cornea includes human Embryonic Stem Cells (ESC) which can be directed to the corneal epithelial lineage. Although ESCs have better differentiation and expansion potential than adult stem cells their use is hampered by ethical issues, regulatory problems and associated funding limitations.

The use of appropriate ECM cellular matrix i.e. collagen IV, laminin or fibronectin in a differential protocol successfully direct human ESC into corneal epithelia [38]. In a mouse derived ESC, the use of collagen IV as a culture substrate has resulted in corneal progenitors which expressed PAX6 and CK12 genes. PAX6 is important for ocular development while CK12 has been regarded as a specific marker of corneal epithelial differentiation. Indeed, transplantation of these corneal progenitors on denuded cornea produced epithelial surface re-epithelisation after 24 hours. However, restrictions surrounding ESC, namely ethical issues, technique of differentiation, accessibility and the costs, have limited the use of

ESC for a larger scale translational approach. Presence of human leucocyte antigen Class I molecules which are a major immunological mediators pose as an immunogeneic challenge which requires tissue tolerance mechanisms before ESC transplantation [39,40].

In the meantime, the advent of human induced Pluripotent Stem Cells (iPSC) has partly resolved the ethical issues surrounding ESC. The use of transcription factors; Oct3/4, Sox2, c-Myc and Klf4 [41], on somatic cells can induce pluripotency in these cells, a process called "reprogramming". Hayashi et al. successfully induced cornea epithelial cells from human adult dermal fibroblast-derived iPSC and human adult corneal limbal-derived iPSC [42] by using the stromal cell-derived inducing activity method. In an animal model, mouse iPSC had been demonstrated to differentiate into corneal epithelial-like cells when co-cultured with corneal stromal cells in the presence of additional factors such as β-Fetal growth factor, Epidermal Growth Factor (EGF) and nerve growth factor [43]. Transcriptionally induced pluripotent cells could be a source of tumour formation [44,45] due to lentiviral integration at the site of gene promoters, and poses the problem of a reliable cellular differentiation. There is also a concern about immunogenicity when used in transplantation [46]. The latter is reported to be related to aberrant methylation and epigenetic memory to their tissue of origin and dependent on the reprogramming methods.

MSCs have tri-lineage potential into adaptogenic, chondrogenic and osteogenic differentiation, have paracrine secretions of immunomodulatory molecules and immune-suppressive properties; ideal for cell-based therapeutic potentials. MSCs were originally isolated from BM, are conveniently isolated from other non-marrow tissue sources such as from the musculoskeletal system [47], adipose tissue [48], oral tissues [49] and umbilical cord blood [50]. Most studies utilizing MSC for LSCD were conducted in animals [51-53]. Almaliotis et al. recently used injectable MSC into the corneal stroma and conjunctiva of alkaline-induced injury in rabbits with a remarkable outcome [54]. Comparative studies between BM-derived and LSC showed comparable results for ocular surface regeneration in a rabbit model [48]. Although MSC-based therapies for the treatment of LSCD are rapidly evolving, the field is in need of further knowledge on the mechanism of action, standardized culture protocols and human clinical trials.

Substrates for Cell-Based Therapy

The second major component in tissue engineering is the biomaterials used as the substrate or scaffolds for cell delivery. The initial focus was to replicate the physical and mechanical properties of the target tissues. Prospectively, more emphasis is being given to develop "biomimetic" substrates which integrate the substrates with the biological environment resembling closely the natural topography of the limbal epithelial crypts as the supportive Extra Cellular Matrix (ECM) [55]. An ideal carrier substrate has often been described to have not only optical clarity, but also able to withstand the culture conditions, flexible to the shape of the cornea and quite tough for surgical manipulation including the suturing.

Biological Substrates

Fibrin sheet

Historically, the use of biological substrates has been the strategy for LSC transplantation when fibrin sheet was first utilized for this indication [15]. A mixture of fibrinogen and thrombin was placed on a plastic ring to allow a coagulation cascade. Primary limbal keratinocytes were grown on feeder layers on this fibrin sheet and the cell to matrix construct were then transplanted to patients' eyes [56]. A clinical trial involving larger number of patients showed a success rate of 76.6% up to 10 years [27]. Fibrin gels are transparent, absorbable and easier to manipulate however, they present a risk of contamination [57] and cause LSC differentiation [58].

Human amniotic membrane

AM as part of the carrier system to transfer limbal epithelial sheets has been the substrate of choice to restore ocular surface disorders [59]. AM facilitates re-epithelisation and has been shown to have anti-inflammatory [60], anti-angiogenic [61,62], and anti-scarring properties [63]. To date, AM has been the most widely used substrate to deliver LSC to the ocular surface. Several modifications have been tried to provide different forms of AM to improve its quality as a carrier, including the use of denuded AM over an intact membrane [33].

The drawbacks of AM are the difficulty to sustain the donor supply and clinical variations in the tissues which might affect the growth conditions. The screening of AM for transmission of diseases is costly and ineffective because it does not totally rule out viral transmission. Additionally, the use of scaffolds or substrates as implants is associated with risk of surgical infections [64]. Hence, researchers have explored the potentials of other materials and used new strategies to develop synthetic tissue-engineered constructs to improve the outcome of LSC transplantation for ocular surface regeneration.

Contact Lens (CL)

Di Girolamo group introduced CL populated by cellular expansion of limbal/conjunctival explants using a xeno-free culture system for autologous transplantation in partial and total LSCD [65]. Sixteen eyes of patients with multiple aetiologies showed an impressive restoration of epithelium in 63% of cases at 2.5 years of follow up. This delivery method of cell-based therapy is attractive in many ways; it uses a regular CL which makes the cost relatively affordable, surgically it is easier to manipulate, and transparency is not a problem. However, the explants method is preferable to the cell suspension method where multiple limbal/conjunctival biopsies from different sites need to be harvested to obtain an adequate size of cell sheet due to poor proliferation of cells from the explants on CL.

Collagen

Collagen forms a major component of the cornea stroma and naturally remodelled by the host cells. Hydrated collagens (hydrogels) are biocompatible, inert, biodegradable and attractive to replace or to complement AM. Hydrogels are more structurally uniformed and the physical and mechanical properties can be modified to suit cellular proliferation [66]. Hydrogels are made up of a 3D network of polymers and water, comprised of macro-molecules connected by electrostatic forces, hydrogen bond, or covalent links. As a scaffold, hydrogels can encapsulate cells and biomolecules as a cellular niche. However, the large composition of water weakens the scaffolds. Cross-linking of collagen with other substances improve the mechanical property of a scaffold [67] however this might alter cellular remodelling and

Table 1: Biomaterials used in corneal epithelial tissue engineering in the clinical studies for LSCD.

Materials	Advantages	Disadvantages
Human Amniotic membrane	Stimulates re-epithelisation, have anti-inflammatory, anti-angiogenic and anti-scarring properties	Semi-transparent, donor to donor variability in the quality of tissues, risk of disease transmission and limited strength
Fibrin sheet	Transparent, absorbable, elastic and easier to manipulate	Risk of contamination and cause early LSC differentiation
Collagen	Biocompatible, relatively cheap, less immunogenic	Collagen hydrogels are mechanically weak, early contraction and easily degraded
Plastically compressed collagen	Transparent, mechanically strong and shaped to the ocular surface. Biocompatible for epithelial growth and stratification	Standardization of methods to achieve desired collagen concentration and density/stiffness to cater to collagen biomimetic properties.
Silk fibroin	Biodegradable, and compatible with cellular growth and proliferation.	Cost of production of natural silk and potential triggering of inflammatory response.
Contact Lens (CL)	Mechanically strong, transparent and easy to handle. Multiple biopsies harvested and grow in cultures is advantageous for repeat procedures.	Synthetic CL may not be an effective biological substrate for cell-based expansion of limbal/conjunctival explants resulting in poor proliferation of cells and loss of SC.
Nanofibers	Three-dimensional structure of nanoscaffolds allows a large surface area and a conducive environment for cellular growth along the fibers.	Elecrospinning fibers consisting of polymers and solvents could be toxic to the cells.
Keratin films	Modification to the structures can produce keratin films which are transparent, absorbable and easy for surgical handling. Cellular growth and proliferation are comparable to AM	Optimisation of the structures of keratin films imperative to achieve a biocompatible construct, better for suturing, non-toxic and non-immunogenic.

impairs the biomimetic property of the substrate [68]. Fibrin, collagen, plastically compressed and alginate hydrogels [69-71] are some of the examples used in clinical studies for LSCD treatment. The use of hydrogels for cellular-based therapies needs a continuous search for the ideal and optimum balance of the composition of the polymer materials i.e. water, ionic cross-linking, pores and permeation. These properties will determine cellular adhesion and the biocompatibility of the materials for tissue engraftment [72].

Plastic compressed collagen

Plastic compression is a technique used to extract water from a collagen hydrogel by downward absorption onto a filter paper [73]. This is performed to increase the mechanical strength of the hydogels. Levis et al demonstrated scaffolds made of compressed collagen gel when compared to denuded AM to be a better biomimetic substrate for the growth of Limbal Epithelial Cells (LEC) [74]. This proved to be mechanically strong, thin and transparent. Additionally, these collagen can be further molded into 3D niche structure mimicking the limbal crypts to allow for better LSC growth [55]. However further standardization of the methods and clinical data is necessary before taking it further as a cell-based therapy for LSCD [75].

Nanofibers

Fabricated nanofibers can be produced by electro spinning methods where biocompatible substrates and polymers are interwoven together [76]. This three-dimensional structure allows a large surface area and a suitable environment for stem cells growth and transfer. In a recent study, fibrous nanoscaffolds from poly-ε-caprolactone demonstrated numerous advantageous properties of controlled shape and porosity, and have a high surface: volume ratio. As a 3D biocompatible structure, this can mimic the physiological ECM cellular matrix, used for synthetic ocular surface regeneration. This scaffold system was shown to be biocompatible with LEC and use of these with cells resulted in good cellular adhesion and cell proliferation [77]. A recent clinical study using MSCs seeded on nanofibers showed significant wound healing from alkaline injury with suppression of inflammation in a rabbit model [78].

Silk fibroin

A biodegradable material has the advantage of variable degradation rate for the viable cells to be released at the site of injury. Silk fibroin which is synthesized from the cocoon of silkworm has been found to be a useful substrate for LEC; it was able to support corneal epithelial proliferation, differentiation and stratification [79,80], although induction of an inflammatory response in the host is a primary concern. The cost of production of a natural silk material for bioengineering is an area of concern. A combination of silk with synthetic materials such as polymers and ceramic for the purpose of cell-based therapy in the cornea is an interesting area for further investigations.

Keratin films

Keratins are a group of structural proteins present on the epithelia of hard or filamentous structures such as the hair, nails, feathers and hoofs of higher vertebrates. Keratin makes these structures to be water insoluble, however its mechanical strength and capability for cells to grow and proliferate on modified keratin-coated culture surfaces have garnered a lot of interest in tissue engineering. Reichl et al. demonstrated in vitro human corneal epithelial growth on modified keratin films that was comparable to AM [81]. By mixing two types of keratin dialysate and subjected it to multiple steps in processing, the keratin films that were produced were mechanically strong for surgical handling but encountered difficulty such as suture loosening; which needs further modification to the keratin structures.

There is an increasing need to develop a synthetic, biocompatible and slowly biodegradable material which could be used as substitute for the AM. The use of a synthetic material would lessen the risk of infection. A substrate which is resilient, biocompatible, and able to adapt to the surface of the eye is an ideal carrier system for delivering of cultured corneal epithelium and a viable option for cellular-based therapy. A summary of tissue-engineered substrates is provided in Table 1.

Emerging Techniques and Future Directions

Decellularised tissues

Strategies involving decellularisation of tissues and organs have been of interest to LSC biologists in the past decade. This technique involves complete removal of cellular components, cellular materials and antigens from tissues to reduce its immunogenicity [82]. This is done while maintaining the corneal structure and the ECM. Decellularisation can be achieved by using chemical and enzymatic

methods, followed by re-seeding with new cells, a process called "scaffold recellularisation" [83,84]. The challenge is to find the right seeding density and the choice of repopulating the surface or via injecting into the scaffold. Maintaining a close balance of preserving structural, mechanical and physiological properties of the decellularised cornea and reducing cellular immunogenicity are among the challenges in this area.

Cell sheet tissue-engineered system

A technique developed to escape from any use of carrier system is a new temperature-responsive polymer e.g. Poly N-isopropylacrylamide (PIPAAm) as a cell sheet engineering system introduced by Okano group and first used in the cornea by Nishida in 2004 [85]. This method allows transfer of cells and the ECM to the ocular surface at different temperature conditions in the absence of a scaffold. PIPAAm polymer and its co-polymer show a hydrophobic state at 37°C and at this temperature cells would adhere and proliferate. At 32°C and below, the cells are detached because of polymer hydration, which allows harvesting of the cells in a mono layer cell sheet while maintaining cell-to-cell and cell-to-ECM contact. Cell sheet fabricated hepatocytes and β-islet cells have been engineered for clinical treatment of liver failure and type I diabetes mellitus [86,87]. As for the treatment of cardiac diseases, a plethora of options are available from cardiomyocytes cell sheets, myoblast sheets, MSC sheets and cardiac progenitor cell sheets for cardiac regeneration [88].

Tissue-engineering in the cornea has often maintained the use of a carrier system for delivery of the cells. A carrier system has the disadvantages of an exposure to infections and transmission of pathogens, especially in the case of biological scaffolds. Both of these factors can hinder tissue integration and affect the survival of tissue transplantation. Suture less techniques as advocated in the transplantation of cell sheets also have the advantages of reducing inflammation post-operatively [85].

The cell sheet engineering system has expanded to include autologous oral mucosa cell sheet fabrication for the treatment of bilateral cases of LSCD; a potential alternative carrier to AM [89,90]. In a study of 26 eyes with bilateral LSCD, transplanted oral mucosa cell sheets supported successful regeneration of the ocular surface with 64% success rate, reduced vascularization and demonstrated a safe and well-tolerated product [90]. This suggests the therapy would also be beneficial for LSCD caused by ocular infection, aggressive surgery at the limbus, contact lens wear and chemical injuries that can contribute to corneal damage. Soon the advances of this system will also breach the barriers surrounding corneal endothelial [91] and retinal pigment epithelial transplantation [92].

Scaffold-free tissue engineered cellular tools have advantages over many scaffolds currently available due to their high cellular availability and long term engraftment. This might be due to the non-invasive thermo-responsive cellular detachment method allowing cells to maintain its ECM, surface proteins, growth factors receptors, ion channel and junctional proteins, which are vital for cellular differentiation [93,94]. However, the disadvantages of cellular sheet approach are possibly the premature detachment of cells or insufficient contact of transplanted cells on the corneal surface. In addition, the costs of the system and the time involved in the manufacturing of autologous cell sheets are part of the disadvantages. These methods need to be further refined to include protocols to assess tissue viability, the quality of tissue constructs and safety assessment. The quality of cells in the tissue construct is vital to ensure permanent tissue repair and successful engraftment.

Conclusion

There have been significant developments in tissue engineering-based therapeutic tools for the treatment of LSCD. *Ex vivo* expanded LSC transplantation has been proven to be able to reconstruct the ocular surface in LSCD eyes using a biological scaffold system which provides transfer of proliferative cells to the target site. The search for the ultimate construct has posed the clinicians, scientists and the industry with many challenges before it can be realized into human clinical trials and clinical practice. Future directions in this field should focus on the development of a high speed, consistent in quality, affordable cell propagation system, accredited by regulatory bodies, and accessible to many users.

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