Investigations of the corneal epithelium in Veterinary Medicine: State of the art on corneal stem cells found in different mammalian species and their putative application

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\textbf{ABSTRACT}

The existence of progenitor cells that can readily differentiate into a specific cell type is a common cellular strategy for physiological tissue growth and repair mechanisms. In the mammalian cornea, many aspects regarding the nature and location of these cells are still unclear. In the human limbus (peripheral area of the cornea) progenitor cells have been found and characterized but in non-human mammals, the picture is not so clear. In this review, we examine current knowledge about the morphology of limbus and the localization of corneal epithelial stem cells in all species studied so far, comparing data with humans. We have also explored different research directions in the veterinary field in order to discuss the: i) currently used protocols and ii) best range of treatments for ocular pathologies in which corneal stem cells are involved.

\section{1. Introduction}

The presence of a population of progenitor cells that can readily differentiate into a specific cell type and provides a functional framework for that tissue is a common cellular strategy for physiological growth and repair. The nature and location of the progenitor cells varies from tissue to tissue and, in some structures such as the mammalian cornea, many aspects regarding the presence of stem cells are still unclear. The eyes of non-human mammals differ in many macroscopically aspects respect to humans. Consequently, researchers have assumed the existence of structural differences between humans and non-human mammals in the composition of cells comprising the central and peripheral cornea. The surface of the eye is a continuous sheet of tissue composed of the transparent cornea and the conjunctiva that overly the white sclera; the peripheral cornea meets the sclera at the corneoscleral junction also known as the limbus. In the latter area, progenitor cells, first named corneal epithelial stem cells or CESC, have been found in humans (Davanger and Evensen, 1971; Schermer et al., 1986). It is known that these cells divide to give rise to progeny that maintain the structure and function of the cornea (Tseng, 1989). Other researchers (Sun and Lavker, 2004) confirmed that CESC of humans are not uniformly distributed in the corneal epithelial basal layer but are specially positioned in the limbal epithelial basal layer only; thus, in humans CESC are now referred as limbal stem cells or LSC (Di Iorio et al., 2005). As veterinarians interested in treating ocular surface pathologies or scientists who use animal models for developing cell-based therapies for use in human diseases, it is important to investigate corneal architecture in as many as species is possible. One of the aims of this review is to provide an overview of the main distinctive structural features of the corneal areas comparing recent results observed in companion/laboratory animals to what is known from human studies. Fig. 1 shows where corneal stem cells have been found in organisms investigated so far; differently from humans, these cells were also found outside the limbus in some mammals so, in this manuscript we referred corneal stem cells as CESC and not LSC. Standard protocols used to isolate these cells will be described and an overview of possible treatments for ocular pathologies, using CESC, will be given.

\section{2. Minimal criteria for the definition of corneal epithelial stem cells}

Corneal epithelial stem cells (CESC) are specialized keratinocytes that are only well described in humans. It is known that human CESC reside in the limbus niche (see the anatomy of the niche in the next chapter) and show the full complement of well-defined keratinocyte stem cell properties; the latter, can be summarised as: i) slow-cycling state as observed in bromodeoxyuridine (BrdU) labelling experiments, also known as label retaining cells or LRC (Bickenbach, 1981), ii) lack...
of the K3/K12 keratin expression which renders these cells biochemically undifferentiated, iii) when cultured, these cells show higher proliferative potential respect than central corneal cells, and possess the ability to grow in colony-forming assays (Lavker et al., 1991). Regarding the molecular machinery that controls and modulates the capacity of CESC to maintain a given state of “stemness”, many specific molecular makers have been described for human limbal stem/progenitor cells: ΔNp63, an isoform of nuclear transcription factor p63 (Pellegrini et al., 2001), ATP-binding cassette protein-B5 and B2 (ABCB5 and ABCB2) (Ksander et al., 2014), integrin-β1 (Li et al., 2014), nerve growth factor (NGF) and its receptor TrKA (Qi et al., 2008a). However, nowadays, none of these molecules alone is recognised as definitive CESC markers, even in humans. Therefore, in Veterinary Medicine it will be important to: i) despite the reservations just noted, use a similar strategy that has been used for the detection of human CESC and ii) define the molecular pathways that determine cell potencies and fates in the corneal epithelium of different species. One of the following chapters will deal with markers used in Veterinary Medicine but it is relevant to state that the described markers in this review, represent the best choice for starting an investigation of CESC in an unfamiliar species.

3. Characterization of CESC in different animals

3.1. Microscopic anatomy

In humans and animals, the peculiar organization of the collagen, together with the absence of blood vessels, maintains cornea transparency to allow the passage of light. Three layers are present in mammals: epithelium, connective stroma and endothelium. In addition, two covering layers are present: the Bowman membrane (between the epithelium and the stroma) and the Descemet membrane (separates the stroma from the endothelium).

In domesticated animals, the existence of a Bowman’s layer is still questionable: in dogs and horses, some electron and in vivo confocal microscopy studies did not show a specific membrane within the corneal layers while it was observed in rodents, primates, and some herbivores (Hayashi et al., 2002). More recently, some authors (Nautscher et al., 2015) confirmed that in many animal species a Bowman’s membrane was not detected by standard histological methods. Probably, this layer is very thin in the majority of animals and only a specific electron microscopy study might shed light about its presence. Regarding the precise location of corneal stem cells in different mammalian species, this is still a controversial topic and remains elusive. Anatomically, the basal layer of the human limbal epithelium appears corrugated because it is arranged in finger-like structures of the epithelium into the stroma; the upward projections of the stroma are described palisades of Vogt (Lavker et al., 2004). The structure formed by the epithelium and the connective tissue represents an ideal environment for maintaining quiescent stem cells and their protection from mechanical and chemical injury. The palisades of Vogt are therefore distinctive features of the human corneoscleral limbus, which serve to maintain the stemness of human corneal stem cells. In contrast, scarce data have resulted from the study of limbus anatomy in animals (Fig. 1). Majo et al. (2008) proposed that murine corneal stem cells are distributed throughout the basal layer of the entire corneal epithelium. Recently, two groups (Nautscher et al., 2015; Patruno et al., 2017) observed that in many animals the epithelium of the cornea is organized in three layers: i) superficial non-keratinizing stratified squamous cells, ii) intermediate cells parallel to the superficial surface and iii) basal cells, perpendicular to the surface layer. Few cell rows constitute the epithelium of cats and dogs (the thinnest) while the pig epithelium consists of 10–12 cell rows; cows, sheep and horses showed a thicker epithelium. Considering the morphology of epithelial cells, the corneal basal layer of dogs and cats is composed of rounded shape cells and nuclei that do not form a columnar layer as observed in other species. In cows, sheep and horses, nuclei of basal cells are elongated with a columnar aspect. Adaptations occurred during evolution surely played an essential role in defining the final structure of the epithelium and further investigations should study the correlation between physiological/behavioural aspects (prey or predator condition i.e.) and anatomical characteristics.

About the microscopic general aspect of the limbal epithelium in studied animals, no palisades of Vogt-like niche structures were observed. One study in equines confirmed that the palisades were not present in horses (Moriyama et al., 2014) although a crypt-like structure was recently observed in this species (Patruno et al., 2017) and in pig (Notara et al., 2011). In the latter species, palisades and niches have
been described with the non-invasive 3D imaging using the full-field optical coherence microscopy method (FFOCM). The quantitative assessment of the limbal crypts showed that stroma represented the most important component of the niche volume (Notara et al., 2011; Grieve et al., 2015). New data by our research group, described in the epithelium of cows and sheep a few invaginations only, without any evidence of real crypts (Fig. 2). Some authors stated that bovine cornea has a structure and biochemical makeup similar to human cornea since as in human cornea, stem cells have been localized in the basal layers of the bovine corneal limbus (Liang et al., 2009) and have been successfully used as a model for human limbal stem cell for basic research (Remtulla and Hallett, 1985; Stepp and Zieske, 2005; Henriksson et al., 2015). New data by our research group, described in the epidermal crypts in sheep (A) and cow (B); new data indicate that some portions of the limbus contains regular invaginations. Scale bars: A = 100 μm; B = 100 μm.

Fig. 2. Morphological details of the limbus area in sheep (A) and cow (B); new data indicates that some portions of the limbus contains regular invaginations. Scale bars: A = 100 μm; B = 100 μm.

3.2. Markers

The phenotypic picture of corneal stem cells relies on a mixture of: i) positive expression of stem cell markers and ii) negative expression of differentiation-associated markers. Indeed, human limbal stem cells lack the differentiation markers such as cytokeratin 3/12 and connexin 43 but instead express p63 and ABCG2. These facts are essential for those who begin studies of new species. The best method to test markers is immunohistochemistry/immunofluorescence but we recommend western blotting and FACS to be used for such investigations in Veterinary Medicine too. The nuclear transcription factor p63 is highly expressed in the basal cells of many human epithelial tissues; it is also known that p63 knockout mice lack stratified epithelia, and the truncated dominant-negative ΔNp63 isoform is the predominant species in stem cells (Di Iorio et al., 2005). Indeed, in the resting ocular epithelium, the α-isoform of ΔNp63 is present only in the basal layer of the limbus and is essential for regenerative proliferation of the ocular surface (Parsa et al., 1999; Pellegrini et al., 2001). ABCG5, another proposed stem cell marker, is a member of ATP-binding cassette transporters (Ksander et al., 2014; Frank and Frank, 2015) and has been proposed as a universal marker for stem cells (Zhou et al., 2001) and corneal epithelial cells (de Paiva et al., 2005). NGF and its receptor TrkA have been identified to be exclusive localized to a subpopulation of basal limbal epithelial cells (Qi et al., 2008b). Integrin β1-enriched human corneal epithelial cells were demonstrated to possess stem cell properties including high colony-forming efficiency and slow-cycling (Jones and Watt, 1993; Li et al., 2005). Importin 13 (IPO13) is a recent nucleocytoplasmic transport receptor protein studied in human corneal and limbal epithelial cells; IPO13 was coexpressed by a part of p63 and ABCG2-positive cells and it may represent a characteristic of limbal progenitor cells (Wang et al., 2009). Other putative “positive” markers can be ΔNp63α, K19, integrin-alpha-9, and vimentin, as (Schlötzéner-Schrehardt and Kruse, 2005; Chee et al., 2006; Shortt et al., 2007; Qi et al., 2008b; Barbaro et al., 2016). However, none of the markers are definitive; thus, additional co-localization studies should be performed with markers of corneal epithelial differentiation (K12 or K3) to demonstrate that the cells positive for the putative stem cell markers are undifferentiated and, where possible, co-stains should also be performed with stem cell markers. (p63 with ABCBS or NGF).

Table 1 summarises the range of markers used in different species. Additional reports revealed that cells positively stained with these markers were spread in the basal layer of limbal and central corneal epithelium (Prontura et al., 2017). In dogs and cats the corneal and limbal conjunctival epithelia share a similar immunophenotype (immunoreactivity for AE1/AE3 and CK18, SMA, and GFAP) although a variable S-100 and desmin immunoreactivity and a remarkable difference of the limbal basal cells for CK8/18 (Chen et al., 2004). In dogs, the distribution of CK8/18 might be related to the unique role of limbal basal cells; here, the cell markers AE 1/3 and CK 5 were evident in central cornea and limbus while the central part expressed CK 3/12. In the pig, fluorescent immunostaining revealed that p63-α + positive cells were within crypts (Grieve et al., 2015). Interestingly, the porcine limbus has been shown to share characteristics with the human limbus since similar observations regarding: i) high colony forming efficiency (CFE) achieved with epithelial cells harvested from crypt regions and ii) expression of the putative stem cell markers markers p63-a and integrin b1 (Notara et al., 2011). In horse, p63 was positive in the nuclei of 2–3 layers of corneal epithelial basal cells located apically in the region where melanocytes are present in the corneal limbus (Moriyama et al., 2014).

Equine corneal immunostaining showed positive expression of p63 and CK14 in the epithelial basal layer but a negative expression of CK3. This expression pattern is similar to CESC in humans, thus indicating that CESC are also distributed in the epithelial basal layer of the equine corneal limbus (Dua et al., 2005; Chen et al., 2006; de Paiva et al., 2006). Specifically, some authors stated that the equine limbal epithelium contains CESC and undifferentiated cells, whereas the central region is composed of advanced differentiated cells closer to terminal differentiated cells (Moriyama et al., 2014). In summary, we suggest that the most reliable markers to be tested in Veterinary Medicine should be p63, ABCBS and K19.

3.3. In-vitro cultures

Primary corneal epithelial cultures are obtained using explants from the central and/or limbal cornea. These explants can be used to
regenerate corneal epithelial sheets suggesting that the high colo- 
genic stem/progenitor cells are possibly located and distributed in the entire cornea or in the limbal area only. In human cornea, only limbal explants grow well to regenerate corneal epithelial sheets, while central corneal explants are less successful (Li et al., 2014); it will be important to perform these tests in animals of veterinary interest too. 

In humans several papers have evaluated corneal epithelial proliferation and preservation of progenitor cells on collagen substrates/anniotic membranes (Tseng, 1989; Sun and Lavker, 2004), while in animals data are scarce. In dogs only, CESC were typically cultured on a substrate or materials such as amniotic membrane, collagen gel, or temperature-responsive culture dish to allow cells to adhere and proliferate easily and to maintain the stem/progenitor phenotype of cultured epithelial cells (Nam et al., 2015).  

3.4. Labelling retaining cells (LRC) 

Potential stem cells present a slow cycling turnover in their corneal and limbal locations and hence can be identified experimentally (Kim et al., 2004; Tumbar et al., 2004; Pajoohesh-Ganjii et al., 2006). To detect LRC, one can label all the cells in the epithelium by a repeated or continuous supply of a deoxynucleoside, tritiated thymidine or BrdU, followed by a long chase period when the label is lost from all the cycling transit-amplifying cells (TAC), while only stem cells that cycle slowly retain the label. In veterinary field, no data are reported in literature about the presence of LRC and their localization in the cornea. The use of BrdU approach might be very useful to better understand the mechanisms of cell repair in the cornea of different animals.  

4. Application of CESC in ocular pathologies 

In humans the partial or total limbal stem cell deficiency (LSCD) is a pathology characterized by a reduction of proliferative ability, resulting in abnormal corneal surface (Di Iorio et al., 2005; Weichsler et al., 2009; Barbaro et al., 2016). In LSCD cases, the corneal surface is covered by conjunctival epithelium and goblet cells become present in the cornea. Corneal conjunctivalization results in an ocular surface that appears irregular, with visual impairment (Vlahović et al., 2010; Ledbetter et al., 2013). LSCD in humans can be a congenital disorder or develop secondary to physical trauma, chemical burns, thermal injury, surgery, autoimmune conditions, and microbial infection (Ledbetter et al., 2013). The best strategy to treat LSCD may be divided in three categories: i) limbal autograft transplantation of expanded or non-cultured cells ii) ex vivo transplantation and iii) alternative approaches such as oxygen therapy, keratoprosthesis implantation, and many others in case of bilateral damages (Utheim, 2013). Overall, cultivated corneal epithelial transplantation represents the best choice since it is a minimally invasive treatment for the donor eyes and because grafts can be prepared from small tissue fragments containing stem cells; it therefore can preserve the structural and functional integrity of the cornea reducing risks of rejection (Gül Sancak et al., 2016).  

In Veterinary medicine, the presence of congenital LSCD with conjunctivalization is still uncertain although a recent case reported by Özgencil et al. (2017) described a probable LSCD in dog. Another report of a dog with possible LSCD and conjunctivalization of the cornea caused by Canine Herpes Virus –1 was also diagnosed with a neurotrophic keratitis (Weichsler et al., 2009; Ledbetter et al., 2013). Moreover, diseases that cause disruption of the transparency of the animal cornea and coverage of its surface by cells and pigments such as pigmentary and lymphocytic-plasmacytic keratitis, keratoconjunctivitis, feline corneal necrosis, symblefaro and feline herpesvirus type-I (FHV-1) are more common than in humans. Interestingly, it is considered that these features observed in companion animals show similarities with primary stem cell deficiency cases of humans (Gül Sancak et al., 2014). However, since there are no reliable links between the listed corneal pathologies in which LSCD is suspected and a possible limbal deficiency, the first step to be taken would be to confirm this correlation, in order to set possible therapies.

The final goal to treat these pathologies would be to apply, efficaciously, corneal stem cells as in humans instead of commonly not successfully used approaches; indeed, most of these treatments use allo-transplantation, which has associated problems with graft rejection and difficulties in maintaining a stable supply of grafts (Moriyama et al., 2014). The use of corneal epithelial cells in corneal operation is still rare in Veterinary Medicine, while surgeons concern procedures such as corneal transplantation, corneal conjunctivalization therapy, or limbal insufficiency and keratopathy (Vlahović et al., 2010). Recently, some veterinarians claimed that corneal ulcers of horses were

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### Table 1  
Markers tested in the cornea of several species.  

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successfully treated using blood derived stem cell injections, although the case numbers were very low and clinical outcomes were evaluated only as case reports (Marfe et al., 2012). The use of corneal stem cells in corneal defects is very effective, although not yet routinely available in veterinary medicine (Gül Sancak et al., 2016). An important advance would be to test the application of in vitro grown stem cells as allografts from animal species of veterinary interest (dogs, cats, horses and pigs) as a model for transplantation research in therapy of corneal defects and deficiencies. Indeed, the application of corneal stem cells to the corneal wound should reduce the time needed for healing corneal opacity. By subconjunctival application of either auto- or allografts of LSC to dogs with corneal injuries, Pirkic et al. (2015) obtained promising results in the healing of corneal defects justifying the introduction of cell-based therapy in veterinary ophthalmology.

5. Conclusions

There are many issues to be addressed that might influence the “panorama” of future veterinary applications of corneal stem cell therapy, including the complete characterization of the corneal epithelium for many species of veterinary interest and the choice of a cell-based versus a non-cell based therapy.

In the last two decades strong evidences from a large number of reports leaves little doubt that human CESC reside in the limbus (Cotsarelis et al., 1989; Zieske, 1994). Moreover, human CESC exhibit the full complement of well-defined keratinocyte stem cell properties, including the lack of the K3/K12 keratin pair in limbal basal cells, their higher proliferative potential compared with central corneal cells, and their ability to grow in colony-forming assays (Scherrer et al., 1986; Tseng, 1989; Nishida et al., 2004; Sun and Lavker, 2004). In contrast, in many studied animals it is not yet confirmed if the epithelial stem cells reside in the limbus or are sparsely distributed in the basal epithelial layer of entire cornea. Some authors demonstrated that murine CESC were distributed throughout the basal layer of entire corneal epithelium (Majo et al., 2008). Here, the palisades of Vogt were detected with limbal crypt features in organ-cultured human corneoscleral rims but not in fresh mouse cornea (Grieve et al., 2015). Regarding the applications of CESC in humans, it is important to remember that in clinical studies alternative cell sources were also used to treat LSCD, including oral mucosal cells (Di Girolamo et al., 2009; Di Girolamo, 2015; Moriyama et al., 2014) or cultured conjunctival epithelial cells (Homma et al., 2004). Veterinarians should take advantage of these reports and test other stem cells to treat ocular pathologies. In our view it will not be useful to look for the best cell sources without a solid scientific database and this can be achieved improving in vitro studies and planning, even in veterinary clinics, a repeatable protocol for each diagnosis; this could lead to the best treatment for each disease. It might be proposed that what is important is not the cell itself but what is secreted from the cell, because this “milieu” (or “secretome”) carries factors expressed at the proper moment; so, to reach the optimal results it will be important to employ a combined approach using eye drops obtained from the secretory milieu of different cultured cells together with a direct application of grafts containing cells and matrix. Final recommendations will regard the use of every possible histological and molecular techniques to fully understand the specific cell location and to clarify signalling circuits involved in the physiology of corneal stem cells.

Therefore, resolving these matters should provide further insights into future regenerative therapies for humans as well as animal ocular tissues. In summary, the regenerative competency of the cornea epithelium of many animal species is probably not restricted to the limbal region but rather includes other cells found throughout the entire basal layer of the cornea; this calls into question previous hypotheses arguing that, in laboratory and companion animals, this process takes place in the limbal region only.

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Localization of corneal stem cells is more difficult to describe; however, it is defined in humans (found in the limbus area only), in pig (similar localization as seen in humans), in rabbit (basal layer of crypt-like structures of the limbus only) and in rats (basal layer of limbus only); in other animals the scarcity of data or ambiguous results suggests the following: mice (stem cells were found in the limbus but also in central cornea, at different layer levels); horse (stem cells were observed in crypt-like structure of the limbus); dog (stem cells were found in the basal layer of limbus but also in central cornea); bovine (stem cells were observed in the limbus but also in central cornea); in cats and sheep no real data were obtained about the localization of putative corneal stem cells.

References


