The Corneal Epithelial Stem Cell Niche

MARY ANN STEPP, PHD1 AND JAMES D. ZIESKE, PHD2

ABSTRACT In recent years, it has become generally accepted that the corneal epithelial stem cells are localized in the basal cell layer of the limbal epithelium. However, a number of questions remain regarding the number, markers, generation, and maintenance of the corneal epithelial stem cells. One of the key questions concerns what makes up the microenvironment or niche that is responsible for allowing the stem cells to remain and function throughout the life of the tissue. This review will consider the unique aspects of the limbus and compare these to what is known about other stem cell niches.

KEY WORDS corneal epithelium, limbal epithelium, stem cell, niche, transient amplifying cell, α9 integrin, α-enolase, microenvironment

I. INTRODUCTION: WHAT IS A STEM CELL?

Although it had been speculated for a number of years that corneal epithelial cells are repopulated by centripetal migration of cells from the limbus or conjunctiva, it was not until 1986 that Schermer et al1 proposed that stem cells localized in the limbus were responsible for generating and maintaining the corneal epithelium. Stem cells are most simply defined as cells that have the capacity to self-renew as well as the ability to generate differentiated cells.2-6 When adult stem cells are considered, a further criterion is added requiring that the cells must self-renew throughout the life span of the animal.

Stem cell research has recently become one of the most active areas in biology research; however, to date, no molecular markers have been recognized that definitively identify stem cells. Therefore, stem cells are usually defined by a group of common characteristics.2-6 First, stem cells are the ultimate precursors for all other cells in a tissue or even in the entire organism. Second, stem cells maintain their own population. They are capable of asymmetric cell division, giving rise to one daughter cell that remains a stem cell and a second daughter cell that will go on to differentiate. Third, stem cells make up only a small percentage of the total cells in the tissue, ranging from as low as 0.01% of the cells in bone marrow to 2-4% of the cells in the small intestine. Fourth, stem cells are relatively undifferentiated. Fifth, stem cells divide infrequently in vivo. Sixth, stem cells have progeny that can rapidly replicate in vitro when placed into cell culture. Many recent definitions of stem cells also require that the cells must be multipotent, i.e., they can differentiate into more than one cell type. These definitions state that cells that give rise to only one cell type are termed progenitor cells. According to this strict definition, corneal epithelial stem cells, which have been reported to give rise only to corneal epithelial cells, would be termed progenitor cells. However, we will use the less restrictive definition in this review and refer to the corneal epithelial precursor cells as stem cells.

A second classification of cells to be defined are the progeny of non-stem daughter cells that result from the asymmetric cell division of a stem cell. These cells are more committed to differentiation than the stem cell and also have a more limited ability to proliferate. They have a range of proliferative capacities. The cell that is the immediate progeny of stem cell division can undergo more rounds of proliferation than the cell that arises after several cell divisions. Regardless, the primary purpose of these cells is to increase the number of cells resulting from each stem cell division ultimately resulting in a tissue. These cells have been traditionally referred to as transient amplifying cell (TAC).1 TACs are also sometimes termed transit amplifying cell (TAC).1 TACs are also sometimes termed transit amplifying cells.3

II. EVIDENCE FOR THE LIMBAL LOCALIZATION OF CORNEAL EPITHELIAL STEM CELLS

Over the past 20 years, research from many laboratories has led to the conclusion that the corneal epithelial stem cells are localized in the limbus.7-12 These cells have been termed both corneal epithelial stem cells (CESC), based on their function, and limbal stem cells (LSC), based on their localization. We will use the term CESC in this review.

The first suggestion that the limbus is the source of CESC was made in 1971 by Davenger and Evensen,13 who showed, occasionally, pigmented cells were observed to migrate from the limbus into the central cornea. It should be noted, however, that the term stem cell is never used in their report. Centripetal migration of cells from the limbus to central cornea has subsequently been documented in animal models.14,15 Schermer et al,1 who demonstrated that the keratin K3 was localized in all corneal and limbal epithelial cells except the limbal basal cells, presented a second line of evidence that the stem cells are localized in the limbus. Coupled with cell culture studies, Schermer's report documented that the limbal basal cells were less differentiated than the other corneal epithelial cells. This report was the first to speculate that the CESC were localized in the limbus. A third line of evidence is that limbal basal cells proliferate less frequently than the rest of the corneal epithelium. Lavker and coworkers16 demonstrated this property by injecting mice with 3H-thymidine and finding that only limbal basal cells retained the label. Fourth, only limbal basal cells are clonogenic in cell culture experiments.17-19 Finally, perhaps the strongest evidence, studies have shown that transplantation of limbal tissue or even cultured limbal basal cells can result in healing of corneal epithelium.20-22

As stated above, one piece of evidence that the CESC are localized in the limbus is the lack of expression of the differentiation marker K3 in these cells.1 Subsequent to this finding, other proteins, including K12,23 aldehyde dehydrogenase class 3,24 and connexin-4325 have been shown to have an expression pattern similar to that of K3. In contrast, several proteins are expressed preferentially in the limbal basal cells. These proteins have been scrutinized for their potential for being CESC markers. Included in this group of potential markers are: 1) metabolic enzymes—α-enolase,26,27 cytochrome oxidase,28 carbonic anhydrase,29 and glucose transporter 130; 2) growth factor receptors—epithelial growth factor receptor,31 TGF-β receptors I and II,32,33 and TrkA34; 3) cytoskeleton components—keratin 1935 and vimentin36; 4) cell cycle components—cyclins D and E,37 and p10738; and 5) others—α9 integrin,39 melanin,40 p63,41 and ATP-binding cassette transporter G2.12,42

Most of the proteins considered as potential markers are expressed in numerous basal cells at the limbus; an example is seen in Figure 1, which shows the co-localization of α-enolase and α9-integrin in the adult rat limbus. The distribution of these two proteins is partially overlapping with some limbal basal cells that express both proteins and others that express more or less of one or the other. This distribution emphasizes the heterogeneity of cellular phenotypes present at the limbus and is somewhat at odds with the findings that only a small popula-

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<tr>
<td>BMP bone morphogenetic protein</td>
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<td>CESC corneal epithelial stem cell</td>
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<td>Dpp Decapentaplegic</td>
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<td>EGF epidermal growth factor</td>
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<td>ESC epidermal stem cell</td>
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<td>FGF fibroblast growth factor</td>
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<td>GFAP glial fibrillary acidic protein</td>
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<td>GISC gastrointestinal stem cell</td>
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<td>GSC germ line stem cell</td>
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<td>HSC hematopoietic stem cell</td>
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<td>IGF insulin-like growth factor</td>
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<td>ISEMF intestinal subepithelial myofibroblast</td>
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<tr>
<td>LSC limbal stem cell</td>
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<td>LSCD limbal stem cell deficiency</td>
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<td>mRNA messenger RNA</td>
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<td>NSC neuronal stem cell</td>
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<td>SGZ subgranular zone</td>
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<td>SVZ subventricular zone</td>
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<td>TAC transient amplifying cell</td>
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<tr>
<td>TGF transforming growth factor</td>
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<tr>
<td>Trn-C Tenacin-C</td>
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<td>VEGF vascular endothelial growth factor</td>
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tion of the limbal basal cells (perhaps as few as 100 cells/limbus) are true CESCs. One possible explanation for these observations involves the niche hypothesis.

According to the niche hypothesis, originally proposed by Schofield, cells are influenced by their microenvironment to become and remain stem cells. Thus, it may be possible that the entire limbus provides a microenvironment that allows the limbal basal cells to maintain stem-like characteristics, and it is not until the cells leave the niche that they differentiate so that they can be readily distinguishable from CESCs by expression of α9 integrin or α-enolase.

In this review, we will examine the niche concept, discussing what is known about niches in other tissues and the microenvironment that may make up the CESC niche.

III. THE ADULT STEM CELL NICHE

Adult stem cells lie within a protected area termed the niche. The niche is made up of three components: the stem cells themselves, supporting cells within the mesenchyme, and the extracellular matrix produced by both stem and support cells. Progress in understanding the molecular and cell biology of several of the adult stem cell niches in mammals has been significant over the past several years. Here, after a brief consideration of what we have learned from drosophila, we will discuss the stem cell niche, as it is currently conceived in mammalian skin, intestine, brain, and in the bone marrow.

A. The germinal niche of the fly—role of cell:cell contact via cadherins

In drosophila, the ovary is an excellent model for the study of support cells and growth factors in the niche. Both appear to be necessary for the maintenance of the germ line stem cells (GSCs) and somatic stem cells that are housed in the fly ovarioles. Lying adjacent to the GSCs are the cap cells; these are mesenchymal cells that form a major part of the germinal niche in the ovary. When one GSC divides, one of its two daughters remains associated with a cap cell and the other ends up one cell away from the cap cell. The cell in contact with the cap cell remains a GSC, whereas the other cell becomes committed to forming a new egg (oogenesis). The cap cells express growth factors required for the maintenance of the GSCs, including Decapentaplegic (Dpp), a fly homologue of bone morphogenetic proteins (BMPs) 2 and 4, Wingless, and Hedgehog. Loss of Dpp or any of the proteins downstream of Dpp in its signaling cascade results in loss of GSCs. Conversely, overexpression of Dpp results in an expansion of the GSCs. These data indicate that gradients of Dpp and other growth factors regulate the numbers of GSCs.

Studies of fly development have also shown that the precise arrangement of cells within the niche is regulated in part by the cell:cell adhesion molecule DE-cadherin. DE-cadherin and Armadillo, the fly homologue of β-catenin, are concentrated at the interface between cap cells and the GSCs and are required for the initial localization of the GSCs within the ovariole. β-catenin not only interacts with cadherins in forming the epithelial cell:cell junctions called adherens junctions; it is also a transcription factor that can regulate the expression of a number of genes important in development, including several that control cell polarity and early segmentation in the embryo. When free in the cytoplasm, β-catenin will bind to a complex that forms after activation of the Wnt signaling pathway. Wnts are ligands that activate Wnt receptors and induce proteolytic breakdown of β-catenin. Their name is derived from the wingless phenotype that mutations in these proteins cause in developing flies. While the niche in the fly ovary has provided insight into the role of Wnt-mediated cell:cell adhesion in the maintenance of the GSCs in the ovary and in the role of morphogens in the initial specification of the GSCs, we still do not know the roles played by extracellular matrix proteins within the niche in the fly.

B. Stem cell niches in non-ocular human tissues

1. The epidermal stem cell niche: the bulge

Remarkable progress in understanding the epidermal stem cell (ESC) niche has been fueled by new methods for creating transgenic mice that can be used for lineage analysis. The work of Tumbar and colleagues and of Morris and colleagues has demonstrated that the cells in the bulge region of the hair follicle are, in fact, pluripotent stem cells that can give rise to epidermis, hair, and the epithelial cells that make up the sebaceous glands. Molecular profiles of ESCs isolated by both groups using two different techniques have yielded similar results and show, indeed, that stem cells are largely quiescent and produce distinctive combinations of messenger RNAs (mRNAs), copies of the information carried by a gene. ESCs are CD34+ and express high levels of α6 integrin as well as specific matrix molecules, including the α1 chain for collagen type VI and tenascin-C. As in fly stem cells, growth factors are crucial for maintaining ESCs. mRNAs encoding molecules mediating Wnt-signaling are generally downregulated in the ESCs, but are upregulated when ESCs are stimulated to divide. ESC survival seems to be associated with upregulation of the transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) pathways and down-regulation of bone morphogenetic protein (BMP)-mediated signaling. Regardless of which of these specific signaling pathways are most critical, it is clear that these important cells possess mRNA profiles distinct from their non-stem cell neighbors.

The identity of the support cells in the mesenchyme that are in contact with the ESCs within the niche in the bulge of the hair follicle is not totally clear. One possibility is that they are mesenchymal cells of the dermal papilla. These cells are clearly important in initiating a new wave of hair follicle growth; however, they are not in direct contact with the bulge during all stages in the growth of the hair follicle. A second potential source of support cells is the arrector pili muscle. The basement membrane beneath the ESC at the bulge is produced by both the ESC themselves and the arrector pili muscle cells.

The elegant transmission electron microscopy (TEM) studies of Akiyama and colleagues show the basement membrane underlying cells of the bulge region adjacent to
the cell membrane of a smooth muscle cell of the arrector pili muscle. Bulge epithelial cells have adhesion complexes similar in appearance to hemidesmosomes along their underlying basement membrane zone; immediately beneath their basement membrane is the cell membrane of the smooth muscle cell. Given the fact that α6 integrin, a component of the hemidesmosomes, keeps appearing in molecular profiles as a possible marker for stem cells, perhaps it is the interaction between the basal lamina of the ESC and the smooth muscle cell membrane via specialized hemidesmosomes that keeps these cells within their niche during quiescence.

The possible involvement of muscle in maintaining stem cells is supported by additional, albeit circumstantial, evidence. Tension in the form of mechanical stress has been shown to mediate lineage commitment decisions in mesenchymal cell populations, including conversion of a stem cell into adipocyte or bone; it is possible that tension applied to the ESCs via their direct attachment to the smooth muscle cells of the arrector pili muscle mediates, via integrins, the transmission of survival signals to the ESC. Further, additional evidence from the study of the gastrointestinal niche show clearly that myofibroblasts play central roles in the maintenance of the stem cell niche in the intestine.

In summary, the ESC is a pluripotent cell capable of repopulating the skin, hair, and sebaceous gland compartments. Commitment to form one of its three differentiated lineages involves activation of Wnt signaling, and the maintenance of quiescence likely involves cell-cell and/or cellsubstrate adhesion and signaling pathways involving TGFβ and/or FGF. The unique matrix of the ECS niche is created by the ESCs themselves and surrounding cells in the mesenchyme, including permanent resident smooth muscles cells that make up the arrector pili muscle as well as the cells of the dermal papilla.

2. The gastrointestinal niche: the crypt base

The gastrointestinal (GI) tract includes the small and large intestine and stomach and is comprised of four distinct cell types: columnar cells, mucin-secreting or goblet cells, endocrine cells, and, in the small intestine, Paneth cells, which secrete lysozyme and the antibacterial cryptins. The columnar cells are most abundant; they possess apical microvilli and are termed enterocytes in the small intestine and colonocytes in the colon. The goblet cells produce mucin to facilitate the passage of food along the intestine, and various endocrine cells are present that produce enzymes and peptide hormones. The four distinct cell types that make up the lining of the GI tract are organized into small crypts, which come together to form larger structures called villi. Paneth cells and the neuroendocrine cells are found at the base of the crypts, where the gastrointestinal stem cells (GISCs) are located.

In the stomach, the tissues are organized differently, and the epithelial lining takes the form of long tubular gland-like structures with foveolar, isthmus, neck, and base regions within each gland. Turnover of the gut lining is very rapid, occurring over a 2-7-day period during homeostasis and more rapidly after tissue damage.

Cheng and Leblond first proposed the Unitarian theory of epithelial cell formation in the small intestine over 40 years ago. This theory states that all of the cell types of the small intestine arise from a single precursor cell. Since those original studies, others have shown that the Unitarian theory holds true for the entire surface of the GI tract. The details are best worked out in the small intestine, where the stem cells are known to be located just superior to the Paneth cells at the base of the crypts of Lieberkuhn.

An important tool for the study of epithelial lining cells in the GI tract is the microcolony assay, first developed over 30 years ago. Animals are given a cytopotoxic dose of radiation. Within 4 days, the GISCs within the crypts undergo apoptosis, but their niche can still be identified by the location of the Paneth cells that are resistant to the effects of irradiation. After all the GISCs have died due to irradiation, the empty crypts have been termed crypt ghosts. These types of studies have shown that crypts are clonal and that survival of one cell within a crypt will allow for regeneration of the entire crypt and overlying villus. Additional studies using mouse embryo aggregation assays and transgenic and mutant mice have confirmed these data. The stem cell niche within the crypt is produced by a fenestrated sheet of intestinal subepithelial myofibroblasts (ISEMFs). These cells are unique in that they form a syncytium that extends throughout the entire lamina propria and, thus, is in close contact with blood vessels. This niche remains within the crypt ghosts after the majority of the stem cells have died following radiation treatment. The ISEMFs make a variety of growth factors that are thought to regulate the proliferation and differentiation of the overlying GISCs.

Although the GISCs have been studied for many years, no markers exist, and progress toward identification of their molecular characteristics has been slow. An exciting report was derived from studies of a mouse that expresses a transgene for the diphtheria toxin A fragment in Paneth cells of the small intestine; as a result of this mutation, the mice lack Paneth cells. Despite the loss of Paneth cells at their crypt base, the GISC cell population expands in these mice. Using laser capture microdissection to isolate progenitor cells from normal crypts and from mutant crypts lacking Paneth cells, Stappenbeck and colleagues were able to generate molecular profiles of gut progenitor cells. Similar studies using isolated stomach progenitor cells revealed significant overlaps in gene expression profiles between stomach and intestine. These profiles showed that c-myc signal transduction pathways were activated in the progenitor cells; this likely reflects the high rate of cell differentiation taking place in the gut and represents a major difference between gut stem cells and those of the skin.

Progress has also been made in evaluating the roles of growth factors, including Wnts, in mediating the proliferation and differentiation of cells within the GI tract. Interest in this area is largely due to the large numbers of cancers that...
arise from these highly proliferative cells. It has been shown that Wnt/β-catenin signaling controls epithelial homeostasis; mutations in molecules involved in Wnt-signaling such as APC, Tcf-4, and Fkh-6, lead to tumor formation. Delta is the name given to a family of transmembrane receptors that are expressed on cell surfaces that bind to ligands on adjacent cells called Notch receptors. The interaction of Delta and Notch has been shown in flies and worms to induce changes in cell fate in the cells expressing Notch receptors. Data from the study of GISCs also show that Notch/Delta signaling pathways regulate the differentiation of goblet, Paneth, and enteroendocrine cells.

In summary, in the GI tract, the GISCs are located at the base of the intestinal crypts and a single GISC can give rise to all the cells of the crypt. Despite the availability of good animal models to study this niche, the rapid turnover of the epithelial lining in the gut has made it hard to isolate and identify markers for these cells in vivo or in vitro. While it is clear that crypts are clonal in vivo and that myofibroblasts provide the stromal support for these cells, the direct molecular level interactions that take place within the niche in the GI tract remain elusive. Since the gut lining turns over more rapidly than do the epithelial cells making up the skin or cornea, it is likely that important differences at the molecular level will be found between the GISCs and other more slowly regenerating epithelia.

3. The germinal niche in the adult brain: the subventricular zone and the subgranular zone

Only within the past several years have we begun to think of the adult brain as having stem cells for the production of new neurons. The brain is formed during development from neuroepithelial stem cells in the ventricular zone that gives rise to both neurons and glial cells. Glia are largely considered to be support cells; two important classes of glia in the central nervous system are astrocytes and oligodendrocytes. Oligodendrocytes function to myelinate the axons of neurons, whereas astrocytes have diverse functions. Astrocytes have a small central cell body with numerous star-like extensions rich in intermediate filament proteins, including glial fibrillary acidic protein (GFAP), that make contact with neurons and endothelial cells of adjacent blood vessels.

The two sites for continued generation of neurons from progenitor cells in the adult brain are the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus of the subgranular zone (SGZ) of the hippocampus. Unlike the other adult tissue specific stem cells characterized to date, the adult neuronal stem cells (NSCs) have the morphology of differentiated astrocytes, and they are intermixed at the NSC niche with other astrocytes and with the endothelial cells that make up the vasculature in the SVZ and SGZ. Within these specific sites, astrocytes appear to serve as both niche and stem cell.

In the SVZ, the lineages of the neural progenitors have been determined. The NSCs themselves are called type B cells; these cells are in direct cell:cell contact with all of the other cell types via their long processes. The B cells give rise to both type C cells that act as transit amplifying cells, as well as type A cells that are a committed population of migratory neuroblasts that will migrate to the olfactory bulb to become interneurons. All of these cells lie adjacent to the lumen of the ventricle and sit in close contact with ependymal cells and are surrounded by a basement membrane arising from endothelial cells of adjacent blood vessels.

NSCs isolated from the mouse SVZ and SGZ can be grown in culture as neurospheres; these are round cell aggregates of neural progenitor cells that grow in a relatively undifferentiated state in culture under defined conditions. Methods for differentiating these cells into specific neural and glial lineages have been developed; these methods involve plating neurospheres on adhesive substrates and giving the cells various combinations of growth factors, including FGF2 and EGF. The development of these in vitro methods for manipulating NSCs has led to many important discoveries, including the elucidation of growth factors affecting neurogenesis, e.g., bFGF, insulin-like growth factor 1 (IGF1), TGFα, vascular endothelial growth factor (VEGF), Eph/ephrin, Shh, and others.

As an example, Garcion and colleagues have shown that tenascin-C is an important regulator of stem cell responses to growth factors. Using neurosphere cultures from wild type and tenascin-C knockout mice, these investigators showed that Tenacin-C (Tn-C)-mediated the conversion of the NSCs from an FGF2 responsive state to an epidermal growth factor (EGF) responsive state. In addition, BMP4 inhibited NSCs from acquiring the expression of the EGF receptor. Tn-C knockout NSCs fail to acquire the EGF receptor unless they are provided with an exogenous source of Tn-C; the failure of the Tn-C knockout cells to acquire EGF receptors is associated with changes in the specification of differentiated cell types. The Tn-C null stem cells generate more neurons and fewer glial cells than do normal cells that express Tn-C. Thus, Tn-C is implicated as an important niche component in the SVZ and SGZ in vitro; in vivo in the normal mammalian brain, it is present at both sites.

Other niche components in the SVZ and SGZ are basement membrane proteins and growth factors that are present around the neural progenitors at the niche. The endothelial cells that form the adjacent blood vessels produce many of these, including VEGF. It has been shown that the NSCs express VEGF receptor 2 (VEGFR2), implicating VEGF signaling in the maintenance of the stem cell phenotype in the brain. The ependymal cells release the morphogen noggin, which antagonizes BMP signaling. BMPs cause the NSCs to differentiate along the glial lineage, and, thus, noggin helps keep the stem cells multipotent.

The basement membrane surrounding the niche contains collagen, laminins, and heparan sulfate and chondroitin sulfate proteoglycans in addition to Tn-C. It acts as a reservoir to allow the formation of morphogen gradients along which differentiating neuronal and glial cells migrate and differentiate. In support of this concept,
when pieces of SVZ containing NSCs are transplanted to another region within the SVZ or SGZ, the cells retain their ability to produce neurons. However, when placed at other sites in the brain distal from the SVZ or SGZ, the ability of this tissue to generate new neurons is lost. When NSCs derived from the SGZ are grown briefly in culture and transplanted back into the SGZ, they retain the ability to make interneurons; placed in non-neuronal sites, they become glial cells exclusively.69,70 Thus, the microenvironment at these two sites not only maintains the stem cell compartment, but it also can direct the differentiation of those stem cells.

4. The hematopoietic stem cell niche: the role of the osteoblast

In many ways, we know more about the stem cells that give rise to the formed elements of the blood than we do about all the other adult stem cells combined. This is because of their use clinically in bone marrow transplants to restore the immune system in patients with metastatic diseases of the circulating cells of the blood, including leukemias. In bone marrow transplants, patients are myeloablated; the cells within their own bone marrow are killed, usually by a high dose of radiation. This kills not
only the hematopoietic stem cells (HSCs) that are located in the bone marrow, but it also kills their progeny, including all the cells of the erythroid and myeloid lineages. The patient is then transfused with cells isolated from a matched donor bone marrow and enriched for the HSCs. The HSCs go into the bone marrow from the circulation, a process called homing, adhere to the marrow niche within the marrow stroma, and initiate hematopoiesis. Bone marrow transplants have been performed for over 100 years, and it has been through attempts to improve their efficiency and reduce mortality that advances in the basic understanding of the HSCs have emerged.

The HSCs are CD34+/Lin–/Sca1+/Kit1+ and can be enriched from whole bone marrow aspirates by cell sorting procedures so that the transfused cells contain primarily cells that will be able to engraft successfully. The HSCs can be grown and maintained in culture. Transgenic and knockout mice can be used for bone marrow transplant studies, and recently, two groups of investigators were able to report the repopulation at high efficiency of the entire bone marrow from a single transplanted HSC.71,72

It had been shown early on that the more primitive of the hematopoietic cells first appear after transplant on the endosteal surfaces of the trabecular bone. The cells that HSCs initially interact with were thought at different times to be stromal fibroblasts, myofibroblasts, and/or endothelial cells. The other cell known to be present at the same site, the osteoblast, was largely ignored by those studying hematopoiesis until about 10 years ago when Taichman and Emerson73 showed that osteoblasts supported hematopoiesis and HSC survival. Recent studies have confirmed those early results, using independent techniques. Zhang and colleagues74 and Calvi and colleagues75 both show that the HSCs bind to osteoblasts directly.

Zhang's studies of a mouse with a conditional ablation of BMP receptor 1A (BMPR1A) revealed increased deposition of trabecular bone, increased endosteal surface, and decreased marrow cavity. Surprisingly, these mice had twice as many HSCs in their marrow as are present in mice without the conditional ablation of BMPR1A. Detailed analyses showed that there were more osteoblasts in these mice and, as a result, there were more HSCs bound within the marrow. These investigators went on to show that the interaction between the HSC and the osteoblasts was in part mediated by N-cadherin-mediated adhesion between osteoblasts and HSCs.

Calvi and colleagues75 studied bone formation and hematopoiesis in another mouse model that was overexpressing an activated receptor for parathyroid hormone under the control of the promoter for collagen α1. They confirmed the association between HSCs and osteoblasts and went on to show that Notch-1 was expressed by HSCs and that osteoblasts expressed the Notch1-ligand, Jagged 1, a protein that is member of the Delta family of Notch ligands. Notch-1 signaling was confirmed to play a role in HSC survival in that addition of Notch-1 inhibitors suppressed HSC proliferation.

Yet another study has emerged that adds to our understanding of the complexity of the signaling between osteoblast and HSC. Arai and colleagues76 believe that the osteoblast:HSC interaction is critical for the induction of survival signals that maintain HSCs in a quiescent state within the niche. The quiescent state protects stem cells from depletion in response to proliferative signals. This group showed that HSCs express the tyrosine kinase receptor Tie2 and that they interact with osteoblasts that express the Tie2 ligand angiopoietin-1 (Ang-1). Tie2:Ang-1 interaction induced HSCs to become quiescent.

While these results clear up many questions, they also leave many questions about the HSC niche unanswered. The exact mechanism of homing is still unclear, but integrin α4β1 is clearly implicated in mediating the interaction between the HSC and the niche. Conditionally ablated mice lacking α4β1 in the HSCs accumulate HSCs in the pe-
ripheral circulation; these data and other data from studies in which normal mice were transplanted with HSCs lacking α4β1 confirmed that α4β1 integrin plays an important role in lymphocyte homing.\textsuperscript{77} One α4β1 ligand is Tn-C. Tn-C is abundant within the bone marrow stroma,\textsuperscript{78} and Tn-C null mice have suppressed hematopoiesis that can be restored by the exogenous addition of Tn-C.\textsuperscript{79} Whether osteoblasts or other stromal cells produce Tn-C in the bone marrow is not clear.

Taken together with previous studies, data from several groups of investigators shows that the osteoblast is an important part of the HSC niche. Cell:cell adhesion, mediated in part by cadherins, and transmission of cell:cell signals via Notch-1:Jagged-1 and Tie2:Ang-1 interactions are important in maintaining HSCs within their niche and regulating their proliferation and differentiation. While the ability of HSCs to home to the bone marrow niche is impaired in mice lacking α4β1 integrin, additional molecules on the HSCs themselves and in the stromal matrix are no doubt involved in the regulation of HSC homing to the bone marrow. Understanding how HSCs home to the marrow is important, as tumor metastasis to peripheral organs is, in essence, a type of homing.

In summary, studies on other niches suggest certain common threads in the generation and maintenance of adult stem cell niches. First, the support cells in the niche often appear to be myofibroblasts, smooth muscle cells, or endothelial cells. Second, a gradient of growth factors produced by these cells appears to be crucial in the maintenance of the stem cells. Third, stem cells frequently appear to adhere to a specialized basement membrane secreted by the stem cells and the support cells. In the remainder of this review, we will examine these commonalities in the limbal stem cell niche.

C. The limbal stem cell niche: a unique microenvironment supporting quiescent corneal epithelial stem cells

The CESC within the limbal stem cell niche are localized there due to a combination of anatomical and biochemical events first set up during development. The niche is maintained by a combination of factors intrinsic to the CESC themselves as adult tissue-specific stem cells, as well as by factors present in the environment. These extrinsic factors include molecules produced by nearby vascular endothelial cells, smooth muscle cells that surround those blood vessels, and/or molecules released from those blood vessels. In addition, factors released by the adjacent conjunctival cells and the TACs intermixed with the CESC also likely play a role in the maintenance of the CESC population. A diagram highlighting the roles of intrinsic versus extrinsic factors in specification and maintenance of the CESC is shown in Figure 2. In this section, we present a model for the CESC niche that is based upon research done on the limbus and also draws upon the research done on other adult stem cell niches.

The stroma that underlies the limbal epithelium is unique in that it is positioned to receive signals from at least six distinct cell populations: the overlying 1) corneal and 2) conjunctival epithelia; the 3) corneal and 4) conjunctival stromal fibroblasts; the 5) vascular endothelial cells; and 6) associated smooth muscle cells of the episcleral blood vessels. All of these cells could secrete growth, differentiation, and/or survival factors that accumulate in the limbal stroma and overlying epithelial basement membrane. Distinct from these factors that are the products of resident cells, there is also the additional influence of proteins from serum that could be released at the limbal vasculature. In response to this unique combination of overlapping signals, we propose that a subpopulation of corneal epithelial cells is induced during early eye development to maintain a quiescent phenotype and functioning as CESC. We believe that the same combination of factors that induce corneal epithelial stem cell quiescence establishes and maintains the limbal stem cell niche.

When the CESC are deprived of their niche, they respond to growth and differentiation signals in ways similar to corneal epithelial cells, and they act as early transiently amplifying cells (eTACs), dividing rapidly for several population doublings to generate large numbers of progeny. This is what happens when human CESC are placed into cell culture.\textsuperscript{80,81} Similarly, if the niche is damaged by severe trauma, changes in the availability of growth/differentiation/survival factors along with changes in the environment itself induce the remaining CESC to differentiate into eTACs, resulting in a reduction of CESC at the site of trauma. This is how trauma to the limbus can result in LSCD. In addition, we know that wounds to the central cornea that spare the cells at the limbus, but require proliferation during active cell migration, place the CESC at risk.\textsuperscript{82}

The limbal stem cell niche can be thought of as having two distinct functional sites within it: a quiescent site and a proliferative site. In homeostatic conditions in adult eyes, the balance of signals is shifted toward maintaining the stem cell in its nondifferentiated state, and survival is favored over cell division and cells are quiescent. CESC cell division is considered to be an asymmetric cell division because it gives rise to one cell that is an eTAC and one cell that remains a CESC. During homeostasis, CESC proliferate, but do so infrequently to replace corneal epithelial cells lost in the tear film. Events or diseases that interfere with either the establishment or the maintenance of gradients of growth and survival factors in the basement membrane and stroma that make up the niche would lead to limbal stem cell deficiency (LSCD). In this view, the CESC are formed from corneal epithelial cells that happen to find themselves in the right place exposed to the right factors at the right time. As long as the corneal epithelial cell has not committed to withdraw from the cell cycle, we believe that it could be induced to revert to a CESC if it were placed within the appropriate site. The conjunctival cells and/or their underlying stromal fibroblasts are proposed to secrete factors that inhibit the immigration of corneal epithelial cells into the conjunctiva. CESC, corneal epithelial basal cells, and their underlying
stromal fibroblasts also likely secrete factors that inhibit the migration of conjunctival epithelial cells into the central cornea.

Survival of the CESC in their nondifferentiated state is most likely regulated via mesenchymal cells in the stroma; these cells likely produce survival factors that maintain the CESC as nondifferentiating cells. In the bone marrow, we know that for stem cell replacement therapy to work well, the niche must be empty; the patient is myeloablated. In procedures involving treatments of human corneas with cultured CESC, care should be taken to remove the damaged or nonfunctional CESC and/or conjunctival cells present at that site, which could block engraftment. The limbal area needs to be free of scar tissue and the adjacent conjunctival epithelium, and the grafted corneal epithelial cells must be in close approximation to the underlying stromal fibroblasts.

During migration of corneal epithelial cells after small wounds, there does not appear to be a loss of CESC,82 suggesting that the CESC niche remains quiescent during re-epithelialization after small wounds. Then, after migration is complete, the niche functions to enhance proliferation, and CESC are signaled to divide until the corneal epithelial cell layer is brought back to its normal thickness. Wounds that are large are more of a challenge to the CESC.82 The CESC niche is forced to remain functionally proliferative for an extended time, and the majority of the TACs near the niche are also actively proliferating and migrating away from the niche toward the central cornea. Because the corneal epithelial sheet is migrating at the same time the CESC are proliferating, the proliferating CESC could be swept into the migratory cell population, where they eventually become post-mitotic and terminally differentiate. Conjunctival epithelial cells would then fill the empty stem cell niche. The lack of CESC at this site would allow for the slow movement of conjunctival cells onto the cornea. Furthermore, the occupation of the niche by conjunctival cells physically prevents neighboring TACs from reoccupying that site after cell migration is complete.

While we have used the term factors repeatedly to signify cues that induce cell proliferation or cell survival, direct cell:cell communication and cell:substrate adhesion to matrix proteins also play important roles as environmental cues to direct cells toward specific fates. Some of the factors we refer to above are likely to be cell:cell adhesion molecules and/or matrix ligands rather than survival/growth factors. Figure 3 shows evidence for the differential localization of α9 and β1 integrins and Tn-C in the normal adult mouse limbus. β1 integrin is less abundant at the limbus compared to the central cornea and conjunctiva, whereas α9 integrin is present exclusively within a subpopulation of the limbal basal cells. Despite the overall reduction in the intensity of cells staining for β1 integrin at the limbus, some cells at this site possess abundant β1 integrin and little α9 integrin. When the α9 integrin ligand Tn-C is colocalized with α9 integrin in the adult mouse cornea, we see Tn-C beneath some but not all of the α9 integrin-positive limbal basal cells. A common theme emerges from Figures 1 and 3: the limbal niche consists of a population of corneal epithelial cells each of which express a unique molecular phenotype.

Regardless of specifics, the model presented in Figure 2 is useful in predicting and testing specific hypotheses regarding causes for the development of limbal stem cell deficiency conditions. Data supporting the above model comes from various sources, and we have built it on the strong foundation of research and insights provided by our colleagues.10,12 We know from work on flies and in skin that niches function in maintaining stem cell quiescence and proliferation. Our own studies of corneal wound healing support the idea that the niche itself is not altered in response to smaller corneal epithelial debridement wounds, but is altered after larger wounds.82 The presence of a specific limbal stem cell niche that functions to maintain these cells in a quiescent state where they are communicating differently with their non-stem cell neighbors is supported by: 1) the recent work of Espana and colleagues83 showing that the limbal stroma enhances CESC survival; 2) past work showing that the basement membrane beneath the limbal basal cells was distinct in terms of matrix composition;84-86 3) and our work showing that integrins were differentially expressed at the limbus.87 Data from the brain and gastric niche suggest that mesenchymal cells, including smooth muscle cells and myofibroblasts, secrete factors that are critical for sustaining the niche both functionally and structurally. Extensive work on HSCs has contributed to our assumptions about the importance of niche-emptying for successful engraftment and to the thinking that successful corneal epithelial cell engraftment likely involves a type of homing wherein the CESC express specific integrins and adhere within the niche to specific ligands.

We have incorporated the findings of Ferrais and colleagues88 and Pearton and colleagues89 into our model. These studies show that central corneal epithelial cells from adult rabbit corneas can be reprogrammed to function as ESCs and produce skin and sebaceous gland when grafted, along with embryonic mouse dermal fibroblasts, back into adult mice. If adult central corneal epithelial cells can be made to adopt an ESC fate, surely they can be made to adopt a CESC fate, if provided the right combination of factors. In the experiments by Ferrais et al,88 those factors were provided by dermal fibroblasts isolated from neonatal mice. If we accept that the corneal TACs can be reprogrammed, then, in order to reprogram them to become CESC and not ESC, it is necessary to identify the factor(s) in mesenchymal cells that are needed to accomplish this transition. It is possible that these factors exist only in neonates and are produced by specific cell types at very specific times during development. However, we know that for some patients with LSCD, the cornea can be successfully treated with transplantation of cultures of corneal TACs.81,90,91 So, the CESC can be functionally restored.
from TACs in adults. One challenge ahead is to determine how this happens and to try to increase its frequency of success. To do that, we need to continue looking carefully at the limbal stem cell niche, both at the level of the CESCs and at the stromal elements that are so important in creating and maintaining it. We need to understand how it functions to maintain the ocular surface and what goes wrong in LSCD.

ACKNOWLEDGEMENTS
We appreciate the excellent technical assistance of Ahdeah Pajoohesh-Ganjii, Sonali Pal Ghosh, and Audrey E.K. Hutcheon. Also, we would like to thank past members of the Stepp and Zieske laboratories who have contributed much of the data that forms the basis for the ideas developed in this review. Finally, we owe a huge debt of gratitude to our numerous colleagues who have listened to and fostered our thinking and our science over the years.

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