Spotlighting adult stem cells: advances, pitfalls & challenges

Anna Altshuler[1], Sara A Wickström[2,#], Ruby Shalom-Feuerstein[1,#]

[1]Department of Genetics & Developmental Biology, The Rappaport Faculty of Medicine & Research Institute, Technion Integrated Cancer Center, Technion - Israel Institute of Technology, 31096 Haifa, Israel. [2] Max Planck Institute for Molecular Biomedicine, Muenster, Germany; Stem Cells and Metabolism Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland; Helsinki Institute of Life Science, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; Wihuri Research Institute, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland;. [#]Corresponding authors.

Abstract

The existence of stem cells (SCs) at the tip of the cellular differentiation hierarchy has fascinated the scientific community ever since their discovery in the early 1950s-60s. Despite the remarkable success of the SC theory and the development of SC-based treatments, fundamental features of SCs remain enigmatic. Recently, advanced single cell imaging, lineage tracing and genomic technologies have been applied to capture the life histories and transcriptional signatures of individual cells, leaving SCs much less space to "hide". Here we discuss different concepts and the implications of new paradigms on the SC niche, dynamics and pathology. Finally, we highlight key questions needed to be addressed for better understanding of the biology of SCs and harnessing their potential in regenerative medicine.

Technological advancements have revolutionized the stem cell research

SCs are often viewed as scarce, virtually immortal cells that are situated at the tip of cellular differentiation hierarchy of somatic tissues, providing the cellular material for tissue growth, renewal and repair. As such, SCs have raised interest in diverse fields including developmental biology, aging, disease pathophysiology and regenerative medicine. The traditional paradigm views SCs as rare, infrequently dividing and long-lived cells. Over the years, scientists' attempts to capture these hypothesized cells have run into unexpected

obstacles. Proving the key tenet of the traditional model of SCs being the only somatic cell type that can proliferate/differentiate for lifetime, would require direct demonstration of such behavior of a cell in vivo, currently an unrealistic fantasy experiment. However, the incredible advancement in single cell fate mapping, imaging and genomic technologies, has improved our position in the pursuit of SCs. These advancements provided strong evidence for alternative hypotheses that have revised the concepts on distribution, proliferation and clonal dynamics of SCs. Clearly, the new concepts have profound impact on the nature of the SC niche, mutation accumulation, SC's clonal evolution in aging and pathologies, and design of SC-based therapy. Here, we describe the different paradigms, the significance of SC hypotheses and current challenges.

Traditional methods used to expose stemness

In 1953, Charles Leblond and Yves Clermont described the renewal of spermatogonia as the "*Stem Cell Renewal Theory*", noting "*the reappearance at each cycle of a new dormant cell which acts as the stem cell of spermatocytes*"[1 2]. While adult tissues were considered as static, Leblond and coworkers established nucleotide incorporation assay to uncover the dynamic cellular turnover, the location of proliferative compartments and the existence of cellular heterogeneity within adult tissues[3 4 5]. In the 1960s, McCulloch and Till developed a marrow cell transplantation assay that evaluates the ability of the grafted cells to prevent the death of irradiated mice[6]. Engrafted marrow cells unexpectedly formed macroscopic nodules on the surface of the spleen of the recipient animals[7] (Box 1 and Fig. 1A). They showed that each nodule is derived from a single marrow cell that produces millions cells with histological morphology of undifferentiated and differentiated cells and colony cells could be serially transplanted[8] . Since only ~1 nodule was developed per 10^4 injected marrow cells, they proposed that the colony forming cells (i.e. SCs) are scarce. In line with the new SC model (Fig. 1D), Green and colleagues reported that only rare keratinocytes possess long-term colony formation capacity in vitro (Box1 and Fig. 1B)[9]. They concluded that a SC is the cell of origin of the largest clones (known as "holoclones") while clones with reduced size are derivatives of short-lived progenitor cells (also known as *transient amplifying cells*) that are committed to differentiation. Remarkably, the holoclone culture

has been successfully applied for treating skin burns[10 11], skin gene therapy[12 13] and to cure corneal blindness[14].

The traditional model considers that the dormant SC divide asymmetrically to produce, an identical daughter SC and a short-lived progenitor cell that is committed to differentiate. The latter is undergoes only few cell divisions before it terminal differentiates[15 16 17]. Based on the assumed infrequent division of SCs in vivo, SC identification based on its proliferative index was established. Nucleotide pulse/chase experiments have been extensively applied for identifying infrequently dividing cells ("slow-cycling" cells), as nucleotide label retaining cells (LRCs) which were often believed to represent "true" quiescent SCs (Box1 and Fig. 1C). This methodology led to the identification of discrete population of LRCs in different tissues[18 19 20], their purification, and importantly, revealed new SC populations in the limbus of the cornea[21] and the bulge of the hair follicle[22].

Concerns and controversy

Quiescence is often viewed as a defining feature of SCs and it is hypothesized that by this way SCs minimize the replication-associated mutational burden. Together with clonal fate mapping, identification of LRCs supported a hierarchical model of rare SCs with a unidirectional trajectory of differentiation (Fig. 1D). However, it is not clear whether LRCs possess superior potency over their non-LRCs neighbors, the presumed short-lived progenitors. If SCs are slow-cycling, their molecular should markedly differ from that of their earliest progeny, the fast-dividing progenitor cells which are expected to express substantially higher activity of cell cycle promoting factors and/or metabolism regulators (reviewed in[23]). Disappointingly, however, many of the proposed markers label an apparently "too large" cell population that does not support SC rareness. To avoid over-interpretation, the term "stem/progenitor" cell population has often been used to describe the targeted cell population. None of the proposed markers could faithfully reliably identify LRCs, nor would the surface expression of such a marker guarantee stemness function of an individual cell in traditional clonal assays (e.g. Fig. 1)[24]. This picture did not significantly change even when single cell RNA sequencing has been applied to capture the presumed scarce, slow-cycling SC. On the other hand, a firm correlation between the expression of various "stem/progenitor" cell markers and the probability to "pass" a

stemness test has been demonstrated. For example, repression of specific "stem/progenitor" marker genes frequently led to reduced colony formation, and/or reduced number of LRCs and/or reduced expression of differentiation genes[25 26 27].

Perhaps few of the proposed "stem/progenitor" population represent a rather more uniform cell population? The colony formation and grafting assays provided seminal findings on SC existence and location. These assays may induce stress, due to digestion of cell surface proteins by trypsin protease, stimulation of detachment-induced apoptosis due to keeping cells in suspension, deprivation of niche signals and more. These conditions largely deviate from the environment within intact tissues. This type of limitations have been recognized already by Till & McCulloch, for example, when they stated that "*cells may behave differently in culture from the way they do in the animal*"[6].

Stress or trauma have been shown to trigger drastic changes in cell potency and behavior. For example, upon injury and in contrast to homeostasis, bulge hair follicle SCs heal of the wounded epidermis and may convert into a epidermal-like transcriptional cell state[28 29 30]. Further, although SC differentiation has traditionally been viewed as an irreversible process, cells display significant plasticity in response to stressors (e.g. grafting, injury of growth in vitro). For example, experimental depletion of SCs in the gut[31], hair follicle[32] and corneal[33] epithelia resulted in recovery of the SC pool by differentiated cells that undergo de-differentiation into SC-like cells (reviewed in[34 35]). In the cornea, the dedifferentiation process did not occur if the niche itself was damaged, suggesting the importance of signals from the niche microenvironment in this process[33]. SC niches contain various cell types such as immune cells or fibroblasts that could potentially secrete factors, provide cellular interactions, or modulate the extracellular matrix configuration to control this process. These findings have encouraged the study of SCs under conditions with minimal interference to homeostasis (e.g. genetic lineage tracing, see below), for avoiding unwanted distresses, or if not feasible, conclusions should be drawn cautiously.

The assumption that each cell type (i.e. SC, progenitor, differentiated) has fundamentally distinct proliferation properties predicts a clearly distinct behavior in a colony formation assay[36]. However, colony formation tests do not produce 3 distinct colony types but rather a continuum of variance in colony size/potency. Till & McCulloch that appreciated this problem proposed a stochastic SC model in which cell fate decision between self-renewal

and differentiation is random[37]. In the following decades, the stochastic aspect of their model has been abandoned, however, it has recently regained substantial support (discussed later).

Are the colony forming cells, LRCs indeed "true" SCs? Are these overlapping entities or a distinct populations? Would every single SC effectually form a large and sustainable clone? Could a non-SC, sometimes, form a large colony or be detected as a LRC? What molecular feature distinguishes between a LRC and its neighboring non-LRCs (presumed progenitor cells)? The lack of convincing evidence for the existence of focalized niches that would expectedly be needed to support rare sporadic SCs (Fig. 1D), further challenges the traditional SC dogma.

Genetic lineage tracing experiments provide direct evidence for SC location

These open questions and the concern on stress-inducing assays emphasized the necessity to follow the dynamics of cells under physiological conditions in vivo. An ultimate proof of stemness property of a given cell population is the direct experimental evidence that SCs generate the relevant progeny in vivo, and that this cellular hierarchy is sustainable for the lifetime of the organism. Genetic lineage tracing experiments provided an opportunity to explore cellular hierarchy and dynamics with least interruption to homeostasis. In these experiments, selected cell populations are specifically targeted using a selective promoter. This facilitates Cre-recombinase mediated stable expression of a reporter gene(s) at preferable developmental stage. Fig. 2 illustrates the outcome of typical multi-color cell fate mapping of the basal epidermal cell layer (the K14-positive proliferative compartment). Basal cells are induced to randomly and irreversibly express a specific fluorescent gene. Clonal survival and clonal size distribution can be followed for prolonged period of time post tissue turnover (for review see[38]). Sustainable clones are considered as SC derived ones, providing compelling evidence for the specific anatomical location of SCs. In the gut epithelium, for example, based on LRC assays, the dogma placed SCs at position +4 relative to the crypt bottom whereas terminally differentiated cells occupied positions 1-3. Potten and colleagues had identified LRCs at position +4[39] and demonstrated that these cells were extremely sensitive to radiation[40]. They further proposed that radiation induces loss of damaged SCs that are replaced by progenitor cells, which would have better

repair capacity, and which would fall back into the +4 position while regaining SC properties. However, there was no direct functional evidence for this hypothesis. Thirty years later, Clevers and colleagues performed lineage tracing and revealed that the Lgr5-positive crypt base columnar cells (positions 1-3) generated all gut small intestine epithelial lineages[41], in line with initial proposition by Leblond[3 42]. They proposed that although label retention correctly identified the location of SCs in few tissues (e.g. cornea and hair), these experiments should be interpreted with caution as terminally differentiating cells may also retain DNA label and may, sometimes, persist in tissues for long periods of time.

Another striking example for confusion that arose from traditional SC assays comes from the study of SCs that renew the corneal epithelium (Fig. 3). A long-standing paradigm placed the corneal epithelial SCs exclusively in the limbus, a ring-shaped zone at corneal-conjunctival boundary, whereas the central cornea was viewed as the differentiation compartment. The limbal SC model gained extensive experimental support and efficient limbal SC therapies have been designed to cure blindness. However, an alternative model positioned SCs over the entire corneal epithelium and not the limbus[43 44]. The data was based on 3 classical stemness assays that generated remarkable results although all tests involved stress. First, surgical or thermal depletion on the entire limbus epithelium which was performed to destroy the entire SC population, had no detrimental impact on the cornea that remained healthy and transparent for 4.5 months (Fig. 3A). How could one explain the sustainability of healthy appearance of tissue that lack SCs for many months? Second, genetically labeled transplanted limbus did not contribute to corneal homeostasis (Fig. 3B), suggesting that the limbus does not participate at all in corneal renewal. Third, central corneal epithelial cells displayed high colony formation in vitro (Fig. 3C) and proliferation and wound healing following transplantation in vivo, suggesting for existence of SCs in the cornea.

These evidence contrasted the leading dogma and raised intense debate[45]. However, genetic lineage tracing experiments provided clear evidence for corneal renewal by limbal SCs. Multi-color genetic lineage tracing of basal limbal and corneal epithelial cells, revealed that while most corneal clones extinct within few weeks, sustainable limbal radial clones replenished the corneal epithelium with a limbal-corneal turnover of 4-months (Fig. 3D)[46 47 48]. While these studies confirmed the location of SCs to the limbus, they could not explain

the reported lack pathogenesis following total limbal epithelia (and SC) depletion[43 44]. A potential explanation would be that the limbal epithelium removal was only partial, leaving even few unaffected SC that allow recovery. To address this hypothesis, a live limbal SC reporter transgene gene (K15-GFP) was identified, allowing a more controlled SC while monitoring SC recovery in living animals (Fig. 3E-F)[33]. One day post SC depletion, the limbus was fully healed by RFP-positive corneal committed cells. Strikingly, corneal committed cells became K15-GFP-positive by day 10. In the last decade, accumulating data indicated that differentiation trajectory is not irreversible and that plasticity of differentiated cells and their ability to regenerate experimental SC loss has been elegantly demonstrated in different tissues[31 32 33 34 35] . This emphasizes the notion that although injury, grafting or colony formation tests may be valuable for testing cell potency, these conditions do not mirror resting conditions.

The hematopoietic lineage has traditionally relied on transplantation methodology[7 49 50]. The potential and hierarchy of hematopoietic cell populations can be assessed by following the progeny of stably labelled sorted cells after engraftment. This strategy suggested a model in which "long-term" bone marrow hematopoietic SCs give rise to "short-term" hematopoietic SCs that further undergo commitment to differentiation into all blood lineage cells. While both SC types display full differentiation capacity following grafting, it is only the "long-term" hematopoietic SCs that would rescue the irradiated animal when transplanted. Recently, to avoid the bias of grafting and to validate the hematopoietic SC hypothesis, a number of studies performed genetic lineage tracing. Surprisingly, cell fate mapping using genetic barcoding[51 52 53] or Cre/loxP recombinase[54 55], suggested that although the "long-term" hematopoietic SCs can reconstitute the entire blood lineage following grafting, they only minimally contribute to the murine adult hematopoiesis. Lineage tracing of the putative "short-term" hematopoietic SCs suggested that these cell population sustain hematopoiesis for up to 330 days of the performed tracing[55]. These results led to the proposition of a revised hematopoiesis SC model[56]. If the "short-lived" hematopoietic SCs are really the workhorse renewing population, the function of "long-term" hematopoietic SCs may be to protect genomic integrity. This later role probably not so key in a short-lived organism like mouse, but in a long-lived human, it may be key.

Although powerful, these genetic mouse models also have limitations needed to be considered. For example, the activity of Cre recombinase may induce cleavage at off-target sites that might cause genomic instability, cytotoxic effects, abnormalities in cell cycle and apoptosis, and it is not clear how representative the labeled cell population is from the overall population being studied[57]. Corroborating evidence using Cre-independent methods are needed to ensure that the cells that are being traced do not represent damaged or stress cells. For example, DNA or RNA sequencing technologies can expose the lineage relations and mitotic histories of cells. These approaches can rely on experimental genetic barcoding, or completely without cell labeling, by analyzing naturally occurring genetic mutations by single cell DNA or RNA sequencing[58 59 60 61]. The latter retrospective lineage tracing approaches are also suitable for studying human samples, although they have their limitations as well as it may be that mutations themselves are non-neutral.

Evidence for abundant SCs with unpredictable persistence

Identification of SC location or niches has become relatively straight forward using lineage tracing. However, capturing SC prevalence, heterogeneity and dynamics of tissue regeneration within these niches is more challenging. For example, the question of whether a SC divides symmetrically, asymmetrically or whether these decisions are taken by a stochastic or predetermined manner has not been easy to tackle. It is often assumed that fate is assigned prior or at the time of division, but it could be that all divisions are symmetric but asymmetrical environmental cues push one of the two sibling cells to differentiate. Are these fate-determination processes controlled at the single SC or population level? Addressing these types of questions would ideally involve recording the life histories of a large population of individual SCs (and their progeny) in real time and for prolonged periods of time under unperturbed homeostatic conditions in vivo. While these types of experiments were long unrealistic, there has been recent progress in this area with the development of synthetic genetic recording systems that use engineered mutagenesis to trace clonal histories of cells, hopefully providing answers to these questions in the near future[62 63 64]. Meanwhile, the question has been approached by utilizing mathematical analysis of quantitative lineage tracing data of “snapshots” in individual animals across various tracing time points. As illustrated in Fig. 2 (exemplified

by the skin epidermis), specific promoters have been used for genetically labeling SC and progenitor cells in basal epidermal layer. A SC-derived clone would expectedly expand over time reaching a maximal average clonal size dictated by the tissue turnover rate. According to a simple SC hypothesis (Fig. 2A), the average size of SC-derived clones would remain constant at post turnover periods and the percentage of persisting clones would uncover SC prevalence. In contrast, a labeled progenitor cell is predicted to initially expand but should be gradually be removed through tissue turnover. However, as illustrated in Fig. 2B, the results of lineage tracing experiments performed in different tissues did not fit with these predictions[65 66 67 68 69]. For example, the average clone size was not stabilized over time but rather continuously increased. Heterogeneity in clonal size distribution increased over time, as some clones became larger in size while others shrunk or disappeared. Mathematical analysis implied that these clonal dynamics cannot be explained by the traditional SC model, but are rather consistent with a neutral drift model (Fig. 2C) that considers the existence of abundant equipotent SCs that neutrally compete between each other[65 66 67 69]. In the gut epithelium, long-term lineage tracing showed gradual progression toward monoclonality of the crypt[67]. According to the alternative neutral drift SC model (Fig. 2C), SC fate choice is unpredictable. SCs may be destined to self-renew or differentiate and exit the niche. It seems that the apparent random (refers to unpredictable) clonal behavior in genetic lineage tracing fits well with the reported variability of clones in the original studies of Till and McChuloch[6 7], that proposed a stochastic SC cell fate choice[37].

The traditional SC model considers that invariant asymmetry is needed to maintain the balance between SC and their progeny, a mechanism controlled at the level of single SC division[70]. By contrary, in the alternative neutral drift SC paradigm, asymmetry is achieved at the population level. In other words, a SCs have on average the same probability to self-renew (produce two SCs) or differentiate[71 72] and the unpredictable fate of single SC may be dictated by external, population-level regulation. Finally, the neutral drift SC model explains the difficulty to predict the performance of an individual cell based on marker expression in label retention or colony formation tests. The plasticity and responsiveness of cells to environmental cues fits well with this model.

Capturing the lifetimes of individual SC in their native niche

Mathematical analysis and computerized simulation of SC dynamics commonly requires making certain assumptions that may be incorrect or overly simplified, and assessment of the degree of confidence from such analysis requires specialized expertise. However, analysis of individual SCs by real time imaging provides robust data. A series of studies by Greco and colleagues, have reported the development and use of an extraordinary system for intravital microscopy. Using the skin SC model, they specifically labeled single SC and monitored its lineage development over time by re-visiting the same niche in live mice[32]. By following the fate and lifetimes of many sibling SCs (Fig. 4A), for example, they provided key evidence in support of neutral drift SC paradigm. The traditional SC model assumes asymmetric SC division giving rise to 2 daughter cells with anti-correlated fates/lifetimes, namely, a long-lived self-renewing daughter SC that would remain in the basal layer, and a short-lived progenitor cell that would differentiate, leave the basal layer and shed-off. Live imaging of many sisters' basal cells revealed that fates were either independent (ear epidermis) or positively correlated (paw epidermis), and lifetimes were strongly correlated in both tissues. Light inducible labeling (without Cre-recombinase) showed similar results. Secondly, recording proliferation dynamics of large zones of the epidermis led to the conclusion that all basal cells cycle at comparable rates with no LRCs[73 74], although this topic is controversial (Fig. 4B). The transparency of the cornea also provided an opportunity to follow SC in live and indeed, the new SC model describes well the behavior of two recently identified SC populations in the cornea epithelium[69 75]. A key caveat of live imaging is that it typically constitutes a short-term assay and thus subtle long-term behavioral features might be missed, including existence of subtle proliferative heterogeneity. Thus, the precise underlying rules that govern SC fate require further investigation.

How would a stochastic mode of cell fate decision accurately balance tissue structure, size and efficiently respond to environmental changes? It turns out that the balance between epidermal SC differentiation (and consequent cell loss) and SC proliferation must be linked by a simple, robust mechanism. During development, basal cell division leads to crowding, and a decrease in cortical tension and increased cell–cell adhesion that triggers differentiation and subsequent delamination[76]. By following the lifetimes of individual

adult epidermal SCs in real time, spatial coupling between proliferation and differentiation has been further demonstrated[77] (Fig. 5A). In adulthood, however, it is the differentiation of an individual SC that triggers the symmetric division of the neighboring SC and not vice versa (Fig. 5A). It has been proposed that due to the constant need to expand the embryonic organ, cells favorably divide. But when tissue reaches its final size, the need to compensate cell loss reverses the coordination strategy, and SC differentiation becomes upstream to SC proliferation. A key open question remains in where the information of organ size and final size encoded? The Mesa and Miroshnikova studies suggest that sensing the size of a single cell is a central node of SC fate decision making on whether to divide or to commit to differentiation. In addition, local topological cues such as collagen fiber orientation might guide the behavior of SCs[78]. However, given that cell fate commitment in the adult epidermis occurs 1-2 rounds of cell division prior to delamination[79], it is unlikely that a single parameter such as mechanics regulates this process, and chemical signaling most likely plays a role. An interesting mechanism of regulation of SC pool size came from the murine testis. A communication mechanism of "quorum sensing" allows taking decisions at a population level maintaining the balance between SC self-renewal and differentiation by resource availability in the niche[80]. In the future, it will be important to understand which factors dictate the balance of SC fate decisions in different tissues and what are the functional implications of specific regulatory principles. For example, how and why a tissue switches from embryonic mode of coordinated proliferation/differentiation to the adult coupling of differentiation/proliferation, what is the relevance of these finding to SC related pathologies like cancer and others are important open questions to address.

Dynamics of SC competition, mutations and clonal evolution

Lgr5+ intestinal SCs divide once a day, generating all intestinal epithelial cell types, replacing the tissue with a turnover of 4 days. By random genetic labeling of individual SCs with one out of four fluorescent reporter genes, the fate of single SCs was traced over time. Interestingly, a drift to monoclonality of crypts was observed[67] suggesting that SC clonal evolution may play a role in the life of the organism. The few SC may persist by chance ("winners") while the others that randomly fated to differentiation ("losers"). However, SCs with specific phenotypic advantages (e.g. superior adhesion, enhanced

proliferation rate) may out compete their less fit SC neighbors. This phenomenon of cell competition based on differential fitness of cells within a seemingly homogenous population was initially been demonstrated in flies, but has more recently also been documented in mammalian development[81 82 83 84 85]. Theoretically, mutations that reduce SC fitness or induce SC loss would be compensated by expansion of neighboring SCs. Interestingly, assuming that a SC that acquires a mutation is surrounded by many non-mutated SCs, it will likely be stochastically replaced by abundant neighboring healthy SCs (Fig. 5B). This view suggest that cell competition may be beneficial to the organism, providing a defense against abnormal development or neoplastic cell transformation[86 87]. However, upon gaining a somatic "super competitor" mutation, the rare mutated SC would outcompete abundant wild type SCs and expand its proportion in the niche, a phenomenon observed following experimental exposure to a carcinogen[88 89]. Indeed, mosaicism and clonal mutations have been reported in apparently healthy tissues[90]. During aging, the clones that acquire mutations in common cancer driver genes may become "winners" and dominant in the tissue, according to genetic drift rules. Such phenomenon can eventually lead to cancer. Indeed, the esophageal epithelium may be largely enriched with mutant clones in aging[91]. Frequently occurring mutations that lead to colon cancer (Apc, Kras and P53 mutations) conferred a competitive advantage, yet limited, to SC in the mouse intestine[92]. Mathematical analysis of lineage-tracing data suggested that the balanced stochastic fate of squamous SCs is could partially be a cell-intrinsic property, but the molecular machinery that achieves this is unknown[65 93].

What are the mechanisms that safeguard the genomic integrity of abundant actively cycling SCs? Kato et al[94] demonstrated that clearance of mutated SCs is caused by selective differentiation and delamination through the DNA damage response-p53-Notch/p21 axis, with the downregulation of Integrin β1. Strikingly, concomitant to SC elimination was the symmetric divisions of nearby SCs. While this mechanism brings important insight into clearing dangerous SCs, it opens the question of a possible failure in this removal mechanism in aging or in pathology.

Notably, the tissue architecture and segregation of SCs and differentiated cells into spatially distinct compartments in some tissues may provide a barrier for expansion of mutated SC. For example, gut epithelial SCs reside in glandular crypt and they generate

differentiated cells that progress upwards along the villus. The differentiation compartments, therefore, provide a physical barrier needed to be overridden by the mutated SC to invade the neighboring villi. Altogether, the paradigm of abundant SCs with stochastic competition have implications for dynamics of tissue development, homeostasis and pathology.

Most of the abovementioned studies were performed on mouse models. Evidence for human epidermal SC clonal dynamics came from a pioneering transgenic SC therapy[12]. Patient's isolated keratinocytes were retro-virally transduced to restore LAMB gene expression. The transgenic keratinocytes were expanded in culture before their engraftment to replace some 80% of the epidermis. They report that transgenic cells were maintained in vivo and epidermal renewal did not cause clonal selection. To illuminate SC clonal dynamics in human, authors followed the genetic traces of viral integration. They proposed that the human epidermis is sustained by a limited number of rare long-lived SCs. Of note, as discussed above, keratinocyte expansion in culture typically results in generation of diverse clones with a variety of sizes. Therefore, these results could also be explained by early variation in ex vivo expansion of keratinocytes leading to non-uniform clone sizes. In other words, larger clones would be more durable and possess a long-term survival advantage even when competition is neutral. In the future the cell lineage trajectory of the human epidermis needs investigation through methods that avoid in vitro cell expansion (e.g. by DNA sequencing of native unperturbed epidermal cells)[95]. In the absence of conclusive evidence, the hierarchal model may thus still be relevant to describe the homeostasis human epidermis.

The implication of the new SC model on the niche

Schofield and colleagues proposed that the niche, the local microenvironment of SCs, dictates their behavior[96]. While much focus has been given to uncovering the identity and behavior of SCs themselves, our understanding of niche – SC regulation is relatively poor. Consequently, primary cultures lack critical niche factors, cell types that provide essential reciprocal signaling, and SC expansion ex vivo is very limited. Therefore, in vivo studies remain the golden standard for studies on SC – niche interactions.

How will the revision of the SC hypothesis impact the view of the SC niche? The traditional assays that have been used to support the hierarchical SC paradigm are based on the anticipation that the innate potency of cells would be preserved independently of even drastic alterations in the extrinsic environment and signals (e.g. when assaying cell potency in vitro or by grafting). This model often considers a fixed road of unidirectional cell differentiation and genetically hard-wired cell states. Although a specific differentiation trajectory is likely the dominating process during homeostasis, a reversal of this process referred as dedifferentiation or reprogramming certainly occurs under stress, and potentially to some extent even under homeostasis. Emerging data assigns a central role for the niche in regulating the directionality of transition between cell states. This notion is supported by the observed adaptability of differentiated cells to changes in their local environment, which reflects their unexpected sensitivity to changing tissue demands[97 32 33 34 98].The plasticity and responsiveness of SC and differentiated cells to their surroundings implies that there may also be dynamic, in addition to fixed, interactions between SCs and their niche. Under homeostasis, fluctuation in the size of the niche or the SC pool may be counterbalanced by feedback mechanisms, such as coupling cell fate decisions to cell density, cell size or tissue tension[76 77 99]. On the other hand, following injury, one could hypothesize that the niches gain new properties, allowing them to promote reprogramming of differentiated cells into SC-like cells (Fig. 3F). Additionally, it will be interesting to uncover the role of the niche in pathology. Aging or mutations may perturb the niche "field" and in turn dysregulate the SC pool[100 101 102 103]. Importantly, the proposed abundance of SCs suggests that the niche is not a focalized zone but rather large in size (Fig. 2C).

These considerations may be of relevance for the design of functional studies of niche factors. The divergence of niche elements includes unique niche cells, soluble factors, extracellular matrix proteins, metabolites, biophysical properties and more[104 105 100 106 107]. Up to date, our knowledge on function of niche factors is relatively limited, but numerous studies have indicated niche-resident cells as important sources for growth factors and cytokines. For example, specific depletion of Kit ligand or CXCL12 in endothelial bone marrow niche cells decreased the SC pool[108 108 109 110]. VEGFR2 expressing sinusoidal endothelial cells have been shown to be necessary for stem/progenitor cell function

following engraftment post irradiation[110] whereas endothelial cells enhanced the engraftment efficiency[111], indicating that endothelial cells play an important role as niche cells. In epithelial tissues that are exposed to the external environment, immune cells may possess dual role in both immunity as well as SC niche. In the gut model, myofibroblasts and muscularis mucosae cells surrounding the crypt base secrete Bmp inhibitors (gremlin1-2, chordin-like 1) to maintain stemness[112]. In the differentiation compartment, stromal lamina propria niche cells produce Bmp4 to antagonizes Wnt signaling, inhibiting SC expansion and promoting cell differentiation[113]. Interactions between intestinal SCs and T helper subsets modulate SC fate decisions whereas regulatory T cells secret cytokines to support SC renewal[114]. In the limbus, the niche for SCs that renew the corneal epithelium, copious immune cells have been specifically identified. The inhibition or deficiency of immune cells resulted abnormal SC hallmarks[69]. Also the extracellular matrix provides important signals, as for example in the skin, the laminin composition of the basement membrane is critical for regulating the state of the hair follicle SC function[115]. Interestingly, SCs can serve as a niche for their progeny. In the airway epithelium, basal SCs continuously supply Notch ligand to their daughter secretory cells. This signal is essential for secretory cell fate maintenance[116]. Therefore, bidirectional signals between SCs and their progeny may orchestrate the fidelity and balance between cell states within a given lineage. Collectively, deeper characterization of the niche – SC crosstalk under homeostasis, repair, aging and in pathology is instrumental for developing new therapies.

Concluding remarks

Much of the effort in the SC field has been focused on studying specific tissue models. It is not clear, however, if the conclusions made in a given tissue can be generalized. Is there a universal SC model, or perhaps tissue-specific dogmas apply? Future experiments should address this question by exploring the traits of different tissue-specific SCs. It is reasonable to assume that each tissue in our body has evolved specialized SC regulatory strategies compatible with their specialized functions and distinct sources of stress. However, the questions of how tissue-specific SCs deal with these type of challenges is poorly understood[117 118 119]. For example, extensive SC activity is potentially required for frequent cell replacement in harsh environments (e.g. intestinal lumen, skin surface) enriched with toxicants, microorganisms or exposed to physical injury. The transparent cornea is very

proliferative and constantly exposed to ultraviolet irradiation. Specific physiological conditions probably influence the wiring of the regenerative strategy for the particular tissue. For example, localizing the SCs at the most well-protected zone (e.g. protected from toxicants, irradiation, supported by nutrients etc), accrediting SCs with special protection from DNA damage (e.g. pigment, radiation absorbing molecules), efficient DNA repair mechanisms or escape mechanisms (e.g. death or differentiation).

There may be trade-offs between having a quiescent and/or an active SC population. Muscle or brain SCs function in tissues that exhibit little cell replacement while the hair follicle cycles between phases of organ growth, regression and quiescence. The benefits of quiescence may be paramount and include reduced biochemical damage associated with the production of toxic agents or biosynthesis of macromolecules, and minimized accumulation of disease-causing mutations. The quiescent state of SCs is accompanied by reduced metabolic activity, transcription and translation[120]. These biological processes have been associated with molecular damage due to the production of toxic agents or errors in biosynthesis of macromolecules[121 118] whereas attenuation of these activities delayed SC aging[122]. Interestingly, although the reduced division rates decrease prevalence of replication associated mutations, quiescent SC preferentially employ an error-prone mechanism of non-homologous end joining to repair their DNA[123 124]. In contrast, frequently dividing SCs favorably engage a non-error prone mechanism of homologous recombination for repairing DNA breaks[125], and frequent division rate was shown to be required for the activation of repair pathways[126].

The discovery of SCs and their research was full of promises, surprises and challenges. Recently, key SC properties have been revealed, many decades after the SC theory was first formulated. This ongoing progress will provide the fundament to uncover the involvement of SCs in aging and pathology. However, to fulfil the promise of SC in regenerative medicine it will be necessary to re-focus some of the efforts of SC tracing from murine models to human biology. From the practical standpoint of regenerative medicine, understanding of the niche – SC crosstalk in human models would facilitate efficient expansion of human SCs, potentially allowing reconstruction of functional tissues[127 128 129] while allowing genome editing of mutated SCs[11 12].

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Figure Legend

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