



Advancing insights into stem cell niche complexities with next-generation technologies

Nicholas Heitman^{1,2,4,5}, Nivedita Saxena^{1,2,4,5} and Michael Rendl^{1,2,3,4}

Adult tissue-specific stem cells are essential for homeostatic tissue maintenance and key to regeneration during injury repair or disease. Many critical stem cell functions rely on the presence of well-timed cues from the microenvironment or niche, which includes a diverse range of components, including neuronal, circulating and extracellular matrix inputs as well as an array of neighboring niche cells directly interacting with the stem cells. However, studies of stem cells and their niche have been challenging due to the complexity of adult stem cell functions, their intrinsic controls and the multiple regulatory niche components. Here, we review recent major advances in our understanding of the complex interplay between stem cells and their niche that were enabled by the tremendous technological leaps in single-cell transcriptome analyses, 3D *in vitro* cultures and 4D *in vivo* microscopy of stem cell niches.

Addresses

¹ Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, Atran Building AB7-10C, Box 1020, 1428 Madison Ave, New York, NY 10029, USA

² Department of Cell, Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, Atran Building AB7-10C, Box 1020, 1428 Madison Ave, New York, NY 10029, USA

³ Department of Dermatology, Icahn School of Medicine at Mount Sinai, Box 1047, One Gustave L. Levy Place, New York, NY 10029, USA

⁴ Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, Box 1022, One Gustave L. Levy Place, New York, NY 10029, USA

Corresponding author: Rendl, Michael (michael.rendl@mssm.edu)

⁵ Both authors contributed equally to this work.

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Introduction

Each day, an adult human generates billions of new cells to replace those that are lost naturally or by damage. At the top of the production hierarchies are adult stem cells (SCs), defined by their abilities for long-term self-renewal and

multipotent differentiation into several different lineage-restricted cell types. The first definitive proof of the existence of adult SCs came from work by Till, McCulloch and others in the 1960s demonstrating the existence of a hematopoietic SC (HSC) pool responsible for maintaining the entire blood-lineage throughout life [1–3]. Since then, multiple adult SCs have been discovered in several tissues and organs, such as the intestine [4,5], brain [6,7], mammary gland [8,9], and skin [10] including hair follicles [11–13].

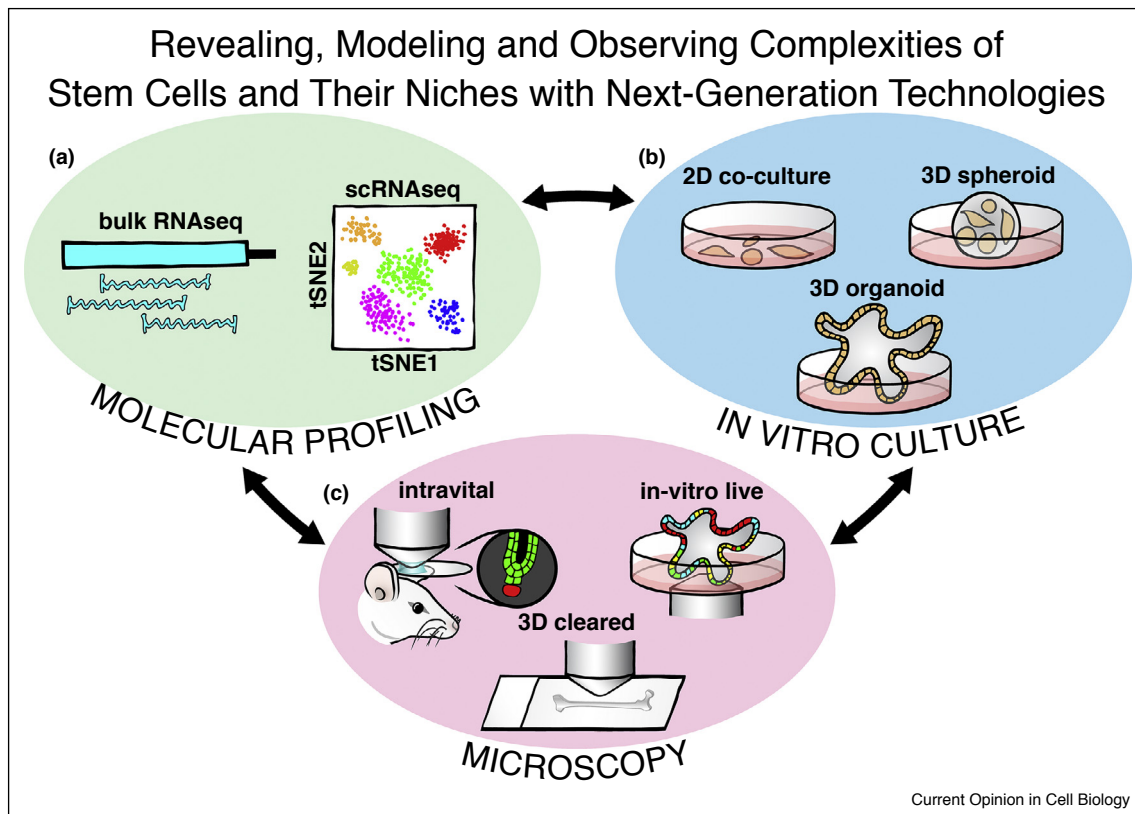
Although many of the special characteristics of SCs are intrinsic, no completely autonomous adult SC has been discovered. Rather, all known adult SCs rely to a large extent for proper function on external signals from its surroundings, termed the SC niche [14–16]. The niche communicates vital information regarding the regenerative needs of the tissue, the importance of which is exemplified by the detrimental effects of deviations from the crucial loss/production balance, as defects of SCs and their niches have been implicated in multiple human disorders and diseases [17].

The HSC sits atop a hierarchy of fate-committed multipotent progenitors (MPP) and terminally differentiated cells of the entire blood-lineage [18–20]. Self-renewal of HSCs and lineage-committed progenitors and their differentiation towards diverse blood lineages are regulated by multiple niche inputs from non-hematopoietic cell types, such as osteoblasts [21,22], peri-sinusoidal [23,24] and peri-arteriolar stromal cells [25], and endothelial cells [23,26], as well as from hematopoietic lineages, such as macrophages [27–29] and megakaryocytes [30].

Hair follicle SCs (HFSC) and downstream progenitors give rise to seven cell lineages that make up the hair shaft and its supporting channel during hair growth, a process that is interrupted by a naturally occurring hair cycle of cyclical bouts of follicle destruction, a resting phase of relative quiescence, and re-growth [11–13,31]. Distinct niche signals controlling the balance of SC rest and activation are thought to emanate from essential niche components that include specialized mesenchymal dermal papilla cells [32–35], direct SC progeny of multipotent progenitors [36,37] and neighboring nerves [38], as well as longer-range inputs from fibroblasts deep in the dermis [39], cells of the dermal adipocyte-lineage [40], and immune cells [41–43].

Intestinal SCs (ISC) residing in the intestinal crypt base constantly replenish the villus epithelium of rapidly

Figure 1



Next-generation technologies, often used in combination or complementation, advance insights into SC niche complexities. **(a)** Molecular profiling by population-based RNA sequencing and by single-cell RNA sequencing reveal unprecedented level of gene expression complexities and cell heterogeneity in SCs and their niches. **(b)** *In vitro* cultures with 2D engineered matrices and co-culture, 3D spheroid aggregates, and structured 3D organoids enable modeling of SC interactions with their niches. **(c)** 3D multicolor light microscopy of 3D cleared tissues and 4D *intravital* and *in vitro* live imaging allow observation of SC niche complexities *in vivo* and in real time.

turned-over enterocytes, goblet and enteroendocrine cells, and other SC progeny that are lost by conveyor belt-like upward displacement [4,5,44]. The intestinal regulatory niche contains pericryptal mesenchymal fibroblasts [45,46], myofibroblasts and smooth muscle cells [47,48], as well as gut lumen microbiota [49,50]. Paneth cells, the only differentiated SC progeny that migrate to the crypt base, also provide regulatory niche signals to ISCs [51–53].

In this review we highlight recent major discoveries into the SC regulation by the niche that were made possible by groundbreaking new technological innovations of single cell-level profiling, complex cell and organoid cultures and major advances in light microscopy (Figure 1). As we are not able to cover the entire extensive body of new knowledge gained in the SC field within the past few years, we refer to excellent recent reviews containing comprehensive updates in several SC niche systems [54–58].

Revealing complexity: single-cell profiling of organs

Precise regulation of gene expression in SCs and their niche is paramount for executing the molecular programs of SC quiescence, self-renewal and lineage differentiation. Specific sets of expressed genes and epigenetic configurations underlie functional distinctions between different cell types within complex tissues, including SCs and the cellular niche components. Since large-scale transcriptome analysis became technically feasible with the establishment of microarrays in *Arabidopsis* [59], it has been used with great impact as a window into SC and niche-specific properties and as a basis for discovering targets for functional studies in multiple SC niche systems [11,12,60–62]. Since then, technologies committed to monitoring the transcriptome of cells, as surrogate for protein expression, have flourished. RNA-sequencing (RNAseq) was established in rapid succession in *Arabidopsis* [63], yeast [64] and mammalian cells [65] that surveys mRNA content in a manner that is relatively

unbiased when compared to microarrays and with superior sensitivity [65,66], and was quickly utilized to analyze transcriptional patterns in mammalian cells, including SCs [67–69]. Purification of SCs and the diverse cell types of their niche through cell sorting for transcriptomic analysis of bulk populations by RNAseq enabled sensitive detection of gene expression, revealing their molecular identities with superior resolution and identifying the expression of ligand–receptor pairs between the SCs and their niche [70*,71]. Although the sensitivity of this approach provides highly detailed molecular descriptions of the cell types of interest, the data cannot resolve subtle heterogeneity within populations and detect the existence of rare subtypes. Immunofluorescence, flow cytometry, and mass cytometry [72] can enable deeper investigation of heterogeneity by interrogating single cells but are often limited by availability of detectors and/or antibodies.

With the advent of single-cell transcriptome analysis the transcriptional profiling power of RNAseq can be combined with the ability to interrogate single cells [73]. Single-cell RNA-sequencing (scRNAseq) and developed analysis algorithms that compress high-dimensional data into two or three dimensions, like t-stochastic neighbor embedding [74] or principle component analysis, allow for efficient identification of heterogeneous cell subtypes. In addition, pseudotime [75] and FateID [76] algorithms enable prediction of differentiation trajectories and reveal stepwise transcriptional changes as cell fates are determined. As a result, SCs, niche cells and other cell types previously considered as relatively homogenous have been shown to be remarkably complex and heterogeneous, pointing to a diversity of unique cellular functions and processes. In the epidermis and hair follicle, pseudotime and pseudospace, a closely related algorithm predictive of spatial localization, were used in tandem to construct a map of HFSC differentiation and discover changes in expression of key signaling, extracellular matrix, and cell adhesion components [77]. A more recent study by Fuchs and colleagues identified heterogeneity amongst HF progenitors, and cognate heterogeneity in the dermal papilla, forming ‘micro-niches’ along the epithelial–mesenchymal interface [37**]. Similar efforts have enabled the discovery of new, rare cell types in both intestinal organoids and the endogenous tissue, as well as heterogeneity amongst ISCs [78,79**]. Other studies have combined scRNAseq with *in vivo* and *in vitro* observations of cells, to link transcriptome data with SC quiescence. Unique molecular profiles of isolated single HSCs and progenitors were associated with divisional history in culture, and identified niche components necessary for maintenance of dormancy [80*]. Additional scRNAseq studies to identify cell-intrinsic factors regulating HSC quiescence revealed retinoic acid signaling as crucial for dormancy transcriptional programs [81]. Heterogeneity amongst niche components revealed the differential

capacity to maintain adult SCs. Stromal osteolineage cells co-transplanted with HSCs/progenitors revealed distinct transcriptional signatures of these cells proximal and distal to engrafted HSCs/progenitors, and identified novel niche factors regulating HSC quiescence [82*]. Overall, scRNAseq has revealed previously unknown complex spatial and temporal heterogeneity of both SCs and niche components and promises future discovery of key effectors of SC quiescence, maintenance and differentiation, as well as additions to the wide array of niche cell types and functions.

Modeling complexity: 2D co-cultures and 3D tissue-recapitulating organoids

Two-dimensional flat cell culture systems have long been used to model the cell relationships within tissues, while enabling relatively straightforward experimental manipulation. The simplicity of 2D cultures enables functional interrogation of identified targets — including those from scRNAseq studies — in the SC niche for quiescence and differentiation programs. Critical niche components for maintaining murine HSCs have been identified in 2D culture using engineered hydrogel microwells that displayed scRNAseq-identified adhesion receptors JamC and Esam [80*]. In similar assays, differentiation of T-lymphocytes from both murine and human HSCs was regulated by fibronectin-immobilized VCAM-1 and Delta-like 4 [83].

Whereas flat cultures lack the cellular and spatial complexity of tissues, three-dimensional cultures have made great strides to recapitulate elements of *in vivo* spatial and structural organization by generating 3D structures out of biomaterials or by seeding cells within them. Seeded murine or human mesenchymal SCs onto 3D silk scaffolds generated a model for bone marrow adipose tissue (BMAT) and its bidirectional relationship with myeloma cell lines and for exploring BMAT-associated homeostatic and disease processes in the bone marrow [84*]. Another recent study showed that 3D epidermal and dermal clusters from young mouse back skin had the ability to self-organize and induce hair follicle formation. This capacity was lost in 3D clusters from old skin but could be rescued by addition of matrix metalloproteinases and other cytokines to the culture [85*].

While 3D cultures require artificial engineering, organoids, which can be generated from a single pluripotent or adult SC, have inherent self-organizing capabilities — reflective of *in vivo* cell rearrangement — thus enabling simultaneous study of structure and function [86]. Organoids have great utility in screening genes and drug compounds but have also been used extensively in evaluating the ‘stemness’ of adult SCs. Early work showed that mouse and human mammary epithelial SCs are capable of forming spheroids in culture, permitting efficient mammosphere production [87,88]. Many other

organ systems have since established organoid protocols, including brain organoids derived from mouse and human induced pluripotent SCs [89,90]. A protocol for generating murine intestinal organoids was first described by the Clevers lab, in which a single ISC could differentiate into distinct crypt-villi structures of solely epithelial lineages [91]. Intestinal organoids have since been used to study many aspects of ISC and niche biology, utilizing both murine and human organoid systems, including coordinate metabolic requirements of ISCs and differentiated progeny [92] and heterogeneity of differentiation programs [93]. Gene manipulation using CRISPR has also been effectively utilized in intestinal organoids; multi-genic knockout of Wnt components in organoids demonstrated their requirement for organoid maintenance [94], and deletions of DNA repair-associated genes were made in ISCs to identify which of these genes can recapitulate mutational signatures often found in human colon cancers [95]. Supplementation of intestinal epithelial organoids with niche components mimicked elements of *in vivo* SC interactions with the niche; addition of intestinal stromal cells to intestinal organoid cultures has elucidated key secreted factors from the gut mesenchyme for SC maintenance [96]. Recently, the Koehler lab defined a protocol for generating murine skin organoids from pluripotent SCs, comprising of both epithelial and mesenchymal cell types, capable of making hair, sebaceous glands, and other accessory structures [97]. In this system, HFSC precursors co-develop with dermal papilla niche precursors, reminiscent of *in vivo* HF morphogenesis. Although starting as a system to evaluate SC characteristics, organoid protocols have been adapted to tractably explore complex intercellular relationships, including SC interactions with their niche.

Observing complexity: advances in *in situ*, *intravital* and *ex vivo* imaging

New techniques in light microscopy have begun to reveal the 3D spatial complexities of SC niches to complement the heterogeneity and rare populations identified by single-cell gene expression profiling. Through the use of multiplexed immunofluorescence with optical tissue clearing for extended imaging depths, Schroeder and colleagues were able to describe the spatial organization of the bone marrow HSC niche within an entire rodent femur [98]. With the advantages of more sensitive and quantitative measures of gene expression compared to immunofluorescence, single molecule fluorescence *in situ* hybridization (FISH) on intestinal crypts enabled expression analysis of several SC markers in spatially distinct ISCs and the verification of rare lineages at single-transcript resolution [78,99]. Multiplexed error-robust FISH made further improvements by enabling detection of up to 1000 distinct mRNA targets within single cells and tissues greatly expanding the power of this technique to visualize the spatial complexities of gene expression within tissues including SC niches [100,101].

The ability to observe living cells in real time can provide a myriad of information of cellular dynamics often not easily captured by the snapshot methods that freeze cells and their molecular machinery in time, but imaging living tissue with traditional widefield and confocal microscopy has been challenging due to light scattering. Multiphoton (MP) microscopy with infrared light greatly expands tissue imaging depths and has enabled real-time observation of live SCs in their native environments. MP imaging of surgically exposed calvariae first provided short-term live glimpses of the cellular dynamics during HSC homing to the bone marrow niche [102,103]. Shortly after, Van Rheenen and colleagues visualized intestine with MP imaging through an implanted abdominal imaging window for lineage tracing of ISCs over multiple days. This uncovered a passive displacement of crypt base columnar SCs in the upper niche, which were functionally replaced by ISC progeny from the lower crypt base indicating a positional competitive advantage and an uncoupling of SC fate decisions from division [104].

Recently the Greco lab has utilized MP imaging to great effect in studying the dynamics of epidermal and HFSCs in their natural state in living mice [33,105,106]. Since the skin and hair are easily accessible for imaging and do not require surgical preparation, the same field and even individual hair follicles could be re-identified with the guidance of visible landmarks such as blood vessel patterns and tattooed dots and tracked over a span of many months in revisit experiments [107]. This enabled the discovery of a precise spatial organization of SC progeny divisions and coordinated cell movements that control the rapid expansion of growing hair follicles [105], and revealed the control of SC fate by the cell position within the niche [33]. In addition to deep tissue imaging, the infrared laser was repurposed to heat-ablate cells with surgical precision and its effects were observed immediately after or over a longer time span. Laser ablation of HFSCs or niche components of individual follicles and later revisits using adjacent unperturbed follicles as a built-in control uncovered a plasticity between different HFSC domains and demonstrated an essential role of dermal papilla niche cells for control of hair regression and HFSC activation during the hair cycle [105,108].

The combined approach of utilizing live imaging on organoid cultures further alleviates the drawbacks of studying fixed tissues, which lack real-time visualization of intra-cellular and intercellular dynamics, or of 2D cultures that do not necessarily recapitulate the complexities of *in vivo* tissues, while adding the great advantage of scalability for high-throughput assays. For example, through live imaging of intestinal organoids and the use of Ca^{2+} -activated fluorescent reporters Julius and co-workers recently identified enterochromaffin cells as responding to neural inputs and relaying information about luminal metabolites to the enteric nervous system via synapses [93]. Another recent study showed a

Figure 2

Highlights of recent SC niche papers utilizing next-generation technologies				
System	Modality	Method	Highlights	Reference
Blood	Molecular profiling	scRNAseq	MPP heterogeneity, fate bias	[76] Herman et al. 2018
		scRNAseq	HSC-maintenance niche factors	[80*] Roch et al. 2017
		scRNAseq	HSC quiescence/activation	[81] Cabezas et al. 2017
		scRNAseq	HSC niche spatial heterogeneity	[82*] Silberstein et al. 2016
		RNAseq	HSC/MPP gene regulation/expression atlas	[71] Cabezas-Wallscheid et al. 2014
	Cell culture	Engineered ECM	HSC to T-cell directed differentiation in-vitro	[83] Shukla et al. 2017
	Microscopy	Cleared multicolor	Whole femur BM imaging cytometry	[98] Coutu et al. 2017
Intestine	Microscopy	Intravital multiphoton	HSC homing dynamics to calvaria BM	[103] Lo Celso et al. 2009
		scRNAseq	ISC + lineages heterogeneity, rare cell identification	[78] Grün et al. 2015
	Cell culture	Organoid	ISC metabolic identity, niche-supplied metabolites	[92*] Rodríguez-Colman et al. 2017
		Organoid	Human ISC tumorigenesis	[95] Drost et al. 2017
		Live organoid	ISC lineages heterogeneity	[93] Bellono et al. 2017
Skin/Hair	Microscopy	Intravital multiphoton	ISC spatial heterogeneity, lineage tracing	[104] Ritsma et al. 2014
		scRNAseq	HFSC/progenitor + micro-niche spatial heterogeneity	[37**] Yang et al. 2017
		scRNAseq	Epidermal/HFSC + lineages spatial heterogeneity	[77] Joost et al. 2016
	Cell culture	RNAseq	HFSC/progenitor + niche transcriptome atlas	[70*] Rezza et al. 2016
		Organoid	Pluripotent SC-derived skin + appendages	[97**] Lee et al. 2018
		3D spheroid	Dermal progenitor self-organization dynamics	[85*] Lei et al. 2017
	Microscopy	Intravital multiphoton	Epidermal SC lineage tracing/clonal analysis	[106**] Rompolas et al. 2016
		Intravital multiphoton	HF epithelial efferocytosis, DP niche laser ablation	[108] Mesa et al. 2015
		Intravital multiphoton	Epidermal/HFSC plasticity, fate determination	[33] Rompolas et al. 2013
		Intravital multiphoton	HFSC/progeny dynamics, DP niche laser ablation	[105] Rompolas et al. 2012

Current Opinion in Cell Biology

Recent insights into hematopoietic, intestinal and skin/hair follicle SCs and their niches by new technologies. Major new advances from recent papers using new technologies are grouped by SC niche system and technology.

distinct heterogeneity in oxygenation between intestinal organoids through live-imaging using a phosphorescent O_2 probe [109]. Finally, the ability to generate organoids from human SCs for *in vitro* live imaging combined with the efficiency of generating fluorescent reporter lines with CRISPR-Cas9 genome editing greatly bolsters studies in human systems [110]. As a recent example, live imaging during the development of human brain organoids was used to model the biomechanics of cerebral folding, a characteristic of humans not shared with standard model rodent species [111*]. This study showcases the power of human organoid systems to pick up when there are limitations to animal models, static imaging and traditional cell culture in order to better recapitulate aspects of the complexity of SCs and the niche during development, homeostasis and disease.

Conclusions and outlook

Advances in single-cell profiling, *in vitro* culture and advanced light microscopy have further enabled SC researchers in the dissection of the complex intricacies of adult SC interactions with the niche on a larger scale and at a more refined level than ever before. These emerging technologies have begun to provide powerful new avenues to interrogate heterogeneity and complexity, diversity in function in SCs and niche components, and their essential cross-talk (Figure 2). However, as newly developed methods and technologies have to fully pass rigorous scrutiny and validation before ubiquitous acceptance and implementation to

the modern biologist's toolkit, researchers should carefully consider their limitations when drawing conclusions and support findings with more traditional, well-established techniques. Regardless, these new technologies open the door to exploration of the complexities of SC functions and the regulation by the niche that was previously not thought possible and promise to continue the successful journey towards elucidating the key regulation behind SC identity and behavior that may allow for the development of regenerative therapies and treatment of disease.

Conflict of interest statement

Nothing declared.

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