

Stem Cell Heterogeneity, Plasticity, and Regulation

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Abstract

As a population of homogeneous cells with both self-renewal and differentiation potential, stem cell pools are highly compartmentalized and contain distinct subsets that exhibit stable but limited heterogeneity during homeostasis. However, their striking plasticity is showcased under natural or artificial stress, such as injury, transplantation, cancer, and aging, leading to changes in their phenotype, constitution, metabolism, and function. The complex and diverse network of cell-extrinsic niches and signaling pathways, together with cell-intrinsic genetic and epigenetic regulators, tightly regulate both the heterogeneity during homeostasis and the plasticity under perturbation. Manipulating these factors offers better control of stem cell behavior and a potential revolution in the current state of regenerative medicine. However, disruptions of normal regulation by genetic mutation or excessive plasticity acquisition may contribute to the formation of tumors. By harnessing innovative techniques that enhance our understanding of stem cell heterogeneity and employing novel approaches to maximize the utilization of stem cell plasticity, stem cell therapy holds immense promise for revolutionizing the future of medicine.

Keywords: Stem Cell, Heterogeneity, Plasticity, stem cell cellular therapy, gene therapy, Hematopoietic stem cells.

1. Introduction

The term 'stem cell', first used by Artur Pappenheim in the 1890s(Ramalho-Santos and Willenbring, 2007), was defined by their ability to consistently self-renew and their potential to differentiate into multiple lineages. Decades ago, research on stem cells was primarily limited to transplantation assays using the hematopoietic system and defined by their functional output. At that time, stem cells were perceived as a homogeneous population that gave rise to progenitors, precursors, and mature cells successively, with an equal propensity. However, research was mainly focused on identifying the true stem cells in specific tissues.

Recent advancements in technology, such as single-cell genome sequencing and lineage tracing, have made it possible to trace stem cell differentiation in vivo. Additionally, gene-editing technology has facilitated the establishment of relationships between genotype and phenotype by knocking out essential genes. High-throughput strategies using fluidic and robotic systems have also enabled the analysis of hundreds of individual cells, providing greater insights into stem cell heterogeneity(Streets et al. , 2014). Through these technologies, it has become clear that stem cells are a diverse population with distinct subsets that are preprogrammed to give rise to different lineages. Each subtype possesses a unique capacity for regeneration and differentiation(Muller-Sieburg et al. , 2002), dynamic behavior, repopulation kinetics, life span, as well as requirements for proliferation(Dykstra et al. , 2007).

In this review, we will discuss recent advances in tissue-specific and pluripotent stem cells, focusing on their heterogeneity. Under homeostasis, this heterogeneity plays an essential role in development, fate decisions, dynamic behavior, and regeneration. During perturbation, aging, and cancer, the striking plasticity of stem cells is provoked. We will also highlight the regulatory network within the stem cell pool, exploring the relationship between intrinsic regulators, extrinsic niches, and signaling pathways.

Additionally, the techniques that enable the characterization of stem cell heterogeneity will be summarized. By understanding the bi-directional interplay between regulatory events within diverse cell niches, we can enable precise control of stem cells, leading to both effective regenerative medicine and a deeper understanding of cancer progression and therapy resistance.

2. General features of stem cell heterogeneity

Stem cells possess two critical capabilities: the ability to self-renew and the ability to differentiate into various lineages(Post and Clevers, 2019). For a long time, scientists believed that there was only one homogenous stem cell pool. However, recent research has demonstrated that stem cell pools contain a consortium of different cell types, each with distinct functional steady-state characteristics. These characteristics include self-renewal, proliferation, differentiation, and lifespan(Haas et al. , 2018). Stem cell therapy requires the guaranteed development of pluripotent stem cells into specific cell and tissue types. Small differences and changes in the pluripotent state of stem cells are sufficient to influence the developmental pathways of stem cells.

Stem cell heterogeneity involves multiple layers, including the stem cells themselves, their surrounding niches, and related signals, all of which determine their functions and fates. For example, the heterogeneity of adipose stem cells (ASCs) could explain differences in fat depots. When exposed to obesogenic stimuli, SDC1+ ASCs in visceral epididymal tissues enhance fibrotic remodeling, while CXCL14+ ASCs in subcutaneous inguinal tissues repress macrophage infiltration (Nahmgoong et al. , 2022). Similarly, three subpopulations of mesenchymal stem cells (MSCs) have been identified, each with distinct characteristics: the stemness subpopulation, functional subpopulation, and proliferative subpopulation (Xie et al. , 2022). In comparison to stem cells, subpopulations of mesenchymal niches in the intestine with distinct expression of Wnt2b, Wnt4, Wnt5a, and other genes were discovered in recent studies

of the embryonic gut (McCarthy et al. , 2020). Single-cell analysis revealed that the anterior (fore-/midgut) and posterior (mid-/hindgut) mesenchymal niches in the embryonic gut exhibit heterogeneity as early as mouse embryonic (E) day 8.75(Nowotschin et al. , 2019), and their complexity doubles in only 24 hours(Han et al. , 2020). However, the inherited heterogeneity of MSCs has led to inconsistent identification and the failure of most clinical trials applying MSC therapy(Zhou et al. , 2021). Signals from the environment and the specific response to them are essential for stem cell fate decisions. Time-lapse imaging has recently revealed that extracellular signal-regulated kinase (ERK) pathway dynamics are highly heterogeneous among different hematopoietic stem cells (HSCs) and with various cytokines, which may provide a novel way to distinguish HSC subtypes aside from surface markers or morphology. For instance, in HSCs, ERK dynamics were activated by 39% and 18%, respectively, with SCF and FLT3L stimuli. Besides, HSCs with intermediate ERK dynamics increased CD45RA expression in the next generation, while those with intermediate and transient ERK dynamics possessed higher CD34 expression in the daughter generation (Wang et al. , 2021b). Importantly, heterogeneity in signaling is beneficial for the maintenance and stability of a population since a homogeneous subgroup of stem cells reacts differently to the same stimuli. By activating the same NGF stimulus in a homogeneous P12 cell population in the pERK-pAKT plane, the pERK and pAKT signal distribution showed apparent variation with a sharp boundary, allowing for further subgrouping by signal maps. (Chen et al. , 2012)

Furthermore, it is evident that in many tissues, heterogeneous populations of stem cells are in constant competition with each other. For instance, in the early blastomere, opposing lineage specifiers trigger a ratio-dependent lineage battle, which pushes the blastomere onto a predisposed yet flexible lineage track, until one side eventually dominates the other and determines the lineage fate (Shi et al. , 2015). In this system, the relative ratio of *Carm1* and *Cdx2* (which guide ICM and TE specifications respectively) in each eight-cell blastomere is different, revealing the predisposed lineage strengths formed in these blastomeres(Shi et al., 2015). Similarly, in the

intestine epithelium, stem cell subsets constantly compete to colonize each crypt, until homogeneity is achieved within one to six months(Ritsma et al. , 2014, Snippert et al. , 2010b). In the hematopoietic system, the lineage bias of stem cells can be exacerbated in the presence of competing Hematopoietic Stem Cells (HSCs), suggesting a competitive relationship between Myeloid-biased and Lymphoid-biased HSCs. For instance, in a mixture of My-bi and Ly-bi HSCs, almost all lymphocytes are generated from Ly-bi HSCs, while almost all myeloid lineages come from My-bi HSCs (Figure 1)(Challen et al. , 2010). Additionally, stem cells could also compete with progenitor cells. HSCs and proximal hematopoietic progenitors are constantly competing for limiting membrane-bound stem cell factors on Leptin receptor+ mesenchymal stem/progenitor cells and endothelial cells to restrict their expansion(Miao et al. , 2022).

However, it is worth noting that the heterogeneity of the stem cell compartment is not infinite. A thorough mathematical analysis of hematopoietic stem cells (HSCs) has revealed that out of all the 54 possible subsets of repopulation kinetics, only 16 actually exist. Therefore, all HSC clones can be assigned to these sixteen groups, and it is highly unlikely to discover new patterns beyond them(Sieburg et al. , 2006).

Despite the extensive heterogeneity found in many aspects of stem cells, it is important to note that once initiated during the developmental process, heterogeneity becomes fixed and remains stable under physiological conditions. For instance, serial transplantation of HSCs indicates that regenerated HSCs retain the characteristics of the original transplanted cell. In fact, all daughter cells derived from the same transplanted HSC show the same life span in serial transplants, even when separated in different hosts(Sieburg et al. , 2011). This suggests that the features of the mother HSC are stably inherited by all daughter HSCs. Similarly, the biased differentiation potential of My-bi and Ly-bi HSCs is stably inherited during constant self-renewal (Muller-Sieburg et al. , 2004). Additionally, the stability of heterogeneity has been found in HSCs derived from mouse fetuses, indicating that the generation of HSC diversity occurs during the developmental process(Benz et al. , 2012). Thus, there must be a

universal, cell-intrinsic program that maintains a fixed degree of heterogeneity under homeostasis. A deeper understanding of stem cell heterogeneity is crucial for precise intervention in diseases and can facilitate the clinical use of stem cells.

3. Heterogeneity directs cell development and determines cell fate under physiological conditions (homeostasis)

Emerging evidence indicates that multiple aspects of cellular physiology, including epigenetics, transcription, mitosis, signal transduction, and metabolic regulation, differ among heterogeneous stem cells. These differences enable stem cells to participate in multilineage differentiation throughout life in numerous tissues and maintain homeostasis or remodel relevant tissues in response to physiological changes.

3.1 Stem cell heterogeneity follows a hierarchy in the hematopoietic system

The discovery of heterogeneity within hematopoietic stem cells (HSCs) began with the observation that purified mCD34^{lo/-}, c-Kit⁺, Sca-1⁺, lineage marker negative (Lin⁻) HSCs exhibit a significant variation in their contribution to the myeloid and lymphoid lineages when transplanted into recipient mice (Osawa et al. , 1996), Analysis of retrovirally marked HSCs(Jordan and Lemischka, 1990) and c-kit (neg) HSCs(Ortiz et al. , 1999) in transplanted mice revealed a skewed differentiation program. Similar experiments demonstrated variation in the repopulation kinetics of transplanted HSCs(Muller-Sieburg et al., 2002). Serial single-cell transplantation of HSCs provided a comprehensive view of the mouse HSC pool, revealing various HSC types(Dykstra et al., 2007), including myeloid- and lymphoid-biased HSCs found in human bone marrow(Mazurier et al. , 2004, Pang et al. , 2011). Further studies have classified HSC subgroups using various markers under different conditions. Under regeneration-mediated stress, long-term (LT)-HSCs are divided into CD112^{lo} and CD112^{hi} subsets by an immune checkpoint ligand CD112. The CD112^{hi} subset responds immediately to blood cell demands, while the CD112^{lo} subset enters a latent state before undertaking

hematopoietic reconstruction to preserve a stem cell reservoir(Kaufmann et al. , 2021). MHCII^{hi} HSC is a subset of quiescent stem cells with increased MHCII expression, displaying reduced cycling and apoptosis characteristics(Li et al. , 2022a), In contrast, the subset of CD34⁺CD49f⁺EPCR⁺ HSC population has high self-renewal abilities and defines the most primitive CD34⁺ HSPCs(Anjos-Afonso et al. , 2022).

Initially, HSCs are formed through endothelial-to-hematopoietic transition (EHT) from endothelial cells in major arteries at E10.5(Yokomizo and Dzierzak, 2010), specified by the factor Evi1(Yokomizo et al. , 2022). Traditionally, it was believed that HSCs gave rise to short-term HSCs (ST-HSCs), common lymphoid progenitors (CLP), common myeloid progenitors (CMP), followed by granulocyte–monocyte progenitors (MEP), and megakaryocyte–erythrocyte progenitors (GMP). However, recent discoveries have unveiled two waves of HSC-independent hematopoiesis. Erythro-myeloid progenitors (EMPs) and lympho-myeloid progenitors (LMPs) are now understood to be derived directly from endothelial cells through EHT, much like the process for HSCs (Neo et al. , 2021). Furthermore, recent studies have shown that hematopoietic progenitors can also originate directly from endothelial cells through EHT, bypassing the need for HSC involvement (Yokomizo et al., 2022). The specific fate of these cells, whether they develop into HSCs, multipotent progenitors (MPPs), or CMPs, is believed to be determined by the expression of Evi1 (Yokomizo et al., 2022).

Furthermore, HSCs exhibit heterogeneity in their proliferative potential. It is suggested that HSCs encompass a dormant compartment that preserves their long-term self-renewal potential, alongside an active compartment that drives hematopoiesis (Trumpp et al. , 2010). Additionally, the active compartment of HSCs displays its own heterogeneity, with predominantly frequently dividing HSCs contributing to self-renewal, and infrequently dividing HSCs participating in the differentiation process (Grinenko et al. , 2018). Among these subpopulations, there is mounting evidence placing Tie2⁺ HSCs at the apex of the hematopoietic hierarchy in terms of

reconstitution potential, signifying their capacity to generate steady-state output, but at a very low frequency (Höfer and Rodewald, 2018). Both the rates of proliferation and differentiation increase dramatically as we descend the hierarchy from the apex (Tie2+ HSCs) to Short-Term Hematopoietic Stem Cells (ST-HSCs), and finally to Multipotent Progenitor Cells (MPPs) (Busch et al. , 2015, Ito et al. , 2016).

Heterogeneity is also evident in the lineage bias of HSCs, encompassing platelet-primed, lympho-myeloid, My-bi, and Ly-bi HSCs. The hematopoietic stem cell (HSC) compartment is organized into a cellular hierarchy that is influenced by the phases of bone turnover(Christodoulou et al. , 2020), At the apex of this hierarchy are platelet-primed HSCs (vWF+ HSCs), which have a megakaryocyte-erythroid potential (Carrelha et al. , 2018, Sanjuan-Pla et al. , 2013). Megakaryocytes differentiate via two distinct pathways, one of which is Myb-independent, bypassing bipotent megakaryocyte-erythroid progenitors. The other pathway involves Csf2rb+ Myb-dependent bipotent progenitors that give rise to both megakaryocytes and erythrocytes(Iturri et al. , 2021). Following the downregulation of megakaryocyte-erythroid genes, lympho-myeloid stem cells (vWF- HSCs) emerge with lymphoid or myeloid biases (Mansson et al. , 2007). However, the myeloid or lymphoid gene programming is not altered during this process(Sanjuan-Pla et al., 2013). Additionally, during the transition between these two subsets, HSCs exhibit combined granulocyte-macrophage and lymphoid potentials but show little to no megakaryocyte-erythroid potential(Adolfsson et al. , 2005a). After some time, vWF+ HSCs can be both platelet and myeloid-biased, whereas vWF- HSCs are predominantly lymphoid-biased (Sanjuan-Pla et al., 2013). Several other subpopulations of HSPCs have also been identified, although debates have arisen regarding their capacity for unilineage or bilineage differentiation (Miyawaki et al. , 2017, Notta et al. , 2016, Psaila et al. , 2016). Supported by single-cell culture assays and computational predictions, it is suggested that unilineage cells emerge directly from a continuum of HSPCs, without transitioning through the multi- and bipotent stages (Grün et al. , 2016, Notta et al., 2016). The apparent multipotency observed at the population level may result from the abundance

of unipotent progenitors within specific compartments (Laurenti and Göttgens, 2018).

Lympho-myeloid stem cells comprise three subsets of HSCs that are defined by their potential to differentiate into either lymphoid or myeloid cells. Myeloid-biased HSCs, also known as alpha-cells, generate normal levels of myeloid progenitors but only produce a limited number of lymphoid progenitors. On the other hand, lymphoid-biased HSCs, also referred to as beta-cells, produce normal levels of lymphoid progenitors but have a reduced capacity to generate myeloid progenitors. In contrast, balanced HSCs, also called Bala HSCs, can generate normal levels of both myeloid and lymphoid progenitors without attenuation, meaning that the ratio of lymphoid to myeloid cells they produce is the same as that found in the peripheral blood of normal mice (Dykstra et al., 2007, Haas et al., 2018, Muller-Sieburg et al., 2004) (Figure 1). These three subsets are considered bona fide HSCs, not just progenitors, because they have the potential to generate all blood lineages in the long term. Furthermore, research shows that the biased differentiation program is not the result of an overproduction of the biased lineage, but rather the attenuated generation of other lineages (Challen et al., 2010).

My-bi and Ly-bi HSCs differ in many aspects, including surface markers, dynamic behavior, and reactions to various factors. The signaling lymphocytic activation molecule (SLAM) family includes CD150, which is expressed at a higher level in My-bi HSCs, CD48, CD229, which is involved in a lymphocyte-activated signal transduction pathway and can largely distinguish Ly-bi HSCs from rarely dividing My-bi HSCs, and CD244. All of these markers distinguish HSCs from restricted progenitors and can subdivide them (Oguro et al., 2013). CD41⁺ HSCs show a myeloid bias, are quiescent, and possess long-term repopulation capacity, as CD41 is associated with exposure to important platelet-released cytokines that enable HSC maintenance and hematopoietic homeostasis (Gekas and Graf, 2013). Moreover, the same type of progeny generated from a different subset of HSCs may express distinct surface markers. Gene array analysis has revealed that the lymphoid progeny of My-bi HSCs

express lower levels of interleukin-7 (IL-7) receptor than those of Ly-bi HSCs, which renders them unresponsive to IL-7 and results in reduced lymphogenesis from My-bi HSCs (Muller-Sieburg et al., 2004). Furthermore, it has been discovered that the vWF-eGFP reporter is expressed in only 60% of HSCs, which may have megakaryocyte-erythroid potential and exist at the top of the hematopoietic hierarchy (Kiel et al., 2005, Osawa et al., 1996).

My-bi HSCs have a longer lifespan than Ly-bi HSCs (Muller-Sieburg et al., 2004), despite a delayed onset of repopulation. This phenomenon may be attributed to changes in the composition of blood as mice age, where blood transitions from lymphoid to distinctly myeloid (Mazurier et al., 2004); In younger D2 mice, blood is composed of almost 80% lymphocytes and 20% myeloid cells, whereas in aged D2 mice, the blood contains about 80% myeloid cells and 20% lymphocytes (Cho et al., 2008). During the early stages of life, Ly-bi HSCs repopulate more frequently than My-bi HSCs, making lymphoid lineages dominant. However, as mice age, My-bi HSCs gradually outlive Ly-bi HSCs, resulting in the production of more myeloid lineages than lymphoid ones. The exact reason for this difference in behavior is still unclear, but it is believed that My-bi HSCs have a slower turnover rate, making them relatively quiescent and able to survive longer (Muller-Sieburg and Sieburg, 2008). Additionally, the aging process may be related to reduced mitochondrial membrane potential in mice. Studies have shown that enhancement of MMP with mitoquinol in old HSCs can reduce the proportion of myeloid cells in peripheral blood and bone marrow (Mansell et al., 2021). More recently, it has been revealed that the interaction between *Atf7ip* and *Setdb1* maintains HSC stemness and hampers myeloid potential. Depletion of either of these genes would induce myeloid differentiation (Wu et al., 2023).

The heterogeneity of HSCs is not only demonstrated by differences in subgroups with varying surface biomarkers, cell fates, and regulation by different signaling pathways. It has also been found that different subsets of HSCs exhibit distinct reactions to stimulation by cytokines and growth factors. For example, a low dose of TGF- β is

stimulatory to My-bi HSCs due to the more rapid formation of CSF-GM colonies, while it is inhibitory to Ly-bi HSCs because of impeded CSF-GM differentiation(Mallaney et al. , 2014). Tetraspanin CD63, which interacts with TGF β receptors I and II, is essential for TGF- β signaling. HSC subgroups with high CD63 expression have been found to be more quiescent and possess long-term self-renewal capacities and myeloid differentiation potential (Hu et al. , 2022). Additionally, My-bi HSCs and Ly-bi HSCs can be distinguished by their capacity for Hoechst dye efflux (Adolfsson et al. , 2005b). (Table 1).

3.2 Competitive heterogeneity in the gut

The intestine contains several types of intestinal stem cells (ISCs). Crypt base columnar cells (CBCs) reside in the Paneth cell zone at the base of the intestinal crypt and are interdigitated with Paneth cells. They are characterized by positive expression of Lgr5 and are considered at the top of the cellular hierarchy in the intestine(Ritsma et al., 2014). Quiescent ISCs (qISCs), also known as +4 cells, are located just above the Paneth cell zone at the base of the crypt. Tier 1 CBCs, also known as center cells, are located at the baseline of the crypt base, while tier 2 CBCs, also known as border cells, reside at the upper part of the crypt base. The location of CBC cells is closely related to their self-renewal and differential functions. Under homeostatic conditions, heterogeneous CBC cells exhibit varying degrees of self-renewal capabilities. Some CBC cells have the potential to generate multiple differentiated progeny, including endocrine and absorptive cells(Barker et al. , 2007), while qISCs divide infrequently(Gracz and Magness, 2014). These two kinds of self-renewal potential are also able to be transferred to each other, but the mechanisms remain unclear(Takeda et al. , 2011). For instance, The Mex3a⁺ cells(Barriga et al. , 2017) in mice and LGR5⁺p27⁺ cells (Ishikawa et al. , 2022) in the human colon are slowly cycling subtypes of Lgr5⁺ qISCs that retain the multipotency to generate all intestinal lineages. Msi1⁺ cells, also known as cycling stem cells, are located right above Lgr5^{high} cells in crypts and can generate Paneth cells at an early stage of injury(Sheng et al. , 2020).

Quantitative clonal fate analyses have suggested that CBC cells engage in neutral competition during cell division for space and niche support. (Lopez-Garcia et al. , 2010, Snippert et al., 2010b). As a result, Tier 1 CBCs have a self-renewal advantage over Tier 2 CBCs due to their location, enabling them to continue dividing while pushing Tier 2 CBCs out of the niches into the transit-amplifying (TA) domain, where they begin to differentiate. (Ritsma et al., 2014).

The heterogeneity of intestinal stem cells (ISCs) is typically regulated by transcriptional, microenvironmental, and growth factors that collaborate to control their self-renewal and cellular fates. Along the cephalocaudal axis, ISCs display obvious polarity due to heterogeneous transcription factor regulation, such as GATA4, which is correlated with specialized regional function from the duodenum to the ileum(Middendorp et al. , 2014). GATA4 expression decreases from the proximal to the distal intestine to inhibit the expression of ileum-specific genes(Bosse et al. , 2006). However, within each specific crypt, there appears to be a preference for a program towards fewer but larger clones. Around 14-16 CBC cells compete with one another over niche signals via neutral drift dynamics(Ritsma et al., 2014, Snippert et al., 2010b), but only 5-7 of these cells are functionally active and are tier 1 CBC cells with self-renewal advantages since they reside closer to the crypt base(Kozar et al. , 2013). Eventually, the winner will generate the entire crypt-villus unit and create a single homogeneous crypt with just a few outliers, as demonstrated by sequencing of green fluorescent protein (GFP)-marked Lgr5-positive cells (Figure 2)(Grun et al. , 2015).

Like other rapidly cycling tissues such as the intestine, the mouse esophagus contains a heterogeneous population of epithelial stem cells, which are hierarchically organized. These distinct stem cell populations possess a slow cell cycle, generating more differentiated transit-amplifying cells. Maintenance of esophageal stem-ness and self-renewal is thought to require Wnt and noggin/BMP signals, which are also essential in intestinal crypts(DeWard et al. , 2014). However, the origin of these signals remains unclear. It is postulated that stem cells may compete for niche signals to maintain their

stem-ness, similar to the mechanism found in the intestine(Jacobs et al. , 2012), Alternatively, they may generate Wnt signals themselves, similar to the autocrine mechanism identified in some interfollicular epidermal stem cells(Lim et al. , 2013).

In mammary glands, clonal epithelial patches that include both myoepithelial and luminal cells are produced from bipotent mammary stem cells (MaSCs), and a few stem cell clones can form a large area of the mammary epithelium, much like the intestine(Rios et al. , 2014). However, MaSCs typically remain dormant until activated by hormones during puberty and pregnancy(Visvader and Stingl, 2014).

3.3 Heterogeneity of highly compartmentalized tissue

The heterogeneity of other highly compartmentalized tissues has also been characterized, including the epidermis, dermis, hair follicles, and others. The interfollicular epidermis (IFE) is generated by stem cells in the basal layer of the IFE(Clayton et al. , 2007) with four distinct subsets(Haensel et al. , 2020, Wang et al. , 2020b). There are two programs of terminal differentiation: the parakeratotic program and the orthokeratotic program. The former, also known as scale, proliferates more rapidly and requires Wnt signaling and Edaradd for enlargement. It gives rise to scale IFE, which retains nuclei in cornified layers but lacks a granular layer. The latter, also known as interscale, proliferates much more slowly and generates interscale IFE characterized by a granular layer and the loss of nuclei in the cornified layer(Gomez et al. , 2013).

The stem cell compartment of hair follicles is much more complex. During embryogenesis, both the entire hair follicle and the sebaceous gland are generated from hair follicle buds(Levy et al. , 2005, Nowak et al. , 2008). However, in adults, separate compartments are derived from distinct stem cell pools, each possessing specific cell markers (Table 2). Lineage tracing has suggested that Lrig1⁺ stem cells in the junctional zone, the basal layer of the sebaceous gland, together with Blimp⁺ cells adjacent to the

sebaceous gland, are responsible for the maintenance of the sebaceous gland. Lrig1+ stem cells in the junctional zone also give rise to the infundibulum(Horsley et al. , 2006, Page et al. , 2013). Cells located in the upper bulge are quiescent during homeostasis, while lower cell bulge stem cells tend to regenerate the outer root sheath of the hair follicle(Rompolas et al. , 2013). Additionally, stem cells located at the hair germ mainly produce lower hair follicles(Greco et al. , 2009), but can also contribute to the outer root sheath. They are considered to be the bona fide stem cells at the top of the epidermal hierarchy for some time, since they can give rise to all skin epithelial lineages *in vitro*, including IFE, hair follicles, and sebaceous glands(Claudinot et al. , 2005, Morris et al. , 2004).

The dermis, in addition to the epidermis, is also heterogeneous and originates from two distinct fibroblast lineages. The upper dermal lineage gives rise to the dermal papilla, arrector pili muscle, and papillary fibroblasts, which are crucial for the formation of hair follicles. On the other hand, the lower dermal lineage generates reticular fibroblasts that give rise to adipocytes and fibrillar extracellular matrix. They also play a key role in the initial dermal repair following injury, as depicted in Figure 3(Driskell et al. , 2013).

Different parts of the lung are also renewed by distinct stem cells under physiological conditions. In mouse airways, the tracheal epithelium comprises mainly Clara cells, ciliated cells, and basal cells. As the basal membrane becomes thinner in bronchioles, Clara and ciliated cells are interspersed with neuroendocrine cells, while the alveolar epithelium is composed of type 1 (squamous) and type 2 (surfactant-secreting) cells. In bronchioles, Clara cells with heterogeneous subpopulations act as stem cells, which self-renew and give rise to different ciliated cells but do not contribute significantly to alveoli, neuroendocrine cells, mesenchymal, or endothelial compartments. In the trachea, both ciliated and Clara cells arise from tracheal basal cells, which behave as stem cells with self-renewal ability. However, most Clara cells only serve as TA progenitors, with only a small population of self-renewing Clara cells, as found in

bronchioles(Rawlins et al. , 2009). In the alveoli, a bipotential progenitor for both type 1 and type 2 cells has been identified during development, which co-express the marker genes of these two cell types. After birth, a switch occurs, and long-lived type 2 cell stem cells produce new type 1 cells(Desai et al. , 2014, Treutlein et al. , 2014). (Figure 3)

3.4 Heterogeneity in pluripotent stem cells

Consistent with the unipotent stem cells, the pluripotent stem cells are also heterogeneous. Demonstrated by transcriptional(Rosas-Canyelles et al. , 2020, Shi et al., 2015) as well as direct developmental evidence(Guo et al. , 2020), initial inter-blastomere heterogeneity is apparent as early as the four-cell stage of embryo development, and is considered the first cell fate decision, which occurs much earlier than morphological distinction. Subsequent transcriptional differences follow a binomial distribution pattern and may be regulated by negative or positive feedback: for some genes, transcriptional asymmetry gradually decreases, while for others, transcriptional asymmetry amplifies over time, possibly due to lineage specifiers. Moreover, the differences observed at the four-cell stage exhibit consistent expression differences between inner cell mass (ICM) and trophectoderm (TE), indicating that it may be relevant to the segregation between TE and the ICM later at the morula stage(Biase et al. , 2014). However, the heterogeneity of blastomeres is still reversible, and there is enough plasticity for them to change their fate before reaching the 32-cell stage(Toyooka, 2020).

During the 16-to-32 cell stage, TE cells can be identified by the presence of *Id2*, whereas ICM cells are marked by *Sox2*(Guo et al. , 2010). Later in the ICM, driven by *Fgf4*, instead of arising from two distinct dividing histories, pluripotent epiblast (EPI) and primitive endoderm (PE) lineages stochastically emerge, and EPI will generate all cell lineages of the embryo(Ohnishi et al. , 2014). Under pluripotency maintenance conditions, the ICM can also generate embryonic stem cells (ESCs), wherein the normal

developmental program is replaced by infinite self-renewal capacity and pluripotency(Tang et al. , 2010). ESCs also experience state transitions under cultural conditions(Chakraborty et al. , 2020). Instead of a completely stochastic process, historical state memories affect further state transitions in the future(Udomlumleart et al. , 2021). The pluripotency of ESCs can be further induced with yolk sac-like cells, directing them toward an anterior ectoderm fate instead of mesoderm and endoderm(Mackinlay et al. , 2021). Although both human EPI and ESC are pluripotent, these properties are underpinned by distinct mechanisms. Influenced by different environments, EPI is enriched for oxidative phosphorylation, while ESC is enriched for the regulation of proliferation, such as the Wnt and MAPK signaling pathways(Blakeley et al. , 2015). Furthermore, single-cell analysis has identified several sub-populations within mouse ESCs, including an epiblast-like subset, a Prdm1-high subset, an Hsp9-high subset, and several subsets that do not match any known cell type(Klein et al. , 2015) (Figure 4).

4. Heterogeneous stem cells exhibit striking plasticity during stress and perturbation (pathological conditions)

Although stem cell heterogeneity and stem cell compartments are stable during homeostasis, recent research has shown that stem cells are more adaptable than previously believed, with broader differentiating potential and stronger self-renewal ability in response to natural (aging) or artificial (perturbation) stress. During the differentiation and self-renew process induced by aging or perturbation, stem cells can switch their function, phenotype, and/or composition to adopt new identities and/or phenotypes, resulting in what is known as stem cell plasticity.

4. 1 Cell functional changes

The function changes of heterogeneous stem cells are typically induced during the

tissue injury and repair process. The self-renewal and differentiation potential of stem cells depends on the properties and extent of the injury.

Different types of injury can induce varying degrees of plasticity in stem cells. There are three main methods of conducting reconstitution assays under perturbation: 1) injury followed by tissue repair, which typically involves the destruction of a large area of cells; 2) targeted laser ablation of stem cells, which focuses on the destruction of only the basal layer of stem cells; and 3) transplantation, where tissue is reconstituted from a mixture of disaggregated cells or even a single cell, which would not typically occur under physiological conditions. Despite the varying degrees of cell loss, plasticity can be evoked under all three conditions. After perturbation, remaining stem cells in the vicinity may exhibit significantly stronger self-renewal and differentiation potential. For example, after tracheal injury, Clara cells demonstrate an increased capacity for both proliferation and self-renewal (Rawlins et al. , 2009). In the intestine, ablation of Lgr5⁺ stem cells does not disrupt homeostasis since other stem cell pools, such as Bmi⁺ cells, can colonize the intestine (Tian et al. , 2011), and quiescent intestinal stem cells (qISCs) can enter the cell cycle and generate new Crypt base columnar cells (CBCs) (Kurokawa et al. , 2020, Yan et al. , 2012). Similarly, hair follicle fibroblasts can be regenerated by quiescent Hic1⁺ stem cells after injury (Abbasi et al. , 2021). Additionally, highly compartmentalized stem cells can broaden their range of progeny and diverge into different lineages under perturbation. For instance, transplantation assays have shown broader plasticity of skin epithelial cells than under steady-state conditions (Prater et al. , 2014). Laser ablation of the hair bulge has revealed that surrounding cells from hair germ can give rise to hair bulge and acquire bulge cell identity, capable of generating the lower hair follicle. In contrast, ablation of hair germ cells enables bulge cells to repopulate hair germ and acquire germ cell characteristics (Rompolas et al., 2013). Lrig1⁺ skin epidermal stem cells typically give rise to progeny that is restricted to sebaceous glands, but they can generate all of the epithelial compartments in reconstitution assays (Jensen et al. , 2009, Page et al., 2013). Nevertheless, despite some stem cells possessing a multipotent differentiation potential,

specific lineages are still preferred during tissue repair. For example, after removing a full-thickness piece of skin including the epidermis and dermis, although the hair follicles contributed to the repopulation of the interfollicular epidermis (IFE), the majority of the reconstituted IFE is derived from nearby IFE basal stem cells (Figure 5)(Mascre et al. , 2012).

The extent of the injury is a critical factor that induces different plasticity in stem cells. For example, in cases of chronic liver injury resulting from a choline-deficient, ethionine-supplemented diet, biliary cells undergo terminal differentiation into functional hepatocytes. This efficiency is further increased when there is a reduction in extracellular matrix and laminin deposition(Espanol-Suner et al. , 2012). The co-opting of differentiated cells to regenerate tissue is also observed following partial hepatectomy in which the regenerative process is achieved by hypertrophy and proliferation of mature hepatocytes(Miyaoka et al. , 2012). Moreover, in models of toxic, cholestatic, and fatty liver disease, hepatic stellate cells repair the liver by giving rise to 82-96% of myofibroblasts (Mederacke et al. , 2013). In the lung, highly focal lung injury is repaired by type two pneumocytes, whereas acute lung damage is regenerated by p63/Krt5+ distal airway stem cells (Hogan et al. , 2014).

During the injury repair, non-stem cells like differentiated cells or progenitors may undergo de-differentiation or transdifferentiation to generate stem cells or converse from one cell type to another. For instance, in hair follicles, cells that have left the stem cell compartment and undergone differentiation can revert to bulge stem cells following laser ablation of stem cells (Rompolas et al., 2013). In the lung, luminal secretory cells can de-differentiate into basal stem cells and repair the epithelium after stem cell ablation (Tata et al. , 2013). Similarly, secretory lineage precursors of the intestine that express DII1 (van Es et al. , 2012) as well as secretory progenitors that retain YFP-labeled histone H2B(Buczacki et al. , 2013, van Es et al., 2012) can de-differentiate and become stem cells after tissue damage. Transient amplifying cells can also give rise to basal stem cells in the mouse testis and in *Drosophila* ovaries in response to stem cell

loss(Kai and Spradling, 2004, Nakagawa et al. , 2007). However, it's revealed that the reversal of differentiation could be short-lived. For example, mouse ependymal cell maturation is reversible with downregulation of Sntn in response to injury, but a mature cell state was rapidly re-established a few days later (Rodrigo Albors et al. , 2023). Collectively, These observations suggest that stem cells in different tissue types can be detected and can trigger the de-differentiation of differentiated cells to regenerate the stem cell pool. Additionally, transdifferentiation, the conversion from one cell type to another, is also possible during perturbation. In the adult pancreas, acinar cells can be converted to beta cells when beta cells are lost during injury(Thorel et al. , 2010), and hepatocytes can be transformed into biliary epithelial cells during liver regeneration(Yanger et al. , 2013).

Apart from the tissue injury and repair process, perturbation in the stem cell niches might boost stem cell plasticity and lead to tumorigenesis. In the context of normal aging, the loss of adrenergic nerve cells in the bone marrow leads to a reduction in niche cells, which subsequently results in the development of myeloproliferative neoplasms from HSPCs (Hanoun et al. , 2014). Similarly, the alteration of the stem cell niche due to a deficiency in the retinoic acid receptor is sufficient to induce a myeloproliferative syndrome (Walkley et al. , 2007). Furthermore, perturbations in cancer stem cell niches can influence cancer functions such as growth or dormancy. It's revealed that HSC dormancy niches characterized by NG2+/Nestin+ MSCs can maintain the dormancy of breast cancer (BC) disseminated tumor cells (DTCs). Factors such as aging or extrinsic influences that affect the homeostasis of NG2+/Nestin+ MSC niches may result in bone relapse in breast cancer. (Nobre et al. , 2021). In contrast, Schwann cell niches play a crucial role in tumor growth. Studies have shown that the genetic removal of endogenous Schwann cells within the prostate tumor microenvironment slowed down both tumor growth and angiogenesis (Rocha et al. , 2023). Additionally, the alteration of endothelial cell metabolism through β -adrenergic receptor signaling in the prostate cancer microenvironment can activate the angiogenic switch, thereby promoting tumor growth (Zahalka et al. , 2017).

4.2 Phenotypic and compositional changes

Stem cell phenotypes, including cell surface antigen expression, can change during HSC expansion experiments, aside from functional changes (Randall and Weissman, 1997). The major change revealed during natural stress, such as aging, is the shift in the composition of the stem cell compartment. For instance, the number of CD271^{Bright} mesenchymal stem cells, which have a strong potential for producing fibroblasts, decreases as humans age (Maijenburg et al. , 2012), which supports observations that aged individuals exhibit more adipogenesis but less fibrogenesis and osteogenesis(Moerman et al. , 2004). Similarly, while HSCs are initially balanced in the fetal liver (Benz et al., 2012), an accumulation of My-bi HSCs in the aged HSC compartment leads to more myeloid lineage cells and fewer lymphocytes in the blood, contributing to the elderly population's sluggish immune response and increased susceptibility to infectious diseases and cancer(Mazurier et al., 2004). Likewise, compared to neonatal mice, neural stem cells in aged mice generate more astrocytes than neurons(Lee et al. , 2012). In cases of injury, changes in composition also occur. Single-cell analysis showed that the proportion of dormant neural stem cells (NSCs) decreases while primed-quiescent NSCs and activated NSCs highly increase in ischemic brain injury, thereby promoting the process of tissue repair (Llorens-Bobadilla et al. , 2015). However, cell markers may not be a reliable indicator of cell function of HSC. For example, after two HSC transplantations, an expansion of cells possessing stem cell phenotypes but lacking stem cell function was found (Spangrude et al. , 1995).

5. Regulation of heterogeneity and plasticity

Regulation of stem cell heterogeneous characteristics and functions involves extrinsic factors, intrinsic factors, signaling pathways, and transcription factors. Intrinsic regulators mainly include genetic mutations, epigenetic factors, and chromatin factors. Although changes in intrinsic regulators are often small, they play a crucial role. In the hematopoietic system, stem cell behavior is consistent with the epigenetic landscape

but not always with the transcriptional state (Yu et al. , 2017). Therefore, intrinsic factors are major components of stem cell regulation, determining the stability of stem cell features. Extrinsic regulation, namely stem cell niches, includes cellular components such as stem cell progeny, neighboring epithelial cells, and stromal cells, as well as bacteria, the immune system, and biochemical substances. They restrict lineage choices and control functional heterogeneity of the stem cell population during homeostasis, injury, and cancer (Pinho and Frenette, 2019). Signaling pathways and transcription factors connect extrinsic factors to the epigenetic landscape and transcriptional state, controlling transcriptional networks that drive specific gene expression patterns in distinct micro-environments and different conditions (Figure 6).

5.1 Intrinsic regulation: direction of cell fate and maintenance of distinct but stable characteristics

As demonstrated earlier, the heterogeneity of stem cell features remains stable under homeostasis and even in separate hosts in some transplant assays. This stability suggests that there must be an intrinsic program controlling stem cell characteristics at both the genetic and epigenetic levels.

At the genetic level, clustered genes in the homeobox family can expand and manipulate the fate of stem cells(Abramovich and Humphries, 2005). In embryonic stem cells, different transposable element types are expressed in various stem cell subgroups, modulating the differentiation process and embryogenesis(He et al. , 2021). During the aging process, the deregulated P-selectin gene and other membrane-associated genes have been linked to the functional decline of HSCs(Flohr Svendsen et al. , 2021). Additionally, accumulating genetic mutations within the genome can give rise to heterogeneous cancer compartments(Welch et al. , 2012). However, in normal tissues, such unprecedented hypermutations that are sufficient for the extensive heterogeneity are usually not possible without affecting mature progeny(Muller-Sieburg et al. , 2012). Sometimes, a single gene is enough to determine the fate of a

stem cell. For example, activation of a single *Hoxc* gene can promote hair follicle regeneration by reprogramming the dermal papilla cells(Yu et al. , 2018).

In addition, modulation at the epigenetic level, such as covalent modifications of DNA and histones, plays a critical role in regulating stem cells, including fate determination and stem cell behaviors. Slight modifications in DNA methylation can determine the differentiation of hair follicle stem cells towards distinct progeny, accurately reflecting their differentiated stage(Bock et al. , 2012). In skin epithelial cells, multiple chromatin factors, including RCOR1, KDM1A, and CTBP1/2, interact with transcription factors ZNF750 and KLF4 to inhibit progenitor gene expression and promote differentiation(Boxer et al. , 2014). The differentiation fate of epidermal stem cells is then controlled by a network of epigenetic regulators, including EZH2, UHRF1, ING5, BPTF, and SMARCA5, and the interaction pattern that controls differentiation can be predicted by a Bayesian mixture model(Mulder et al. , 2012). Deletion of the histone methyltransferase *Setd8* can block proliferation and differentiation, causing apoptosis and leading to the loss of sebaceous glands and interfollicular epidermis(Driskell et al. , 2012). CBX4 maintains epidermal stem cells in a slow cell cycle and guards them against differentiation and senescence through its SUMO ligase activity(Luis et al. , 2011). During the conversion from ICM to ESC, the expression of repressive epigenetic regulators increases while that of gene activators decreases, allowing for the stability of the newly acquired epigenotype and is crucial for the maintenance of their undifferentiated pluripotent state and infinite self-renewal capacity(Tang et al., 2010). In the hair follicle, modifications of chromatin dynamics enable stem cells to cycle through quiescent and activated states without losing their infinite self-renewal and differentiating potential(Lien et al. , 2011). *Jarid2* is associated with H3K27 trimethylation and the maintenance of hair follicle stem cell proliferation (Mejetta et al. , 2011). Epigenetic factors also regulate stem cell behavior during tissue damage. After an injury, demethylases are markedly upregulated, and fewer PcGs are bound to the regulatory regions of wound-induced genes, contributing to the proliferation and migration of wound-edge cells(Shaw and Martin, 2009). Furthermore, it has been

revealed that the chromatin state is associated with the epithelial-to-mesenchymal transition (EMT) in tumors. Tumors initiated from IFE and hair follicles possess distinct chromatin landscapes, leading to more frequent EMT and metastasis in hair follicle stem cell-derived squamous cell carcinomas(Latil et al. , 2017).

Epigenetic patterns can provide important insights into stem cell identities. In embryonic stem cells, super-enhancers, a large cluster of enhancers, are bound by chromatin regulators, transcription factors, and cofactors, which play a key role in determining cell identity in both health and disease. These enhancers are related to genes that regulate normal cell biology, while in cancer, they are found at oncogenes and other tumor-associated genes(Hnisz et al. , 2013). Variation of chromatin states is consistent with lineage bias in HSCs(Buenrostro et al. , 2018). Single-cell DNase sequencing (scDNase-seq) of DNase I hypersensitive sites and histone modifications can identify ESCs and other cell types(Jin et al. , 2015), while DNA methylation maps could distinguish among blood and skin cell types(Bock et al., 2012). Human primordial germ cells experience a global DNA demethylation wave at around 10 to 11 weeks after gestation, which can identify them from ESCs and other human-induced pluripotent stem cells(Gkountela et al. , 2015). Additionally, single-cell analysis has revealed subtle differences in epigenetic patterns among stem cell subtypes. Three subsets of Mouse ESCs, identified as ES1, ES2, and ES3, exhibit different levels of histone modifications, with high, intermediate, and low H3 lysine 4 dimethylation (H3K4me2 signaling) for pluripotency signatures respectively (Rotem et al. , 2015).

5.2 Extrinsic regulation: modification of cell behavior by stem cell niches

The stem cell niche is a highly complex microenvironment surrounding stem cell populations that exhibits its own heterogeneity and compartments. It comprises multiple cellular lineages, biochemical molecules, microorganisms, immune systems, and even physical forces(Lane et al. , 2014). All of them will be reviewed here, below. During homeostasis, cell-to-cell interactions between basal stem cells and secretory

cells are critical for maintaining their differentiated states and preventing de-differentiation processes that are triggered during injury(Tata et al., 2013). Certain irreversibly committed cells can also contribute to the stem cell niche. For example, in the hair follicle, a bulge cell progeny called K6⁺ inner bulge cells can home back to the bulge and function as a niche for bulge cells. They can secrete FGF18 and BMP6 to transmit quiescence signals to the stem cells in the hair bulge, halting their proliferation(Hsu et al. , 2011). On the other hand, niches can enhance distinct stem cell identities during steady-state and modify stem cell behavior under perturbation, such as injury, transplantation, and cancer(Rognoni and Watt, 2018). For example, in the epidermis, stem cells leaving the stem cell compartments convert to transit-amplifying cells, which can promote quiescent-stem cell proliferation in return through the expression of Sonic Hedgehog (SHH)(Hsu et al. , 2014). The aggregation of dermal progenitors will activate beta-catenin in adjacent epidermal cells and determine their fates(Shyer et al. , 2017).

During development, thymic epithelial cells play a crucial role in stem cell niches. The cortical and medullary epithelial compartments are responsible for regulating lymphoid lineage fate through specialized antigen presentation(Anderson and Takahama, 2012). Epithelial Paneth cells, interdigitated among CBCs in the small intestine, provide essential ligands for the survival and proliferation of stem cells. These include secreted Wnt ligands and transmembrane Notch ligands. After leaving the crypt, the fate of CBCs is determined by their immediate cellular contact with different epithelial cells. Contact with Notch ligand-expressing secretory cells triggers Notch pathways, specifying these cells as absorptive enterocyte lineage. In contrast, contact with enterocytes, which cannot provide Notch signaling, leads to secretory fate through the expression of ATOH1(Goodell et al. , 2015). In contrast to the restricted niche in the intestine, no discernable epithelial stem cell niche has been previously recognized in the esophagus, so it is speculated that the entire basal cell layer of epithelial constitutes a stem cell niche(Doupe and Jones, 2013).

In addition to stem cell heterogeneity, stromal cell populations are also heterogeneous and possess a distinct ability to interact with stem cells and maintain their repopulation capacity. In the hematopoietic microenvironment, stromal cells have been classified into three subtypes; however, only a relatively small number of them contribute to the maintenance of long-term repopulating stem cells(Wineman et al. , 1996). Similarly, heterogeneity can be observed in both healthy and tumor dermal fibroblasts(Rinkevich et al. , 2015), highlighting the complexity and heterogeneity of stroma, much like that of epithelium. Stromal cells provide various essential signals for stem cell features. In the skin, adipocytes promote stem cell cycling(Festa et al. , 2011), restricted lineages of stem cells gradually form through epithelial-mesenchymal interactions(Yang et al. , 2017), and dermal papilla is responsible for the maintenance of hair follicle identity. Transplantation of stromal cells promotes the generation of sperms and primordial follicles(Bhartiya et al. , 2021), while Apla endothelial cells control the maintenance of HSCs in the hematopoietic system(Chen et al. , 2019). Conversely, the activation of intestinal mesenchymal cells expressing TNFSF14 impairs stem cell proliferation and promotes inflammation(Kinchen et al. , 2018).

Several types of mesenchymal cells have been found to regulate stem cell functions. Even without epithelial Wnt signaling from Paneth cells and other intestinal epithelium, the stromal cells themselves can produce enough Wnt to support normal intestinal proliferation and differentiation, as well as regeneration after injury (Kabiri et al. , 2014). In ablation assays, a subgroup of enteric glial cells marked by glial fibrillary acidic protein (GFAP)+ were found to enhance the self-renewal ability of LGR5+ ISC during injury by expressing WNT ligands (Baghdadi et al. , 2022). The lineage of fibroblasts in the dermis is crucial for dermal(Driskell et al., 2013) and perivascular cell regeneration(Goss et al. , 2021) after injury. Additionally, in the hematopoietic system, osteoblasts can activate Notch pathways in HSCs when stimulated by PTH(Calvi et al. , 2003).

Immune cells can form a dynamic and specific barrier and direct stem cells under

perturbation. For example, during injury, gamma delta T cells promote hair follicle regeneration by secreting fibroblast growth factor 9 (Fgf9), which triggers Wnt expression and activates fibroblasts to also express Fgf9, forming a feedback mechanism that amplifies proliferation signaling (Gay et al. , 2013). Immune cells are also involved in the interaction between bacteria and epithelial cells. Skin-resident dendritic cell subsets sense local microorganisms, such as the colonization of *Staphylococcus epidermidis*, and induce commensal-specific T-cell responses to enhance the anti-bacterial defense of keratinocytes without inflammation formation (Naik et al. , 2015). Moreover, during homeostasis, the immune system can still control stem cell functions through immune molecules like antibodies. For instance, integrin antibodies promote the interactions between stem cells, extracellular matrix, and niche cells, leading to decreased stem cell differentiation in the epidermis (Byron et al. , 2009, Evans et al. , 2003). In the bone marrow niche, regulatory T cells with CD150 maintain HSC quiescence and prevent allogeneic HSC rejection by adenosine (Hirata et al. , 2018), while macrophages play a supportive role in the proliferation of HSCs and multipotent progenitors (Gao et al. , 2022).

Biochemical substances, such as various receptor ligands and cytokines, are important components of stem cell niches and facilitate communication between niche cells and stem cells without direct cell-to-cell contact. These substances can originate from various sources, such as the autocrine signaling of stem cells themselves, paracrine signaling from neighboring niche cells, surfacing bacteria, or the circulating blood.

For example, thrombopoietin is not only the primary extrinsic regulator of platelet development but is also essential for the maintenance of a specific subset of platelet-biased HSCs in mice (de Sauvage et al. , 1996). Overexpression of Dickkopf1 in stem cell niches can increase cell cycling, but also represses the regenerative function of stem cells by inhibiting Wnt signaling (Fleming et al. , 2008), while DL4- μ beads, along with several cytokines, can direct T cell differentiation from the pluripotent stem cells without stromal cells (Trotman-Grant et al. , 2021). Besides, a purine derivative, aryl

hydrocarbon receptor antagonists can promote the expansion of HSCs *in vitro* (Boitano et al. , 2010). However, some biochemical substances can also promote cancer. For instance, epidermal growth factor receptor ligands can selectively promote alveoli type 2 pneumocyte self-renewal *in vitro*, leading to the production of multifocal, clonal adenomas (Desai et al., 2014). Similarly, cancer-associated fibroblasts secrete TGF beta and SDF1, promoting malignancy in a non-cell-autonomous manner (Scherz-Shouval et al. , 2014).

It is intriguing that the regulation mediated by extracellular biochemical substances is far more complex and heterogeneous than we ever thought. Even the effect of a single niche molecule can vary depending on the niche cells that express it and the stem cell subsets it acts upon. For instance, while the removal of Cxcl12 from different niche cell populations produces distinct effects on HSCs, deletion from mineralizing osteoblasts has no impact on HSCs. Endothelial cells and mesenchymal progenitors expressing CXCL12 support HSCs' long-term self-renewal capabilities, while CXCL12-abundant reticular cells and osteoblasts promote B-lymphoid progenitors and retain HPCs in the bone marrow (Greenbaum et al. , 2013). Therefore, specific cytokines can elicit varied responses from different subsets of HSCs. For instance, a low dose of TGFβ supports My-bi HSCs while suppressing Ly-bi HSCs (Challen et al., 2010). In contrast, IL-11 increases the Ly-bi lineage while decreasing the My-bi lineages (Dykstra et al., 2007).

Epithelia are colonized by specific bacteria at distinct locations a process that depends on the ecology of the epithelial surface and involves both endogenous host factors and exogenous environmental factors (Grice and Segre, 2011). As a result, heterogeneous bacterial colonies can serve as part of the niches and modulate stem cell function. During early life, the formation of the lung microbiota increases the generation of goblet cells in the epithelial layer, helping to build tolerance towards allergens (Gollwitzer et al. , 2014). Furthermore, bacterial products such as muramyl dipeptide, which is common to all bacteria and acts as an agonist of NOD2, are essential to produce resistance to cytotoxic injury and sustain the survival of ISC in the

intestine(Nigro et al. , 2014).

5.3 Signaling pathway and transcription factors: communications between the extrinsic niches and intrinsic transcription processes

Signaling pathway serves as a bridge between extrinsic niches and the intrinsic transcription process. These pathways consist of various signaling cascades, such as Wnt signaling, Notch signaling, extracellular signal–regulated kinase (ERK) signaling, BMP signaling, and more. When stem cell niches receive stimuli from elements in the environment, they either activate or inhibit certain signaling pathways through direct cell-to-cell contact or the release of secreted biochemical substances. Additionally, changes in transcription factors are often associated with modifications to these signaling pathways. The expression of stem cell markers also reflects the signaling pathways that regulate stem cell lineages. For instance, *Lgr5* is a Wnt target gene that associates with the Frizzled/Lrp Wnt receptor complex, which responds to stimuli from cell niches(de Lau et al. , 2011).

Although stem cells from various tissues display heterogeneity, many of them share common signaling pathways during different physiological and pathological conditions. One of the most prominent examples is the Wnt pathway, which plays a vital role in various tissue development and maintenance processes. For instance, Wnt signaling is responsible for regulating the expansion of scales during epidermal development and maintaining scale-interscale boundaries in adult skin(Gomez et al., 2013). During esophageal development, Wnt pathways are crucial for separating the esophagus and the trachea to form their respective tissues(Jacobs et al., 2012). In the ovary, distinct Wnt signaling activity, organized spatially, determines the fate of ovarian follicle stem cells(Reilein et al. , 2017). Similarly, in the intestine, the Wnt pathway regulates ISC subsets differently, driving the proliferation of CBC cells while qISCs are resistant to those signals and remain quiescent during homeostasis(Yan et al., 2012). In addition to epithelial cells, Wnt signaling also plays a significant role in mesenchymal

compartments. For example, Wnt signaling mediates the communication between different fibroblast subsets involved in epidermal stem cell niches(Kumar et al. , 2014). Different dermal compartments are also regulated by epidermal Wnt signaling(Driskell et al., 2013). During injury, Wnt signaling promotes upper dermal lineage expansion, permitting hair follicle regeneration instead of scar formation(Driskell et al., 2013, Ito et al. , 2007). Moreover, Wnt signaling is essential for basal nail stem cells to orchestrate the regeneration of wounded tissue and attract nerves that stimulate mesenchymal blastema growth(Takeo et al. , 2013). Notably, abnormal Wnt signaling function also affects tumor formation. For instance, RSPO-LGR4 can promote the proliferation of leukemia stem cells by upregulating the Wnt signal(Salik et al. , 2020).

Notch signaling also plays a significant role in determining the behavior and fate of stem cells. In contrast to Wnt signaling, Notch signaling occurs through cell-to-cell interaction by transmembrane ligands(Siebel and Lendahl, 2017). In the intestine, activation of Notch signaling occurs as the nascent progenitor cells leave the stem cell compartment, leading to the development of absorptive lineage. Without Notch signaling, those cells will express Atoh1 and become secretory progeny(He et al. , 2004, Noah and Shroyer, 2013). Similarly, in the salivary glands, the enrichment of Notch signaling in the inner layers of the endbud determines luminal fate instead of acinar fate(Chatzeli et al. , 2023). Moreover, Notch signaling is vital for maintaining normal cell behavior. For example, downregulation of Notch signaling in the skin can cause atrophy, inflammation, and cancer(Hu et al. , 2012). Similarly, loss of Notch signaling in esophageal epithelium leads to excessive proliferation of mutant cells(Adam et al. , 2015). Under stressful conditions, Notch signaling in blood vessels of bone marrow is repressed, leading to a biased differentiation program towards myeloid lineages(Tikhonova et al. , 2019).

The extracellular signal-regulated kinase (ERK) pathway has been found to be associated with the fate and behavior of stem cells. Time-lapse imaging and microfluidics have shown that HSC progeny with intermediate ERK signaling express

more CD45A than those with transient or sustained ERK signaling(Wang et al., 2021b). Moreover, mitochondrial phosphoenolpyruvate carboxykinase (PCK2) has been found to enhance the osteogenic differentiation of mesenchymal stem cells (MSCs) through ERK1/2 cascades(Li et al. , 2022b).

Transcription factors play a crucial role, either directly or indirectly, in regulating stem cell fate and behavior. Both Wnt signals and BMP inhibitory signals contribute to the stabilization of β -catenin(Andl et al. , 2004, Kobiela et al. , 2003, Zhang et al. , 2006), which acts as a transcription cofactor for the TCF/Lef1 family of DNA binding proteins(Clevers, 2006). The discrete expression of transcription factor ASCL2 specifies stem cells, which enables the translation of the Wnt gradient into intestinal stem cell identity(Schuijers et al. , 2015). LHX2 is required for β -catenin function in the Wnt pathway and plays a crucial role in maintaining cortical progenitor proliferation(Hsu et al. , 2015). It also helps balance cell quiescence and lineage differentiation of hair follicle stem cells (Folgueras et al. , 2013). CDX2 is a transcription factor that activates the mTORC1 and Wnt/ β -catenin signaling pathways(Fan et al. , 2017). Lgr5-positive stem cells in the intestine require CDX2 to maintain their identity, and their loss will convert them into gastric stem cells(Simmini et al. , 2014). Similarly, the transcription factor GATA4(Thompson et al. , 2017) is involved in the acquisition of jejunum identity over ileum identity, which only occurs in the absence of Wnt signaling(Middendorp et al., 2014).

The centrosome plays a critical role in the transcriptional regulation of stem cells. For instance, transcription factors such as PAX6 (Walcher et al. , 2013) and SOX4 (Tiwari et al. , 2013) regulate NINEIN and AKNA in neural stem cells. Both of these proteins are essential for microtubules anchoring. have demonstrated the vital role of NINEIN in stem cell maintenance by anchoring microtubules at the subdistal appendage (SDA) (Wang et al. , 2020a). On the other hand, the centrosomal microtubule organizing center (MTOC) activity is crucial for preserving stem cell identity by regulating specific signaling pathways. Impairment of centrosomal MTOC activity leads to a stiffening of

the apical membrane, activating the YAP pathway, which, in turn, induces cell proliferation and overproduction of progenitor cells. (Shao et al. , 2020).

6. Stem cell heterogeneity is related to various diseases and treatment strategies

6.1 Tumor

Single-cell sequencing studies conducted on glioblastoma(Patel et al. , 2014), gastric cancer(Yan et al. , 2018), and liver cancer(Ho et al. , 2019) (Lee et al. , 2022) have revealed that tumors exhibit several characteristics similar to stem cells, including the ability to self-renew, stemness-related expression states, and significant intra-tumoral heterogeneity, including copy number variations, cell cycle, immune response, and hypoxia. Despite these similarities, stem cells and cancer cells differ in their behavior and fate, such as differences in proliferation potential and sensitivity to various stresses. Thus, malignancy can be considered a unique form of stem cell heterogeneity and plasticity (Figure 7).

6.1.1 Cancer in a mutant cell: cancer stem cells and competitive advantage

It is postulated that tumors follow a hierarchical program in which cancer stem cells represent the apex of the cancerous hierarchy due to their highest proliferating capability. Mutations in cancer stem cells typically arise in oncogenes or tumor suppressor genes, making it easier to develop additional mutations. In juvenile myelomonocytic leukemia, for example, all somatic mutations can be traced back to the Ras pathway mutation from the very beginning(Louka et al. , 2021). Multiple secondary or tertiary mutations can create a variety of new cell clones, which coexist to form the cancerous hierarchy(Welch et al., 2012). These mutations provide advantages over normal cells, and the tumor grows through asymmetric competition.

In addition to tumor suppressor genes such as APC, PTEN, and TP53, DNA mutations were found in chromatin modifier genes, and the resulting changes in the epigenome, in turn, affected further accumulation of gene mutations(Heide et al. , 2022). Non-genetic changes in cell phenotype, such as epigenetic factors, can also drive a hierarchy of cancer stem cells where mutations do not play a role. Single-cell sequencing revealed that breast cancer's same chemotherapy-resistant genotype further developed subpopulations with different transcriptional profiles by reprogramming(Kim et al. , 2018). Apart from genetic perturbations, clinical treatment regimens could also induce further plasticity to the heterogeneous tumor population(Tang, 2022). For instance, after being exposed to castration, some of the double knockout prostate tumors changed their identity to become NE-like cells(Ku et al. , 2017).

Otherwise, mutations may occur on bona fide stem cells or progenitor cells, gradually altering their proliferating properties through multi-step transcriptional reprogramming and fate switching(Wang et al. , 2021c). For example, JAK2V617F+ myeloproliferative neoplasms are associated with JAK2 mutations in the megakaryocyte-primed HSC subpopulation(Tong et al. , 2021). Mutations in genes encoding cohesion in HSCs present a pre-leukemic state and generate multiple further mutations, resulting in acute myeloid leukemia(Corces-Zimmerman et al. , 2014, Shlush et al. , 2014). Similarly, a single-cell transcriptomic atlas revealed that colorectal adenomas derive from stem cell amplification driven by Wnt signaling instead of metaplasia in differentiated cells(Chen et al. , 2021a). In this manner, mutations in cancer stem cells disrupt the normal regulation of stem cells and provide a competitive advantage over other stem cells, leading to tumor formation. For instance, inhibition of the Notch pathway in esophageal epithelial stem cells increased their ability to proliferate and help surrounding cells differentiate, leading to tumor formation(Alcolea et al. , 2014). In the mouse intestine, stem cells were tested with mutations in Apc, K-Ras, and TP53 genes, respectively; however, only a TP53 mutation provided a proliferative advantage, and other mutant clones were replaced by wild-type stem cell lineages(Vermeulen et al. , 2013).

Cancer stem cells have been identified in some cases, while in others, many cells may function as stem cells. For example, in the mouse intestine, 5%-10% of the cancer cell population is Lgr5+, responsible for adenoma growth(Schepers et al. , 2012). Similar cell groups have been identified in mammary tumors through lineage tracing(Zomer et al. , 2013). In malignant squamous cell carcinomas, clonal analysis showed cancer stem cell-like behavior, whereas in papillomas, which are benign epithelial tumors, the bulk of the tumor is generated by multiple tumor cells(Driessens et al. , 2012).

6.1.2 Cancer in a non-mutant cell: excessive acquisition of plasticity

Cancer can arise due to changes in regulatory factors such as signaling pathways or cell niches even in the absence of genetic mutations, which may occur spontaneously or during injury.

Exposure to UVA light can downregulate stromal Notch signaling, leading to stromal atrophy and epithelial cancerization(Hu et al., 2012). The paracrine action of PGE2 from pericryptal Ptgs2-expressing fibroblasts can initiate sporadic tumor formation(Roulis et al. , 2020). Cancer-associated fibroblasts (CAFs) expressing HSF1 can promote cancer formation by secreting TGF-beta and SDF1(Scherz-Shouval et al., 2014). Metabolic reprogramming also contributes to tumor initiation. For example, active glycolysis in quiescent cells with +4 ISC and enteroendocrine markers promotes stemness and tumorigenic potential in the intestine(Sebastian et al. , 2022).

In addition to the disruption of metabolic and signaling pathways, the tumor niche plays a crucial role in the initiation and progression of cancer. Cancer-associated fibroblasts (CAFs) rely on the transcription co-activator YAP for several functions such as angiogenesis, ECM deposition, and increased matrix stiffness(Calvo et al. , 2013). This could promote tumor progression by expressing microRNA-dependent PTEN(Mouw et al. , 2014). Furthermore, removing fibroblasts through lineage-specific ablation can decrease melanoma growth(Rinkevich et al., 2015).

6.2 Cellular therapy of stem cell

The concept of harnessing the remarkable plasticity of a patient's own stem cells to obtain a variety of cell types for therapy without the risk of immune rejection is an enticing prospect. However, procuring tissue stem cells is not an easy task, which limits their value for stem cell therapy, and this has redirected our attention towards induced pluripotent stem cells (iPSCs). Not only are they accessible, but they also have the potential to generate all types of tissue stem cells.

iPSCs are patient- and disease-specific pluripotent stem cells derived through reprogramming of differentiated human somatic cells by transduction of four defined transcription factors (Takahashi et al. , 2007). iPSCs have proven to be successful in generating a diverse array of tissues, demonstrating their significant therapeutic potential. For instance, iPSCs can produce all lineages of skin epithelial cells and also generate associated mesenchymal cells like fibroblasts or melanocytes, making them useful in skin regeneration(Bilousova and Roop, 2014). When human endoderm stem cells derived from PSCs are transplanted into the liver of rodents and swine, they can ameliorate hepatic injury(Xu et al. , 2023). The kidney can be produced from PSC by emphasizing the regulation of vascularization(Koning et al. , 2020). Even fine brain subregions like hypothalamic arcuate organoid models have been generated from human iPSCs, offering a promising platform for exploring nucleus-specific features in human arcuate development(Wang et al. , 2021a).

The safety profile of reprogramming is a major concern for the clinical use of iPSCs. Creating iPSCs using genome-integrating viruses poses potential risks. Consequently, various alternative methods have been explored since the inception of iPSC technology in 2007, including the use of nonintegrating adenoviruses (Stadtfield et al. , 2008), non-viral plasmids (Okita et al. , 2008), non-integrating episomal vectors (Yu et al. , 2009), as well as the utilization of proteins (Zhou et al. , 2009) and modified mRNA (Warren et al. , 2010) to generate iPSCs. More recently, chemical reprogramming has emerged

as a safer approach for iPSC generation. By inducing an intermediate plastic state, human chemically reprogrammed pluripotent stem cells can successfully exhibit key characteristics of embryonic stem cells (Guan et al. , 2022). In non-human primates, directed differentiation towards pancreatic islets through chemical reprogramming has restored insulin secretion and improved glycemic control effectively and safely (Du et al. , 2022). Furthermore, *in vivo* chemical reprogramming facilitates tissue repair *in situ* from adjacent mature cell types. For instance, chemical reprogramming from fibroblasts to cardiomyocytes has been induced successfully through oral or intraperitoneal administration in mice (Huang et al. , 2018).

While chemical reprogramming has alleviated concerns about safety, the challenges associated with the use of iPSCs primarily arise from three key aspects. Firstly, it is challenging to direct the differentiation of iPSCs into specific tissue stem cells and to purify the desired cells (Murry and Keller, 2008). For instance, the differentiation potential of HSCs derived from human iPSCs is highly skewed toward the myeloid lineage. This could be due to the fact that ESC preferentially generates My-bi stem cells during development since the differential potential of ESC-derived HSCs *in vivo* is still myeloid skewed (Woods et al. , 2011). Secondly, immaturity is still a concern with ESCs and iPSCs. It is yet to be determined how similar the tissues derived from iPSCs are to adult or fetal tissues (Stutchfield et al. , 2010). For example, fetal liver progenitors and ESC-derived hepatic precursors appear to be less efficient at generating liver tissues than transplantation of adult hepatocytes (Agarwal et al. , 2008). Additionally, in some circumstances, immune rejection is still hard to avoid, as grafting My-bi HSCs will inevitably produce a low level of lymphocytes (Muller-Sieburg et al., 2012).

6.3 Gene therapy of stem cell

The recent advancements in highly precise and safer gene editing technologies have opened up new opportunities to enhance the development of hematopoietic stem/progenitor cell (HSPC) gene therapy (GT). This therapy aims to reconstitute the

hematopoietic system by correcting autologous HSPCs using gene editing techniques. The first licensed ex vivo gene therapy, Strimvelis, introduced ADA genes into HSCs using a viral vector and yielded remarkable clinical responses in a patient with ADA-SCID (Aiuti et al. , 2017). Soon, the first gene therapy trial for sickle cell disease, which involved the lentiviral vector-mediated addition of an anti-sickling β -globin gene into autologous hematopoietic stem cells, resulted in significant clinical improvements (Ribeil et al. , 2017).

Beyond the hematopoietic system, HSPCs can serve as delivery vehicles for inherited neurometabolic disorders. While enzyme replacement therapy is effective for some metabolic disorders, its application in neurological disorders is hindered by the inability of proteins to cross the blood-brain barrier. Fortunately, genetically corrected HSPC-derived myeloid cells can migrate to the central nervous system and secrete the necessary enzymes to correct the functions of surrounding cells (Capotondo et al. , 2012). Building on this concept, gene therapy has enabled 15 out of 17 X-linked adrenoleukodystrophy patients to live free of major functional disabilities (Eichler et al. , 2017). Similar progress has also been observed in the treatment of metachromatic leukodystrophy (Sessa et al. , 2016).

Off-target DNA changes pose a significant safety concern in vector-based gene addition. The emergence of high-fidelity Cas9 nucleases has raised hopes for safer genome engineering through techniques like gene editing, base editing (Rees and Liu, 2018), and prime editing (Chen et al. , 2021b). However, the clinical safety of gene editing in HSPC-GT is yet to be fully established (Tsai et al. , 2015), as DNA double-strand breaks at genomic off-target sites continue to present a potential source of genotoxicity (Kosicki et al. , 2018). Additionally, concerns revolve around the toxic effects of alkylating agents required for most HSCT applications, making it necessary to explore the replacement of alkylating agents with other conditioning regimens (Ferrari et al. , 2021). Furthermore, achieving genetic engineering of human HSPCs in vivo, rather than ex vivo, remains a formidable challenge. For instance, reaching a sufficient

proportion of resident HSCs for therapeutically effective chimerism is difficult. Simultaneously, intrinsic safety challenges associated with biodistribution still persist (Ferrari et al. , 2023).

7. New approaches for the characterization of stem cell heterogeneity

Research on the heterogeneity of stem cells has been ongoing for many years. However, the dyeing technique, which is primarily used to identify and track different skin stem cell subsets, has limitations in terms of specificity and sensitivity due to technical conditions. Furthermore, the results obtained are the average values of multiple cells, making it impossible to exclude the interference of non-stem cell signals and track the evolution of cell fate in pathological conditions. Fortunately, novel techniques have been developed that allow for the accurate differentiation and tracking of the heterogeneity of stem cells and the evolution of a single stem cell's fate in pathological conditions.

7.1 High throughput strategies of single-cell RNA sequencing

In recent years, single-cell RNA sequencing has seen an increase in throughput, allowing for a more comprehensive understanding of stem cell heterogeneity and the identification of stem cell sub-populations in unprecedented detail (Table 3). Compared with tube-based protocols, combining microfluidic technology with single-cell whole-transcriptome analysis has been shown to significantly enhance the precision and sensitivity of transcriptome detection when working with limited amounts of RNA. Meanwhile, by performing the reactions in a microfluidic system, it is much easier to discriminate real biological variability from the technical noise (Streets et al., 2014). With the Disco system, it is now possible to process low-input single-cell suspensions of small tissue samples efficiently instead of requiring large cell inputs (Bues et al. , 2022). The technique has seen further improvements, enabling the detection and

utilization of overloaded microfluidics, optimizing throughput, and reducing confounding effects(Datlinger et al. , 2021, Kang et al. , 2018). High sensitivity can also be achieved by combining sequencing with stochastic barcoding of single cells, which can analyze a few thousand cells simultaneously and can readily scale up to 10,000s or even 100,000s of cells(Fan et al. , 2015).

7.2 Spatial transcriptomics

Spatial heterogeneity of gene expression strongly influences cell function, yet single-cell RNA sequencing fails to provide such information. Early attempts to obtain spatial information relied on in situ hybridization(Eng et al. , 2019, Lee et al. , 2015, Trecek et al. , 2017). However, these techniques require specialized knowledge, sophisticated imaging, and complicated equipment, rendering them impractical for cross-lab validation(Perkel, 2019). Recently, an evolving technology called spatial transcriptomics uses DNA-barcoded beads to provide high-quality spatially resolved gene expression data(Ståhl et al. , 2016). Other emerging methods, such as Slide-seq(Rodriques et al. , 2019), Slide-seqV2(Stickels et al. , 2021) and HDST(Vickovic et al. , 2019) have optimized spatial resolution, detection sensitivity, and provided high-plexity single-cell detection capacity. To simplify the process and make it more user-friendly for any lab, 10X Visium(Maynard et al. , 2021, Salmén et al. , 2018) has made the technique commercially available, while DBiT-seq(Liu et al. , 2020) directly barcodes biomolecules in tissues instead of on a substrate, obviating the sophisticated steps of decoding the beads and enabling multi-omics spatial sequencing of mRNAs and proteins (Table 3).

7.3 Single-cell lineage tracing and barcodes

Lineage tracing is a powerful technique to study stem cell heterogeneity under steady-state conditions, as it can identify stem cells and their progeny without disaggregating tissue or disturbing homeostasis. During the process of proliferation and differentiation, a stem cell population not only gives rise to specific progeny but also produces tiny

replication errors along the way. These errors can be utilized to trace the developmental lineage of cells and reveal stem cell heterogeneity. Lineage tracing is designed to uncover the developmental lineage tree from the genome through whole-genome sequencing of single cells for these replication mutations (Carlson et al. , 2011). This method has been applied to elucidate the contributions of embryonic cells to adult tissues in mice (Behjati et al. , 2014). However, due to the low rate of endogenous somatic mutations, the expensive deep whole-genome sequencing, and the incompatibility with *in situ* readouts, exogenous markers that can mutate at a higher rate are adopted to track the cell lineages (Chen et al. , 2022). Barcodes are specific nucleic acid sequences that can consistently label cells with unlimited sequences and have been used to track cells in lineage tracing and high-throughput sequencing (Kebschull and Zador, 2018). New technologies have emerged based on barcodes to improve the efficiency of cellular labeling and fate mapping. For example, GESTALT, a CRISPR barcode, inserts multiple CRISPR–Cas9 target sites into barcodes to accumulate various mutations during repopulation, enabling high-resolution maps of stem cell lineages (McKenna et al. , 2016). Polylox barcoding has also been developed to achieve large-scale fate mapping with non-invasive techniques (Table 3). By using Cre recombinase, constant random DNA recombination will be produced, resulting in high label diversity (Pei et al. , 2019). With the help of Polylox recombination *in situ*, extreme diversity of barcodes has been realized and the separation between myeloid-erythroid and lymphoid lineage has been revealed (Pei et al. , 2017). More recently, base-editing barcodes have allowed higher resolution mapping of the cell phylogeny with more than 20 mutations per barcode (Liu et al. , 2021). Similar to the lineage map, the metastasis map has also been generated by barcodes to reveal heterogeneous patterns and mechanisms for cancer metastasis (Jin et al. , 2020).

7.4 ‘Pseudotime’ analysis and bioinformatics tools

The concept of pseudotime enables us to capture the dynamic changes in transcriptomes

during development or differentiation processes by sequencing a large number of cells at a given time point. Several bioinformatics tools have been developed to capture the developmental dynamics of heterogeneous stem cell populations (Table 3). The Waterfall bioinformatics pipeline was developed to reconstruct the developmental trajectory de novo and successfully capture molecular cascades during the initiation of neurogenesis(Shin et al. , 2015). Monocle, another unsupervised algorithm, can increase the temporal resolution of the transcriptome and provide a time-serial re-establishment of a developmental process by collecting single-cell RNA sequencing data at multiple time points(Trapnell et al. , 2014). Similar tools have been used to reconstruct the continuous route of differentiation in the lung(Treutlein et al., 2014) as well as the trajectory of neurogenesis in the adult subventricular zone(Llorens-Bobadilla et al., 2015). To reconstruct complex developmental trajectories, SCUBA has utilized nonlinear dynamics and stochastic differential equation theories to model bifurcating developmental trajectories(Marco et al. , 2014), while Monocle 2 has realized an accurate reconstruction of complex trajectories with multiple branches through its Reversed Graph Embedding strategy(Qiu et al. , 2017).

8. Summary

Since the discovery of stem cells over 100 years ago, numerous debates about the interrelationships among various cell types have arisen, and unprecedented insights into the characteristics and regulation of stem cells have been gained. Despite the distinct features of stem cell pools in different tissues, they share some general similarities regardless of their origins. Stem cells themselves, their surrounding niches, and their signals are heterogeneous with a consortium of distinct subsets genetically preprogrammed for different functions, behaviors, and fates. Moreover, each of these heterogeneous subsets exhibits striking adaptability during stress and perturbation.

Given our comprehensive understanding of stem cells and their features, it is essential to identify the specific types of stem cells required for transplantation in distinct circumstances accurately. Furthermore, by delving into the complexity of stem cells' intrinsic regulation network and extrinsic niches, we can modulate the behavior of stem cells more precisely, leading to wider use in regenerative medicine, transplantation, and malignant prevention. Finally, the combination targeting of stem cells themselves, their intrinsic regulation network, and extrinsic niches may provide supplementary opportunities for synergy.

Single-cell sequencing techniques with increasingly higher spatial and temporal resolution, lower technical noise, and lower prices will ultimately generate systemic maps of epigenetic, transcriptional, and lineage information precisely at the single-cell resolution, offering us deeper insights into stem cell heterogeneity and its regulation network under physiological and pathological conditions, and optimizing our understanding of development, disease, and malignancy.

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Tables

Table 1. Differences between Ly-bi and My-bi stem cells

HSC subsets		Ly-bi	My-bi
biased differentiation program	lymphoid progenitor	normal	attenuated
	myeloid progenitor	attenuated	normal
other dynamic behavior	life span	short	long
	onset of repopulation	early	delayed
age-related proportion	younger D2 mouse	80%	20%
	aged D2 mouse	20%	80%
degree of Hoechst dye efflux		least dye efflux	greatest dye efflux
surface marker	CD150	lower	higher
	CD229	higher	lower
	CD41	negative	positive
reaction to various factors	high dose of TGFβ1 (10 ng/ μ L)	inhibitory	inhibitory
	low dose of TGFβ1 (10 ng/ μ L)	inhibitory	stimulatory
	IL-11	stimulatory	inhibitory

Table 2. Surface marker of stem cells in the skin

Location	Surface marker	References
stem cells adjacent to the sebaceous gland	Blimp	(Page et al., 2013)
basal layer of the sebaceous gland	Lrig1	(Nowak et al., 2008)
junctional zone	Lrig1, PLET1	(Jensen et al., 2009)
Isthmus	LGR6	(Snippert et al. , 2010a)
bulge	LGR5, CD34, NFATC1 K15, K19, TCF3, TCF4, LHX2, SOX9, Gli1	(Jaks et al. , 2008, Liu et al. , 2003),(Greco et al., 2009)
hair germ	Lgr5, TCF3, Gli1	(Brownell et al. , 2011, Howard et al. , 2014)

Table 3. Summary of new approaches for stem cell heterogeneity

Method	Advantages	Disadvantages	References
Droplet-based high-throughput single-cell RNA sequencing			
inDrops	A open-source system, possesses flexibility for nonstandard approaches or modifications for different types of protocols.	Higher error rate. Lower sensitivity, might be due to excessive cDNA amplification.	(Zhang et al. , 2019), (Zilionis et al. , 2017)
Drop-seq	Lower experimental cost, especially for a large number of single cells.	Small compromises in sensitivity and precision.	(Zhang et al., 2019)
10X Genomics Chromium	Higher molecular sensitivity and precision. Less technical noise.	Higher price.	(Zhang et al., 2019)
demuxlet	Harness natural genetic variation to demultiplex singlets and detect doublets from multiple individuals, thereby lowering per-sample library preparation cost and reducing confounding effects.	Sample processing and preservation for solid tissues remain challenges.	(Kang et al., 2018)
scifi-RNA-seq	Massively boosts the throughput by resolving transcriptomes from overloaded microfluidics chips. Cost-effectiveness. Not limited to scRNA-seq, but could also be used to enhance the throughput of other single-cell assays such as single-cell ATAC-seq, whole genome sequencing and combine them in the same cell.	Lower quality than the scRNA-seq using fresh intact cells.	(Datlinger et al., 2021)
scNT-seq (single-cell metabolically labeled new RNA tagging sequencing)	Measures the temporal RNA dynamics by joint profiling of newly transcribed and pre-existing mRNAs from the same cell. Highly scalable. Cost-effective. Compatible with cryo-preserved cells.		(Qiu et al. , 2020)
DisCo	Process low-input single-cell suspensions of small tissue samples efficiently. Process low-input samples at a strongly decreased cost.	Possess lower throughput than stochastic droplet systems.	(Bues et al., 2022)
Spatial transcriptomics			
FISSEQ (fluorescent in situ sequencing)	High spatial resolution (subcellular) with theoretical limits over 16,000 genes. Compatible with both formalin-fixed paraffin-embedded (FFPE) tissues and fresh frozen samples.	Laser-induced damage causes image degradation after 25 total cycles. Small scannable areas (4 × 4 mm). Sophisticated and impractical for cross-lab validation, requiring a lengthy and technically demanding imaging process.	(Lee et al., 2015)
Spatial Transcriptomics	Provides visualization and high-quality quantitative gene expression data with spatial resolution. Does not require specialized knowledge or equipment.	Low spatial resolution (hundreds of micrometers).	(Ståhl et al., 2016)
Slide-seq	Spatial analysis of gene expression with high spatial resolution (10-µm). Unbiased and genome-wide analysis. Scalability to large tissue volumes. Does not require advanced knowledge or equipment.	Low transcript detection sensitivity. Does not include histology. Sophisticated steps like decoding the beads.	(Rodrigues et al., 2019)

Slide-seqV2	Magnitude higher sensitivity.	Sophisticated steps like decoding the beads.	(Stickels et al., 2021)
HDST (high-definition spatial transcriptomics)	Highest spatial resolution (2- μ m). Relies on commoditized tissue, molecular, bead-array, and imaging modular tasks, instead of pre-selected markers or nonstandard instrumentation.	HDST data is currently relatively sparse. Sophisticated processes.	(Vickovic et al., 2019)
10X Visium	Commercially available. Requires no special instruments and straightforward to use in any lab. The recently released Visium FFPE is compatible with FFPE tissues and can realize full transcription spectrum detection of more than 18000 genes. Allow for immunofluorescence protein detection as well.	Spatial resolution is limited to 55 μ m. The quality of cryosection massively affects the results.	(Salmén et al., 2018) (Maynard et al., 2021)
DBiT-seq	High spatial resolution and large numbers of detectable genes per 10-mm pixel. Excellent data quality and high genome coverage. Realize multi-omics spatial sequencing of mRNAs and proteins. Does not require specialized knowledge of microfluidic control. No sophisticated processes, and the device is simple. Does not require the lysis of tissues to release mRNAs. Compatible with formaldehyde-fixed tissue slides.	Does not directly resolve single cells, but derives single-cell spatial omics by computation. A limit of 5 μ m pixel size in practice, although theoretical pixel size can be 2 μ m. Currently small scannable areas in 10 mm pixel (1 x 1 mm), but can be improved by a serpentine microfluidic channel design.	(Liu et al., 2020)
Single cell lineage tracing			
Embedded viral barcodes	Provides high sensitivity and throughput, reducing the impact of experimental noise and providing identification of small changes in barcode abundance. Cost efficient. Does not require specialized computational skills, and the genetic barcodes are easy to recover by a single PCR step. It can be adapted to both in vitro and in vivo experiments with high fidelity.	Requires cells to be susceptible to viral transduction, and tolerate cell isolation, short-term culture, and transplantation. In vivo injection of barcode-carrying virus might lead to the label of unwanted cells and uneven transduction. Lentiviral vector, cell isolation, and culture might alter cell behavior and change cell properties. The transduction efficiencies for different cell types are different.	(Bramlett et al., 2020)
Polylox barcodes	Non-invasively labels thousands of individual cells with high barcode diversity and high resolution in vivo. Has a more defined genomic location of the barcode.	Expensive since it requires long-read sequencing. Requires experimental models with inducible Cre to provide transient Cre activity for Polylox barcodes. Rely on the hypothesis that the DNA recombination is random, which may not be entirely true.	(Pei et al., 2017, Pei et al., 2019)
PolyloxExpress	Realizes joint readout of barcodes and single-cell transcriptomes, providing transcriptional signatures of individual cell fates under physiological conditions.	Expensive since it requires long-read sequencing. Requires experimental models with inducible Cre to provide transient Cre activity for Polylox barcodes. Rely on the hypothesis that the DNA recombination is random.	(Pei et al., 2020)

GESTALT: CRISPR barcodes		There are chances that identical edits may recur in distantly related cells, which can confound lineage inference.	(McKenna et al., 2016)
		Nonuniform editing efficiencies may lead to suboptimal sequence diversity.	
		Intertarget deletions may contribute to the loss of information.	
	Application at the level of a whole, complex multicellular organism.	Editing to early embryogenesis may be restricted by transient means of introducing Cas9 and sgRNAs.	
	The rates and patterns of editing are editable by adjusting expression levels and delivery mode of editing reagents.	Isolated tissues are easily contaminated by blood, the cells in which may obstacle the assignment of alleles to specific organs.	
	Efficiently obtain the information of barcodes from RNA or DNA by a single sequencing read from each of hundreds of thousands of cells in single cells.	Editing is not directly coupled to the cell cycle, limiting the resolution of reconstruction of lineage relationships.	
		The anatomical resolution was restricted to organs and tissues.	
	Uses indel mutations, which would lead to missing information by removing earlier mutations already recorded on a barcode.		
SMALT: base-editing barcodes	Uses a short barcode sequence at a single genomic locus instead of a barcode set in separate genomic loci to represent a cell.	The barcode is not recoverable by scRNA-seq by far.	(Liu et al., 2021)
	Achieves 21 mutations per barcode with an effective barcoding space larger than 10^{30} , which is higher than other methods.	The performance at very early nuclear cleavage cycles was not satisfactory.	
	Produces substitution mutations, which is the typical mutation type used and many algorithms can be readily applied to it.	There are many errors in the cell trees.	
'Pseudotime' analysis			
Monocle	Exploit the temporal variability at the single-cell level.		(Trapnell et al., 2014)
	Be able to explore poorly characterized temporal processes since it does not rely on known marker genes.	Only reconstruct linear trajectories.	
Waterfall	Reconstruct continuous biological processes at single-cell resolution.		(Shin et al., 2015)
	Requires very little prior information to perform.		
	Assign each cell a 'pseudotime' based on its proximity to the cluster-derived trajectory, instead of directly connecting each cell to the next.	Only reconstruct linear trajectories.	
	Applicable for not only single-cell RNA-seq datasets, but also single-cell mass-cytometry datasets and two synthetic datasets.		

SCUBA	Identification of complex processes involving multilineage specification with temporal resolution. Automatically detect bifurcations without using prior biological knowledge. Detects bifurcations with as few as 20 cells. Applicable to RT-PCR, RNA-seq, and mass cytometry data.	Considerable loss in temporal resolution and accuracy. Requires temporal information data. Be restricted to identifying a single branch.	(Marco et al., 2014)
Wishbone	Correct identification of bifurcation points. Higher accuracy and temporal resolution.	Require the user to specify the structure of the trajectory. Require the selection of a canonical marker set. Be restricted to identifying a single branch.	(Setty et al., 2016)
Monocle 2	Learns complex cellular trajectories in a fully data-driven, unsupervised fashion by a machine-learning strategy. Requires no prior knowledge about the genes or the branch points in the trajectory. Trajectories are more accurate and robust with only limited assumptions.		(Qiu et al., 2017)

Figure Legends

Figure 1. Speculative model for the hematopoietic system

Apart from HSCs, both LMP and EMP, along with various progenitors, have the capability to originate directly through EHT. Stem cells have distinct lineage biases and generate lineage-restricted progenitors. vWF⁺ HSCs are at the top of the HSC hierarchy. They are mostly platelet-biased, but sometimes tend towards higher myeloid and lower lymphoid reconstitution. However, all mice engrafted with vWF[−] HSCs showed clear lymphoid-biased reconstitution in peripheral circulation (Sanjuan-Pla et al., 2013). During the transition of these two subsets, HSCs exhibit combined granulocyte-macrophage and lymphoid potentials, but little or no megakaryocyte-erythroid potential. Three subsets of lympho-myeloid stem cells give rise to lymphoid and myeloid lineages at different ratios. Mixing the competing HSCs can exacerbate the lineage bias. Whereas during the process of aging, the composition of the stem cell compartment will shift towards the myeloid lineage. In most compartments, HSPCs remain heterogeneous, as indicated by the presence of similar but distinct colors, with a greater number of unilineage cells outnumbering the bilineage cells. The dashed line means during perturbation, such as injury or transplantation, progenitors can regain stem-ness and contribute to the stem cell pool.

Figure 2. Two distinct levels of heterogeneity in the small intestine

On the left side: along the cephalocaudal axis, the expression of GATA4 in crypt stem cell subsets decreases. On the right side: at each specific location, stem cell subsets compete for colonization of the whole crypt. Different color represents different stem cell subtypes. Stem cells at the bottom of the crypt have a competitive self-renewal advantage for niche support.

Figure 3. Stem cell heterogeneity in restrictedly compartmentalized tissues (skin and lung)

The left side of the arrow represents stem cell compartments, which are minor populations attached to the basal membrane, while the right side of the arrow represents

compartmentalized tissue generated by stem cells. Different color represents different stem cell subsets and cell lineages generated from the same stem cell subsets share the same color with the stem cell. (A) Stem cell compartment in the skin. Sebaceous glands are generated from stem cells from the junctional zone, basal layer of sebaceous glands, and adjacent to sebaceous glands. Stem cells from the upper bulge remain quiescent whereas those from the lower bulge as well as hair germ give rise to the lower part of a hair follicle. IFE is generated by stem cells at the basal layer of IFE. Scale IFE has nuclei retained in cornified layers but does not possess a granular layer, whereas Interscale IFE is characterized by a granular layer and loss of nuclei in the cornified layer. Upper dermal lineages give rise to papillary fibroblasts, arrector pili muscle, and dermal papilla while lower dermal lineages produce reticular fibroblasts, which generate adipocyte lineages as well as fibrillar extracellular matrix. (B) Stem cell compartment in the lung. In the trachea, both Clara cells and ciliated cells are generated from tracheal basal cells. In bronchiole, Clara cells both self-renew and give rise to ciliated cells. In the alveoli, Type 1 and type 2 pneumocytes are generated by a bipotential progenitor during development. The arrow on the right side means that type 2 pneumocytes will generate type 1 pneumocytes later in life.

Figure 4. Heterogeneity in pluripotent stem cells

During embryogenesis, pluripotent stem cell heterogeneity first appears at the four-cell stage. Heterogeneity of blastomere is reversible until around the 32-cell stage when TE cells are marked by *Id2* while ICM cells are marked by *Sox2*. *Fgf4* is indispensable for the differentiation of ICM, where EPI and PE lineages stochastically emerge instead of arising from two distinct dividing histories. In the absence of *Fgf4*, lineage segregation is halted without differentiation into either of the two lineages. Meanwhile, ICM can be induced to pluripotent ESC at the proper condition *in vitro*. Both EPI and ESC show pluripotency despite distinct mechanisms. During the process of self-renewal, the phenotype of ESCs is relatively constant, but ICM cells are not *in vivo* because they will undergo further changes according to their developmental program.

Figure 5. Plasticity after the injury

(A) Pathways of normal development (B) Non-stem cells undergo de-differentiation to generate stem cells. (C) Transdifferentiation to another type of epithelium during perturbation. (D) Surrounding stem cells exhibit much stronger self-renewal and differential potential. Different color means generated from different stem cell subsets. During injury, surrounding stem cells from different compartments can compensate for the lost stem cell and gain new identity and functions same as the lost stem cell subtypes. The dashed line represents regenerated cells after injury.

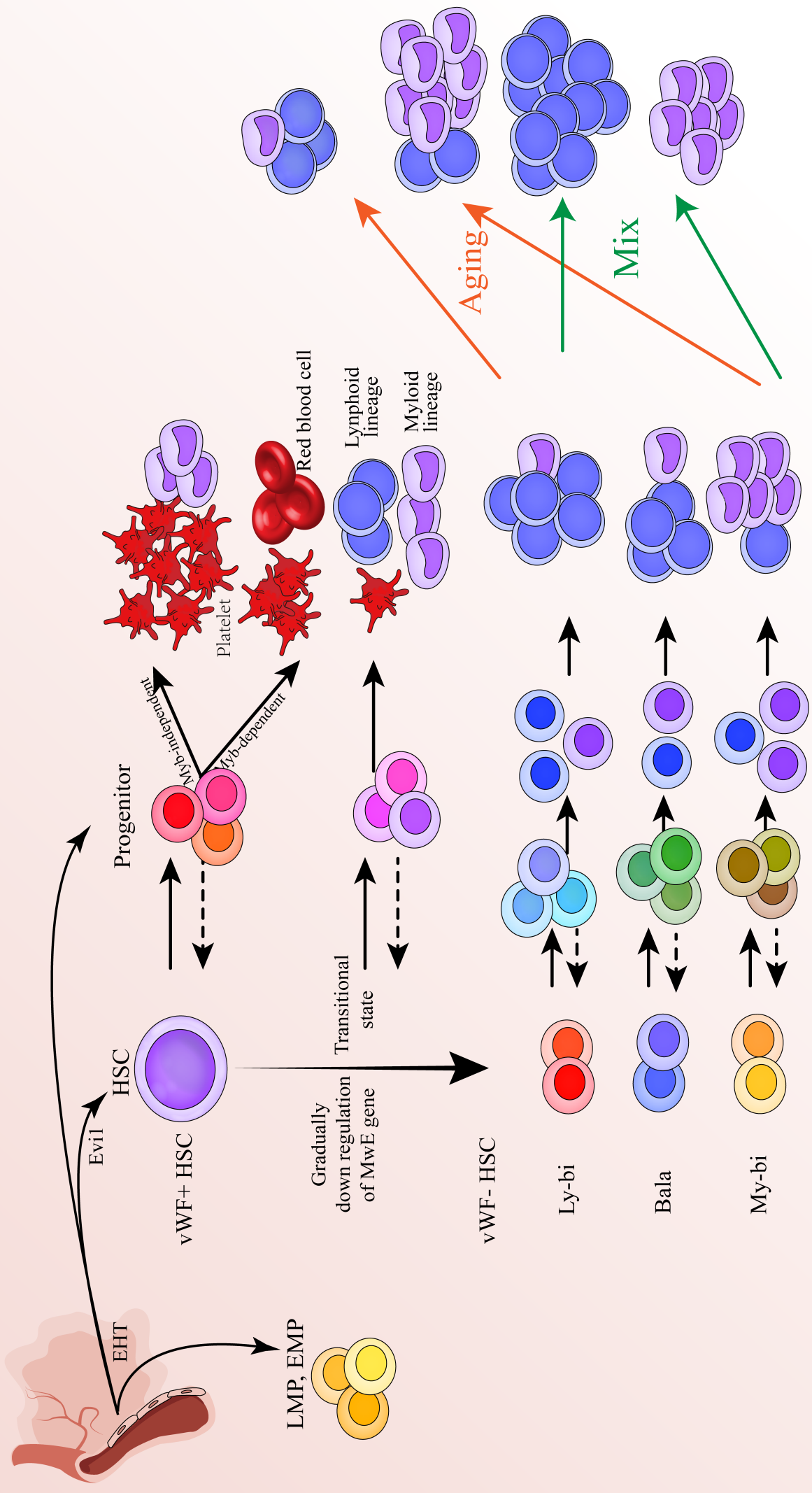
Figure 6. Regulation networks of stem cell heterogeneity and plasticity

Stem cell behaviors are regulated by stem cell niches, signaling pathways, transcription factors, and epigenetic regulators. Stem cell niches are extrinsic factors, which hold considerable promise for artificial manipulation and the evolution of regenerative medicine. Stem cell niches consist of neighboring stem cells and their progeny, epithelial cells nearby, surrounding stromal cells, surfacing bacteria and its products, the immune system, as well as biochemical substances. The niches affect the stem cell population by either cell-to-cell contact or biochemical molecules, which will induce or suppress certain signaling pathways as well as associated transcription factors. Intrinsic regulation at the epigenetic level involves epigenetic factors like gene methylation and chromatin factors like histone modifications. Tiny modification at the epigenetic level contributes to the stability of stem cell heterogeneity. Both intrinsic and extrinsic regulation result in changes of transcription and consequently its downstream translation and stem cell identity, fate, or behavior.

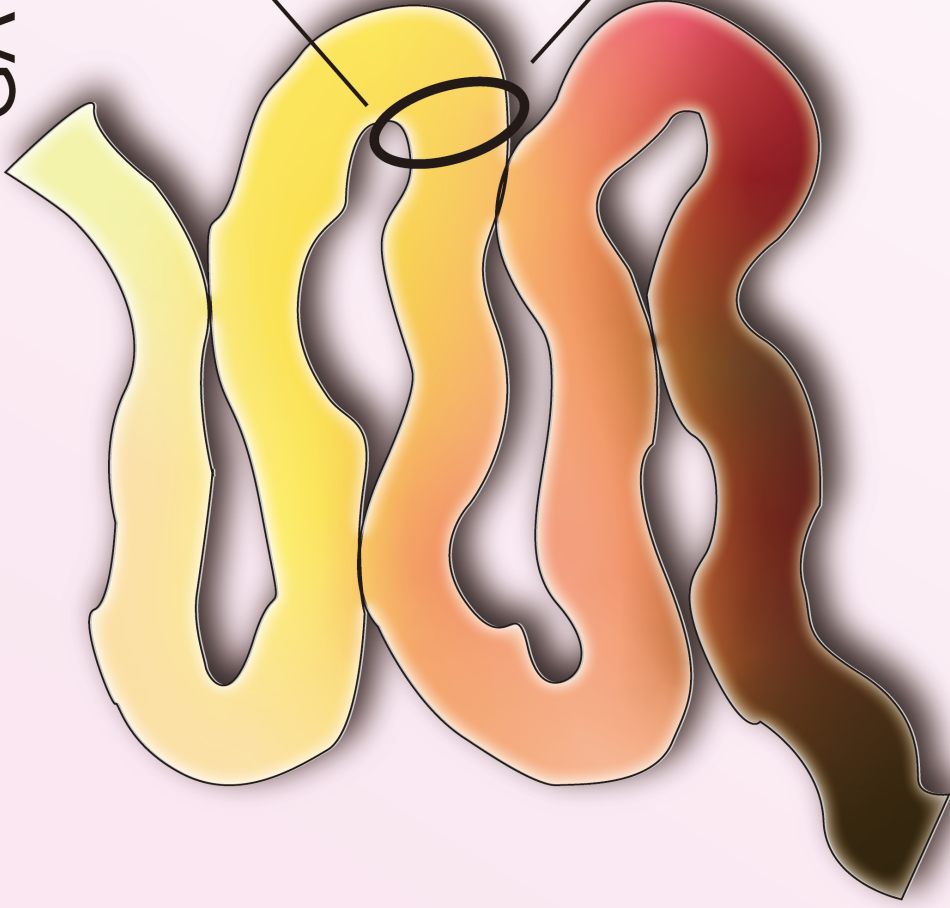
Figure 7. Speculative models of tumor formation

(A-B): Tumor stem cell model. Mutation of key genes in a somatic cell or stem cell itself generates tumor stem cells, which interrupts regulation systems and thus gains competitive advantages over surrounding cells with higher ability of repopulation and mutation. Further mutations generate tumor stem cell hierarchy and form the heterogeneity in the tumor. Different color represents different subtypes of normal stem

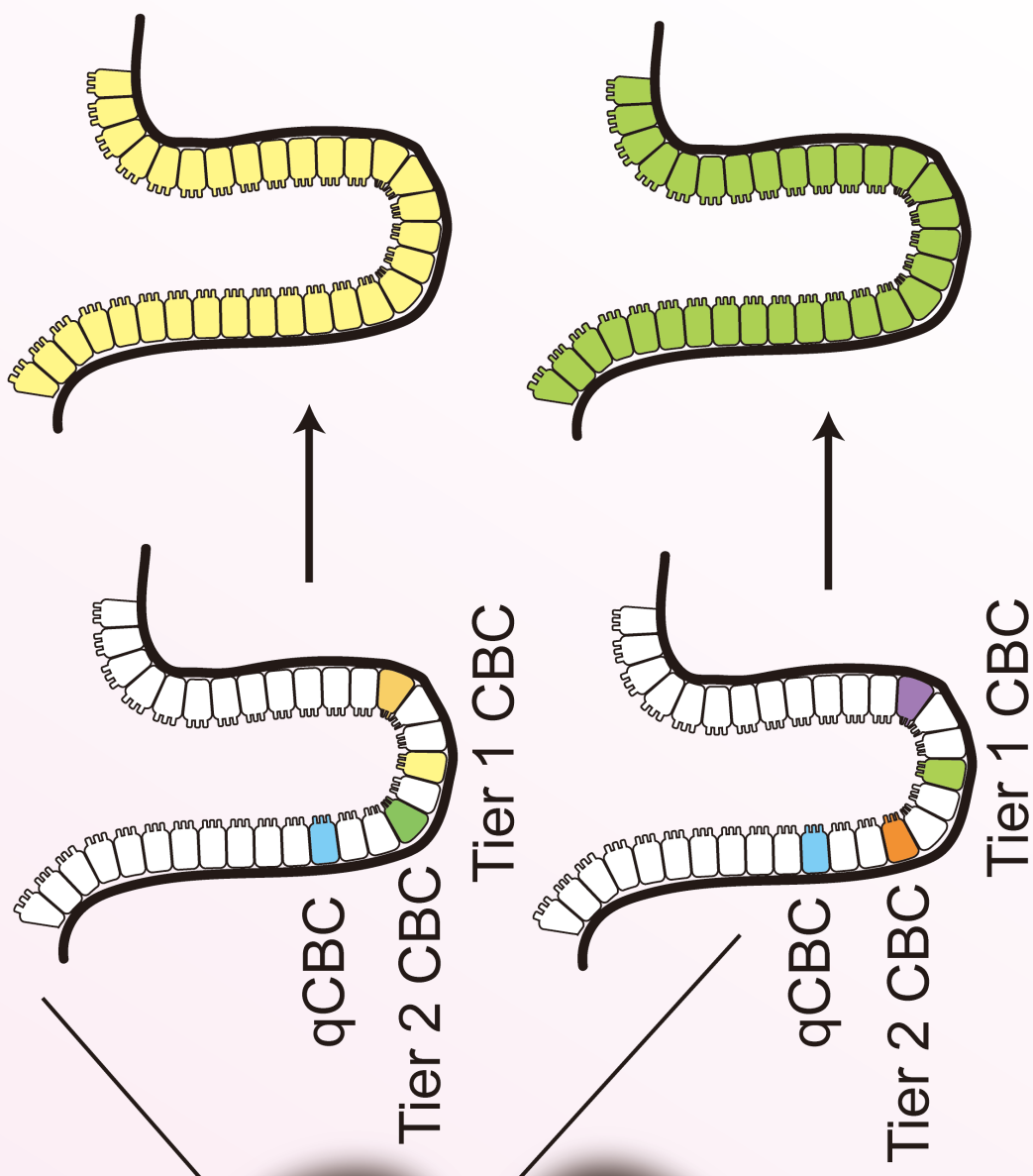
cells or tumor cells. Even though the mutated cell did not reside at the base of the crypt, the mutation provided it with competitive advantages over others. (C-D) Plasticity model. Without mutation, the generation of a tumor can also be initiated by excessive plasticity acquired by a pile of cells after a perturbation or under homeostasis, during which process change of regulation confers cell plasticity.

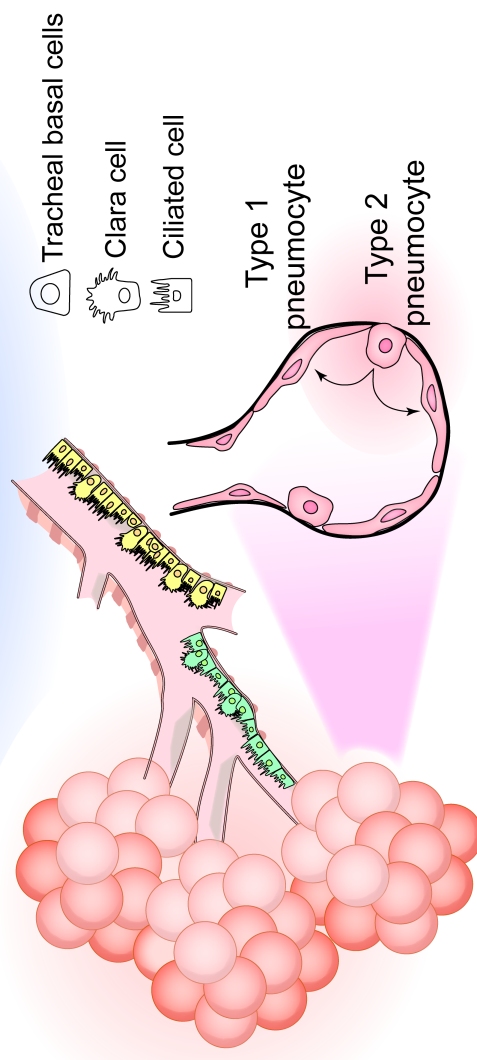
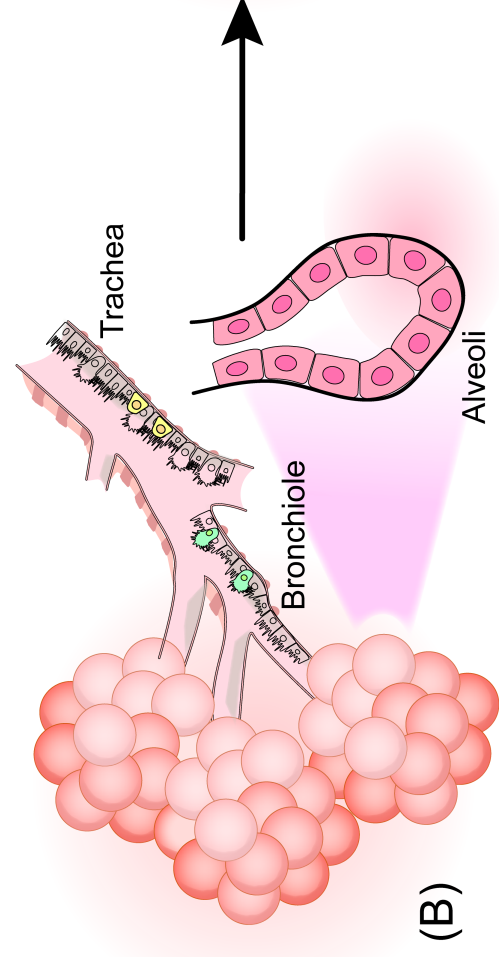
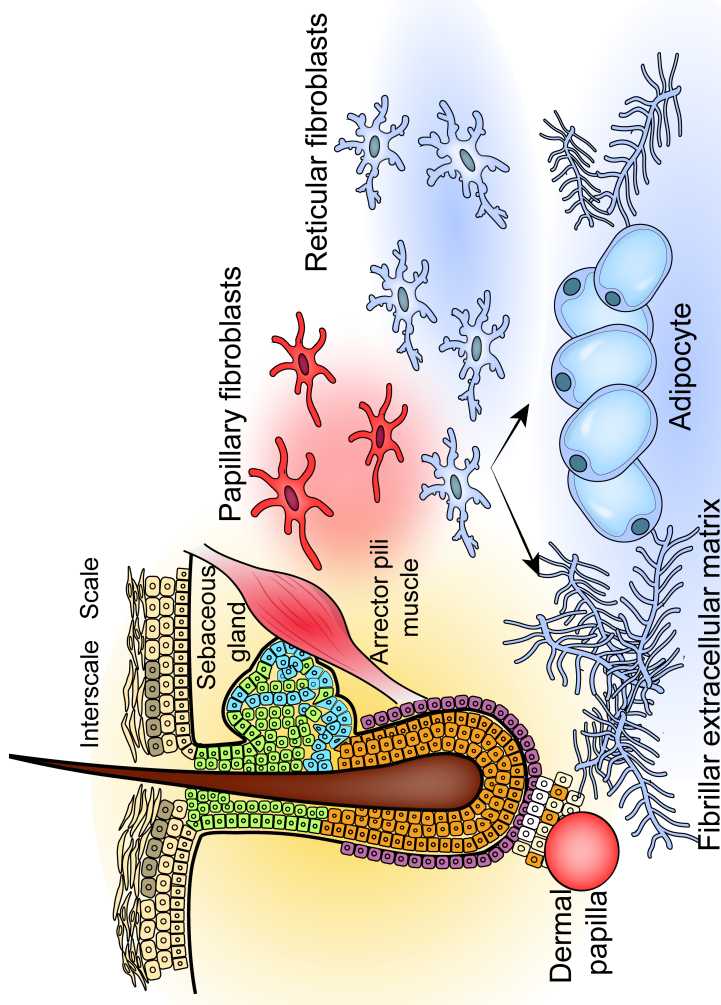
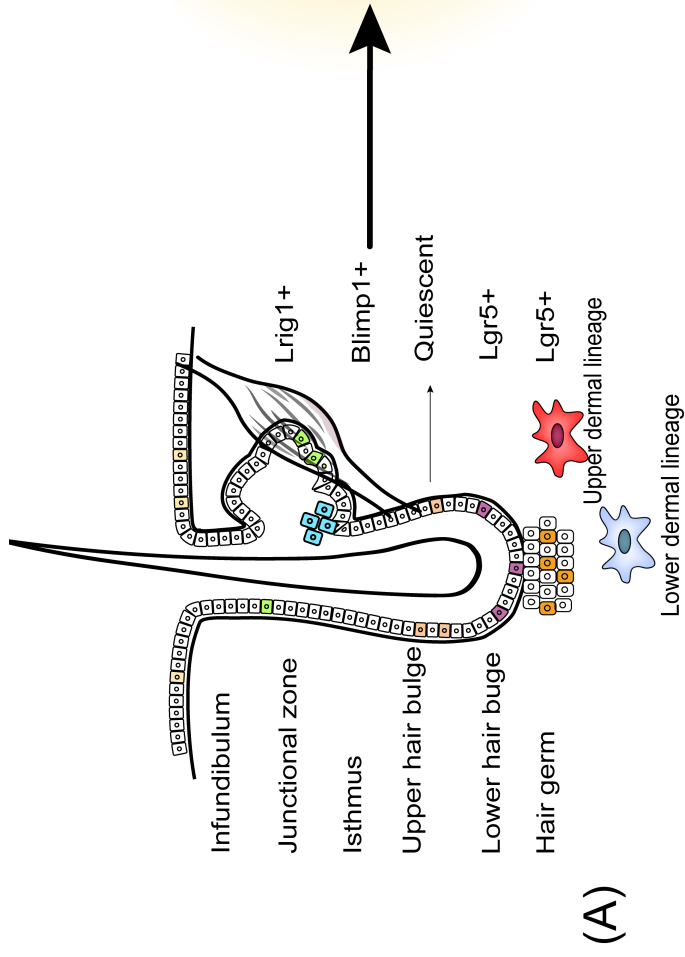


GATA4+



GATA4-





First fate decision

