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Molecular regulation of stem cell quiescence

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Abstract

Subsets of mammalian adult stem cells reside in the quiescent state for prolonged periods of time. This state, which is reversible, has long been viewed as dormant and with minimal basal activity. Recent advances in adult stem cell isolation have provided insights into the epigenetic, transcriptional and post-transcriptional control of quiescence and suggest that quiescence is an actively maintained state in which signalling pathways are involved in maintaining a poised state that allows rapid activation. Deciphering the molecular mechanisms regulating adult stem cell quiescence will increase our understanding of tissue regeneration mechanisms and how they are dysregulated in pathological conditions and in ageing.

Stem cells are undifferentiated, long-lived cells that are unique in their abilities to produce differentiated daughter cells and to retain their stem cell identity by self-renewal[1]. Most mammalian adult tissues contain resident stem cells, which proliferate to compensate for tissue loss throughout the life of the organism. They possess remarkable proliferative capacity, allowing them to engage in massive and repetitive regenerative activities in response to tissue damage. A subset of tissue-specific adult stem cells persists in the quiescent state for prolonged periods of time[2]. Whereas quiescence is not an essential characteristic that defines stem cells, dysregulation and loss of quiescence often results in an imbalance in progenitor cell populations ultimately leading to stem cell depletion[3]. As a result, tissue replenishment is affected during homeostasis and following damage. Thus, deciphering the regulation of quiescence will contribute much to our understanding of how tissue regeneration is accomplished in physiological and pathological settings and may lead to new therapeutic strategies for tissue maintenance or repair.

The concept of cellular quiescence has changed over time. Previously, it was thought that cells become quiescent by default, because of challenges to continued pro liferation such as nutrient deprivation or contact inhibition. Now, it is believed that cells, particularly stem cells, adopt the quiescent state to preserve key functional features. Recently, much attention has focused on the active regulation of the quiescent state as well as the properties of stem

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cells that persist in a quiescent state. Such properties allow them to withstand metabolic stress and to preserve genomic integrity over a lifetime.

In this Review, we summarize recent advances in the field of stem cell quiescence and discuss the characteristics and regulation of the quiescent state. Beginning with a historical summary of studies of the cell cycle and the existence of a quiescent state, we focus on the identification of stem cell populations that reside in the G0 phase of the cell cycle, the molecular signatures of this state and the regulatory mechanisms that maintain cells in the quiescence state. Finally, we examine specific properties of quiescent stem cells that assure survival over extended periods of time, and we present a model of the quiescent state as a 'poised state' rather than a dormant state.

The G0 phase of the cell cycle

Historically, the G0 phase of the cell cycle was referred to as an inactive, non-cycling state. It was first recognized and described as a state in which cells have irreversibly exited the cell cycle, as exemplified by terminally differentiated cells such as neurons or cardiomyocytes or, more recently, senescent cells (BOX 1). Such cells do not re-enter the cell cycle except in response to extraordinary experimental stimuli. By contrast, the discovery of another type of G0 phase, namely the quiescent state, is characterized by the ability of cells to re-enter the cell cycle in response to normal physiological stimuli.

Discovery of quiescence

The existence of a quiescent state was hypothesized on the basis of early cell cycle studies (BOX 1). In 1951, Howard and Pelc used radioactive labelling techniques to study the timing of DNA replication during cell division, thereby defining the four phases of the cell cycle[4]. Interestingly, the concept of quiescence arose from the observation that not all cells in a population proliferate at similar rates. The term 'growth fraction' was used to describe the cell population that is actively proliferating. In somatic tissues, some cells continuously divide, while other cells exist in a non-proliferative state during homeostasis but are able to respond to extrinsic stimuli and re-enter the cell cycle to begin proliferating[5].

For years, debate continued about the nature of the state of cells that are non-cycling but able to proliferate in response to extrinsic stimuli. Some investigators considered these cells to be in a prolonged G1 phase, and others postulated that they could be in a cell cycle phase that is distinct from G1 and termed this non-proliferative state G0 (which is also referred to as the quiescent state)[6]. Subsequent studies demonstrated that sub-optimal conditions such as high cell density[7] or serum insufficiency[8] could drive cells into this quiescent state. In 1974, Pardee provided evidence for a distinct quiescent state and demonstrated the existence of a restriction point (R-point) in G1 that determines cell fates: cells in G1 can become quiescent before the R-point but commit to enter a mitotic cell cycle after the R-point[9] (BOX 1). The author hypothesized that normal mammalian cells possess unique regulatory mechanisms to shift from a quiescent state to a proliferative state and that dysregulation of these mechanisms might result in malignant transformation. In 1985, Zetterberg and Larsson discovered that serum deprivation results in the inhibition of protein synthesis in all cell cycle states but that only cells in early G1 exit the cell cycle and become quiescent[10]. Together, these early studies suggested the existence of a quiescent state, access to which is restricted. To date, the molecular control of quiescence still remains to be fully elucidated.

The diversity of quiescent states

Many unicellular organisms reside in the quiescent state for a prolonged period of time to survive in unfavourable environments[11]. Quiescence is also a state of growth cessation that occurs in multicellular organisms. For example, studies of seed dormancy revealed that

plants utilize this state to preserve the capacity for growth, thereby circumventing an unfavourable environment[12]. In mammals, the ability of tissue stem cells to reside in the quiescent state is crucial for proper homeostasis and regeneration of many tissue types. Quiescent stem cells are able to respond to stimuli that originate from their niche environment by activating and entering the cell cycle (BOX 2). Interestingly, tissue stem cells are not the only population of cells in G0 that are able to resume proliferation and contribute to tissue regeneration. For example, mature hepatocytes are capable of entering the cell cycle and contribute to liver regeneration in the case of partial hepatectomy[13]. Thus, both stem cells and differentiated cells can reside in a reversible G0 phase.

Identification of quiescent stem cells

Our understanding of the characteristics of quiescent stem cells has been limited by the rarity of this population in many tissue compartments. Quiescent stem cells have been identified by their low RNA content[14,15] and their lack of cell proliferation markers[16], as well as by label retention as an indication of low turnover. Label retention as an indication of quiescence is based on the concept that once cells have incorporated a label, rapidly dividing cells lose the label quickly, whereas quiescent or very slowly cycling cells retain the label for extended periods of time. Identification and localization of cells in the quiescent state have relied primarily on techniques that allow the analysis of the incorporation and then retention of labels such as 5′-bromo-2′-deoxyuridine (BrdU)[17,18], tritiated thymidine[19,20] or, more recently, the use of H2B–GFP[21–24] or H2B–YFP[25]. For decades, label retention was considered to be an essential property of adult stem cells[26]. However, it has become increasingly apparent that the use of label retention alone is insufficient to identify adult stem cells. Recently, evidence has suggested the coexistence of reserve (quiescent) and active (proliferating) stem cell pools in high-turnover tissue compartments[2,27]. The use of a lineage tracing approach based on label retention has provided new insights into the nature and function of label-retaining cells (LRCs) in the gut[25]. Whereas active stem cells function during normal homeostasis, quiescent LRCs seem to serve as a reserve pool of stem cells, only called into action upon tissue injury. In addition, interconversion of reserve and active intestinal stem cell (ISC) populations has also been observed previously[25,28–30].

Similar to the gut, skin is another high-turnover tissue in which both quiescent and active stem cells are present. The mammalian epidermis consists of regions that contain hair follicles interspersed with interfollicular epidermis. Hair follicle morphogenesis relies on both quiescent and active stem cells. Quiescent stem cells that are responsible for regenerating the hair follicles lie within the bulge of the hair follicles[21,31,32], which can be visualized using advanced imaging techniques[33]. Interestingly, adult epidermal homeostasis seems to rely solely on active stem cells[34], whereas quiescent stem cells in the bulge are involved in the process of wound healing but not normal homeostasis[35]. Lineage tracing experiments have also facilitated the identification of long-lived stem cells in the mammary epithelium[36,37] and in glandular epithelia such as that found in the prostate[38].

In low-turnover tissues such as liver or muscle, the use of label-retention techniques is well-suited for the identification of quiescent stem cells. However, it has been proposed that more than one type of low-turnover stem cells exist in a given tissue. In the muscle compartment, the existence of a low-turnover population of fibrogenic and adipogenic progenitor cells that is functionally distinct from muscle stem cells has recently been proposed[39,40]. Thus, in either high- or low-turnover tissues, techniques such as lineage tracing are needed to identify quiescent stem cells and to study their function.

Molecular signatures of quiescent stem cells

Recent advances in genetic approaches and high-throughput analyses of various stem cell subpopulations have provide d valuable information on the molecular signatures of quiescent stem cells in different tissue compartments. These findings have not only revealed unique signatures of the quiescent state but also provided potential avenues for identifying and characterizing regulatory pathways, networks and determinants of the quiescent state.

Transcript profiles

Transcript profiling was traditionally limited to bulk-differentiated tissues due to a lack of cell purification techniques and a need for large amounts of RNA to perform such analysis. To understand the transcriptomes of quiescent stem cells, much effort had been focused on various techniques to purify and characterize stem cell populations. Prospective isolation of quiescent stem cells by fluorescence-activated cell sorting (FACS) was first used to purify haematopoietic stem cells (HSCs)[41] and has quickly become a standard technique for isolating stem cells. To date, FACS techniques have been devised for the isolation of muscle stem cells (MuSCs)[15], ISCs[42], hair follicle stem cells (HFSCs)[31,43], neural stem cells (NSCs)[44] and many other stem cell populations. These advances in purifying subpopulations of stem cells have allowed the use of high-throughput techniques such as microarray and RNA-sequencing to further our understanding of the transcriptomes of these stem cells.

Facilitated by advanced isolation techniques, high-throughput gene expression analyses of quiescent stem cells and their differentiated progeny have provided important information regarding the identities of genes that are important for lineage determination and differentiation. In particular, a comparison of gene expression profiles of different types of quiescent stem cells, including HSCs[45], MuSCs[15] and HFSCs[43], reveals a gene signature that is common to these quiescent stem cells (TABLE 1).

As expected from a non-proliferative phenotype, the signature reveals the downregulation of genes that are involved in DNA replication and cell cycle progression. Examples of genes that are downregulated in all three quiescent cell types (HSCs, MuSCs and HFSCs) include genes encoding cyclin A2, cyclin B1, cyclin E2 and survivin, which control various aspects of cell cycle progression[15,43,45]. Cyclin A2 and cyclin E2 are important regulators of cell cycle checkpoints[46,47]. HSCs that lack cyclin A2 are unable to proliferate *in vitro*, indicating the essential role of cyclin A2 in HSC proliferation[48]. Whereas cyclin B1 binds to cyclin-dependent kinase 1 (CDK1) and promotes entry into mitosis[49], survivin has important roles in the regulation of microtubule dynamics during mitosis[50]. Moreover, downregulated genes correlated with the proliferation status (including genes such as proliferating cell nuclear antigen (*PCNA*) and mini chromosome maintenance complex component 4 (*MCM4*)) and with mitochondrial function (for example cytochrome *c* (*CYCS*))[15,43,45]. As mitochondrial biogenesis is required for stem cell activation, low expression of *CYCS* reflects low metabolic activity of the quiescent stem cell. Conversely, genes that are upregulated in quiescent stem cells include genes encoding signalling molecules involved in transcriptional regulation and stem cell fate decisions such as forkhead box O3 (*FOXO3*) and enhancer of zeste homolog 1 (*EZH1*). It is likely that there are transcriptional signatures that are unique to specific populations of quiescent stem cells. However, it is also possible that gene products, the expression levels of which change as stem cells progress from the quiescent state to the activated state, constitute signalling pathways that are common to various different stem cell populations. This may reveal mechanisms that specifically relate to the induction or maintenance of quiescence.

Other than protein-coding genes, profiling of non-coding RNAs such as microRNAs (miRNAs) has also revealed the function of various miRNAs in regulating stem cell

quiescence[51,52]. miRNA signatures have recently been identified in multiple quiescent stem cell populations such as the HSCs[52], NSCs[52], MuSCs[51,52] and HFSCs[53]. Similar to the gene expression analysis, miRNA profiling of HSCs, NSCs and MuSCs and their differentiated progenies has led to a common miRNA signature of stem cell activation from quiescence, which suggests an important role of miRNA pathways in regulating stem cell quiescence post-transcriptionally[52].

Characterizing the transcriptional landscape of quiescent stem cells is likely to provide information on common gene expression patterns that maintain quiescence, such as genes that are involved in cell cycle regulation, as well as specific patterns that relate to quiescent stem cells in particular lineages in various tissue compartments.

Epigenetic profiles

Recent epigenetic studies have shed light on how chromatin states contribute to maintaining stem cells in a poised state for lineage progression. Knowledge gained from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can be applied to quiescent adult stem cells. Studies of histone methylation have revealed the epigenetic landscape as one of the key determinants of gene expression[54,55]. Trimethylation of histone H3 at Lys4 (H3K4me3) and H3K27me3 are of particular interest because of their roles in the positive and negative regulation of transcription, respectively[54]. Chromatin regions that are marked by both H3K4me3 and H3K27me3, which are termed bivalent domains, are frequently located in close proximity to transcription start sites[56]. Many genes that carry such bivalent chromatin patterns are master regulators of cell lineages and are thought to maintain ES cells in a poised state to allow flexibility for lineage choices.

In view of regulators that govern chromatin modifications, conditional knockouts of the H3K27 methyl-transferases EZH1 and EZH2 in HFSCs revealed an essential role of chromatin modification in hair follicle homeostasis and wound repair[57]. In muscle, deletion of EZH2 impairs MuSC proliferation and derepresses gene expressions of non-muscle lineages[58]. By contrast, overexpression of EZH2 in HSCs prevents HSC exhaus-tion[59], whereas HSCs are lost when EZH1 is ablated[60]. Together, these studies suggest important roles of H3K27 methyltransferases in regulating stem cell quiescence in an epigenetic manner.

In tissue compartments such as muscle and skin, where prospective isolation of large quantities of stem cells is feasible, it is now possible to use genome-wide chromatin immunoprecipitation followed by sequencing (ChIP–seq) to obtain epigenetic profiles of the resident stem cells. In contrast to observations in ES cells, few genes are marked by bivalent domains in lineage-restricted, quiescent HFSCs[61]. Intriguingly, in both quiescent HFSCs and MuSCs, thousands of genes are marked by the H3K4me3 mark (which is associated with active transcription)[61,62], suggesting a permissive chromatin state for transcription. However, it has previously been proposed that H3K4me3 marks genes for transcriptional activation but does not necessarily predict whether these genes are being actively transcribed[63]. Given the low transcriptional output in quiescent stem cells, it is likely that not all genes marked by H3K4me3 are indeed being actively transcribed but may reflect the fact that quiescent stem cells are, in general, less differentiated than their proliferating progeny and that this epigenetic mark identifies genes that may be transcribed upon activation. This correlation and the significance of this epigenetic signature remain to be demonstrated experimentally.

Molecular regulation of quiescence

Although transcriptional and epigenetic profiling may be of value to provide molecular signatures of quiescent stem cells and may point to pathways that are important for the induction or maintenance of the quiescent state, each pathway needs to be tested in studies of stem cell quiescence *in vivo* to determine the functional relevance. In the following section, we highlight genes and pathways for which experimental evidence supports an important contribution to the regulation of the quiescent state.

p53 and RB protein

p53, a master regulator of diverse cellular processes, especially those involved in the maintenance of genomic integrity, has an important role in regulating stem cell quiescence[64]. p53 deficiency in HSCs promotes cell cycle entry with a reduction in the number of quiescent HSCs[64]. The mechanism by which p53 mediates HSC quiescence is independent of the CDK inhibitor p21, which is an important regulator of cell cycle progression at G1 that has previously been shown to regulate HSC quiescence[65]. In addition to p53, another crucial regulator of the cell cycle, the tumour suppressor RB, has also been implicated as a regulator of stem cell quiescence. Early studies of RB revealed that one of its major roles is to inhibit cell cycle progression[66]. The cell cycle progresses normally when RB is inactivated by phosphorylation facilitated by different cyclin–CDK complexes[67]. In NSCs, genetic ablation of RB together with p53 triggers NSC over-proliferation, resulting in a brain tumour phenotype[68]. In ES cells, ablation of all three RB family members (RB, p107 and p130) results in impaired differentiation and an increase in cell turnover under growth arrest conditions[69]. Genetic ablation of RB in quiescent MuSCs results in a vast increase of muscle stem and progenitor cells and an acceleration of cell cycle re-entry[70] (FIG. 1a). Muscle progenitors that lack RB do not differentiate due to their inability to exit the cell cycle[70]. Similarly, the quiescent HSC pool is lost when all three RB family proteins are conditionally ablated. This HSC depletion is accompanied by an expansion of early haematopoietic progenitors and an impairment of the reconstitution potential in transplantation experiments[71].

CDK inhibitors (CKIs)

Many CKIs, including p21, p27 and p57, are expressed in quiescent stem cells and promote cell cycle arrest by inhibiting CDKs (FIG. 1a). As shown in stem cell compartments such as HSCs and NSCs, inhibition of p21 results in an increase in stem cell proliferation and in a decrease in the quiescent stem cell population[65,72]. In NSCs, the loss of p21 does not seem to alter the lineage fate. The reduced number of quiescent stem cells correlates with impaired self-renewal capacity of p21-deficient cells, which ultimately results in an exhaustion of the stem cell pool[65,72].

Interestingly, inhibition of p27 does not affect the number or self-renewal of HSCs, but increases the size of the haematopoietic progenitor pool[73]. The fact that p57 deficiency has no effect on HSC quiescence might be due to the functional overlap with other CKIs[74,75]. Previously, it was shown that p27 and p57 bind to heat shock cognate protein 70 (HSC70), a molecular chaperone involved in the nuclear import of specific proteins. p27 and p57 control nuclear transport of the HSC70–cyclin D1 complex and regulate the cell cycle entry of HSCs[75]. In double-knockout mice lacking both p57 and p27, the loss of CKIs promotes nuclear import of the HSC70–cyclin D1 complex and concomitant RB phosphorylation. As a result, HSC quiescence is severely impaired[75]. These studies suggest that CKIs are functionally important for the maintenance of stem cell quiescence.

Notch signaling

Notch signalling is involved in tissue maintenance and contributes to cell fate decisions during tissue regeneration[76,77]. This pathway is an important regulator of proliferation and cell fate commitment of transit amplifying progenitors in many tissue compartments[76,77]. Recent evidence has demonstrated that Notch signalling also has a role in regulating stem cell quiescence. In MuSCs, genetic ablation of RBP-J, the DNA binding factor that is essential for mediating canonical Notch signalling, results in a depletion of the quiescent stem cell pool[78,79]. The loss of quiescence is associated with spontaneous activation and premature differentiation of stem cells[78,79] (FIG. 1a). In adult NSCs, cell fate is determined by the levels of Notch activity, and quiescence is promoted by high Notch activity[80]. In contrast to its role in muscle and brain, Notch signalling is not required for quiescent HSC maintenance[81], highlighting the complex context-dependent role of this pathway in regulating stem cell quiescence. In fact, Notch signalling promotes differentiation of stem cell progeny in the interfollicular epidermis and hair follicles[82,83]. However, whether Notch may also have a role in the regulation of stem cell quiescence in these compartments remains to be determined as, for example in muscle, Notch signalling may both promote quiescence and be important in lineage progression of stem cell progeny[78,79,84].

Post-transcriptional regulation by miRNAs

Since the discovery of the founding miRNA, *lin-4,* a small non-coding RNA that regulates several crucial genes during development in *Caenorhabditis elegans*[85], hundreds of conserved miRNAs have been discovered in vertebrates[86]. Over the past decade, it is clear that these small non-coding RNAs have important roles in the post-transcriptional regulation of diverse cellular processes. miRNAs bind to the 3′ untranslated region (3′ UTR) of target mRNAs, resulting in their cleavage or trans-lational repression[87]. This mode of post-transcriptional regulation has emerged as an important aspect in the control of stem cell quiescence, as recently demonstrated in HSCs[88] and MuSCs[51]. In HSCs, miR-126 controls stem cell quiescence by attenuating multiple components in the PI3K–AKT signalling pathway[88]. Interestingly, reducing miR-126 activity allows HSC proliferation without inducing exhaustion[88]. Conversely, overexpression of miR-126 in HSCs impairs cell cycle entry, resulting in a lower haematopoietic contribu-tion[88]. By contrast, conditional knockout of the miRNA processing factor Dicer triggers spontaneous activation of quiescent MuSCs[51]. MuSCs subsequently undergo apoptosis, which is similar to the finding in HSCs[89]. Furthermore, many quiescence-specific miRNAs have been identified in MuSCs, and it was demonstrated that one miRNA, miR-489, is an important regulator of the quiescent state[51]. miR-489 functions to prevent MuSC proliferation by suppressing the oncogene *DEK*[51,90]. In another study, the *Myf5* (myogenic factor 5) mRNA and its regulatory miRNA miR-31 were found to be sequestered in mRNA ribonucleoprotein particle (mRNP) granules in quiescent MuSCs[91]. This report suggests that quiescent MuSCs are primed for differentiation, as the storage of mRNAs makes then readily available for the activation of differentiation programmes. These studies provide evidence for the spatial and temporal regulation of miRNAs in the quiescent state and the active regulation of stem cell quiescence by post-transcriptional mechanisms (FIG. 1b).

The finding of distinct miRNA expression patterns in quiescent stem cells suggests that miRNAs are important regulatory components of the quiescent state. Although the underlying mechanisms have yet to be determined, the importance of transcript 3′ UTRs as targets for miRNAs suggests that stem cell quiescence is controlled, at least in part, by mechanisms that alter 3′ UTR length and thus the susceptibility to regulation by miRNAs[92–95]. 3′ UTR length of a transcript can be modified by mechanisms such as alternative splicing or alternative cleavage and polyadenylation[96,97]. There is much interest in the role of alternative polyadenylation in controlling several aspects of stem cell function,

many of which specifically relate to changes in 3′ UTRs[98]. The differential susceptibility of a myogenic factor, paired box 3 (PA×3), to miRNA regulation has been reported in quiescent MuSCs that were isolated from different muscle groups[92]. It has previously been shown that T cell activation from the quiescent state is associated with widespread shortening of 3′ UTRs, thereby circumventing the regulatory role of mRNA-targeting miRNAs during activation[94]. Shortening of 3′ UTRs seems to correlate with proliferation in many cell types[94], including aberrant proliferation in the case of cancer cells[95] or during somatic cell reprogramming[99] (FIG. 1c). In addition, miRNA regulation can act as a fine-tuning mechanism to modify target gene expression. mRNAs can be partially repressed when both miRNA and target mRNA are co-expressed[100]. A change in the expression levels of miRNAs can tip the balance and result in repression or activation of many functionally important target genes[101,102]. In the same way, miRNAs may repress genes that are required for stem cell activation. By tipping the balance, genes that are functionally important for activation can be derepressed and participate in the process rapidly.

Survival mechanisms in quiescent cells

Long-lived, non-dividing quiescent stem cells may accumulate damage from environmental stress (for example, oxidative stress caused by the accumulation of reactive oxygen species (ROS)), similarly to any long-lived cell such as a post-mitotic neuron or cardio-myocyte[103]. Environmental stress may lead to damage of cellular constituents, including DNA, a process that has been proposed to underlie the ageing of cells and tissue and to limit lifespan[104,105]. Accordingly, quiescent stem cells seem to have adopted specific mechanisms to respond to environmental stresses and, thus, to maintain cellular integrity and assure long-term survival. These mechanisms are likely to be different from their proliferating progeny, which can be subject to selection during proliferative expansion and are capable of diluting out damaged cellular components during cell division.

Signalling to protect from environmental stress

Studies of the FOXO family of transcription factors have revealed that this pathway is functionally important in quiescent stem cells to safeguard these cells from environmental stress. In the mammalian system, FOXO family members (which are FOXO1, FOXO3, FOXO4 and FOXO6) have important roles in various cellular processes in a PI3K– AKT pathway-dependent manner[106]. HSCs depleted of FOXO1, FOXO3 and FOXO4 exhibit a marked increase in ROS and the propensity to exit from quiescence[107] (FIG. 2a). Interestingly, administration of the antioxidant *N*-acetyl-L-cysteine is able to rescue this FOXO-deficient phenotype in HSCs[107]. In NSCs, FOXO3 regulates the size of the NSC pool[108]. NSCs devoid of FOXO3 are defective in self-renewal, highlighting the importance of this pathway in regulating stem cell quiescence and survival.

Metabolic sensors and response mechanisms

The survival of quiescent cells depends on intrinsic mechanisms to sustain metabolic function during persistent environmental stresses. In an extreme case, quiescent MuSCs and HSCs were found viable in post-mortem tissue[109]. The remarkable ability to survive in such adverse conditions suggests that quiescent stem cells may have unique protective mechanisms, many of which are described above. Recent findings suggest that at least some stem cell populations reside in poorly oxygenated niches[110–112], and this has sparked interests in understanding how stem cells regulate their metabolic demand in such hypoxic environments. Interestingly, quiescence is induced when cultured haematopoietic cells are grown under hypoxic conditions[113,114]. During normal homeostasis, HSCs express hypoxia inducible factor 1α (HIF1α), a basic helix–loop–helix transcription factor that is expressed in mammalian cells growing in hypoxic conditions. The level of HIF1α is important for

HSC quiescence[115]. Inhibition of ubiquitin-mediated degradation of HIF1α results in over-stabilization of HIF1α protein and induction of HSC quiescence[116] (FIG. 2a). Conversely, HSCs in HIF1α-knockout mice are not able to maintain quiescence, and these mice exhibit HSC depletion[116]. Liver kinase B1 (LKB1), a regulator of AMP-activated protein kinase (AMPK), mammalian target of rapamycin complex 1 (mTORC1) and FOXO pathways, links sensing and metabolism and is required for maintaining energy homeostasis. Moreover, LKB1 is thought to be the master regulator of cellular metabolism by limiting cell growth in unfavourable conditions such as hypoxia[117]. Interestingly, upon genetic ablation of LKB1, HSC quiescence is lost and is accompanied by an increase in progenitor cell proliferation and eventual depletion of HSCs[118–120] (FIG. 2a). The effect of LKB1 on HSC quiescence is cell autonomous, as shown by transplantation experiments in which LKB1-deficient HSCs are not able to rescue lethally irradiate d recipient mice[118–120].

In keeping with their ability to sense and respond to environmental cues related to the metabolic state, quiescent cells rely on autophagic processes for survival, and the induction of autophagy seems to be important in the regulation of stem cell activation. Autophagy is a lysosomal degradation pathway that is involved in cytoplasmic organelle recycling, preserving the healthy state of cells by removing damaged components[121]. Conditional knockout of the essential autophagy gene *Atg7* in the haematopoietic system results in a reduced number of stem and progenitor cells of multiple lineages and the accumulation of aberrant mitochondria and ROS. This suggests that autophagy is essential for maintenance of HSC quiescence[122]. Autophagy is also induced in HSCs in which *Lkb1* is conditionally ablated, which implies that autophagy may act as a compensatory mechanism to rescue the metabolic stress in these mutants[118].

Preservation of genomic integrity

In addition to environmental stress, quiescent stem cells can also be subjected to DNA damage during normal homeostatic turnover, and quiescent stem cells depend on DNA repair mechanisms for survival[123]. Among the most detrimental DNA mutations are double-strand breaks (DSB), and cells have specialized mechanisms to repair these mutations. In mammalian systems, two major mechanisms, namely homologous recombination and non-homologous end-joining (NHEJ), mediate DSB repair[124]. Whereas NHEJ is an error-prone DNA repair mechanism, homologous recombination is a high-fidelity DSB repair mechanism. Homologous recombination uses a long homologous sequence to guide repair in the S phase and G2 phase of the cell cycle, in which sister chromatids are available as templates[125]. By contrast, NHEJ does not require a template and is predominantly used in G1 phase of the cell cycle[125]. Consistent with these findings, a recent study demonstrated that quiescent HSCs preferentially use NHEJ to repair DSBs[126], whereas homologous recombination has been reported to occur more predominantly in proliferating progenitor cells. Intriguingly, this suggests that although DSBs are repaired in quiescent stem cells, mutations may accumulate in these cells as a consequence of using error-prone repair mechanisms (FIG. 2b).

In considering mechanisms by which quiescent stem cells preserve genomic integrity, John Cairns proposed the immortal strand hypothesis that was based on the idea that stem cells possess unique mechanism to safeguard their DNA by non-randomly segregating sister chromosomes during mitosis[127]. In this model, the oldest template DNA strands are preferentially segregated to the self-renewed stem cells to avoid the accumulation of replication-induced mutations in the stem cell pool. Emerging evidence has demonstrated that stem cells in different tissue compartments exhibit template strand segregation during cell divisions[128]. There is mounting evidence of asymmetric chromosome segregation in stem cells such as MuSCs[17,18,129], ISCs[20,130,131] and NSCs[132]. However, the mechanisms by which stem cells engage in asymmetric chromosome segregation remain to be

determined[133,134]. Furthermore, asymmetric chromosome segregation does not occur universally in stem cell compartments, and its prevalence may differ depending on the experimental paradigm. During normal tissue homeostasis, neither template strand segregation nor label retention was observed in one study of HSCs[135]. Similarly, evidence of asymmetric chromosome segregation was lacking in certain studies of epidermal stem cells[136,137] and ISCs[138]. Further studies investigating the mechanisms that regulate template strand segregation may reveal how, and the extent to which, stem cell populations use this intriguing cellular function to preserve genomic integrity in the quiescent state.

Quiescence as a poised state

Recent discoveries suggest that the quiescent state is not just a passive state but, instead, actively regulated by different intrinsic mechanisms. It seems that quiescent stem cells have the ability to sense environmental changes and respond by re-entering the cell cycle for proliferation. How does a quiescent stem cell respond to such stimuli rapidly? In one extreme scenario, a quiescent stem cell would maintain the expression of all necessary components that are required for activation and proliferation. However, given the low metabolic state of a quiescent stem cell, this seems unlikely. We thus propose that quiescent stem cells are poised for activation by specific energetically favourable mechanisms that are compatible with the low metabolic state of quiescence and that allow for rapid and global responses needed for activation (FIG. 3). One such example is the regulation of the quiescent state by miRNAs (see above). From an energetics point of view, it seems favourable for a quiescent stem cell to alter the expression of specific miRNAs, as each of these in turn affect a pool of target genes. It has previously been shown that the miR-16 family of miRNAs has a role in regulating G0 to G1 transition[139]. Intriguingly, silencing of transcripts that are downregulated by miR-16 also affects cell cycle progression[139]. As miRNAs can affect a number of target genes that are important in a shared pathway, we propose that this is the case in the regulation of quiescence and activation. Further investigations of the regulatory factors that affect alternative cleavage and polyadenylation of mRNAs or miRNA expression will provide a better understanding of how the quiescent state is actively regulated by these transcriptional and post-transcriptional mechanisms.

In view of the epigenetic control of stem cell quiescence, loci required for stem cell activation are possibly marked by permissive histone marks. Transcriptional activation of loci that are marked by H3K4me3 at their transcription start sites may depend on additional trans-criptional and post-transcriptional mechanisms. To understand whether loci are actively transcribed, it is crucial to consider occupancy of RNA polymerase II (Pol PII) at methylated histones as well as the Pol II phosphorylation status (FIG. 4). Pol II activity is tightly regulated by phosphorylation of its carboxy-terminal domain (CTD)[140]. Phosphorylation of Ser5 of the Pol II CTD by transcription factor IIH (TFIIH) is required for transcription initiation, whereas phosphorylation of Ser2 of the Pol II CTD, of DSIF (DRB sensitivity inducing factor) and of NELF (negative elongation factor) by pTEFb (positive transcription elongation factor) is needed for transcription elongation[141–143]. In various cell types, including ES cells, Pol II often occupies promoter regions and not the gene body. This is consistent with evidence of promoter-proximal pausing which indicates some form of post-initiation regulation[144–146]. Interestingly, Ser2 phosphorylation of the Pol II CTD is absent in many adult quiescent stem cells, which indicates a lack of transcription elongation in these cells[147]. MYC controls Pol II-mediated elongation and pause release, and it can activate a large number of genes that promote rapid proliferation[144]. The existence of mechanisms in ES cells that allow silent genes to be activated in a precise and synchronous fashion may also be applicable to the regulation of adult stem cell activation from the quiescent state. The identification of genes that are poised for stem cell activation may

provide insight into how the quiescent state can rapidly respond to changes in their environment.

In addition, genes that are necessary for lineage progression can be poised by transcriptional and post-transcriptional mechanisms. Epigenetic profiling of the H3K4me3 mark in quiescent HFSCs or quiescent MuSCs (T.H.C and T.A.R, unpublished observations) has revealed a number of genes that are marked by this permissive histone mark[61,62]. In support of the hypothesis that the quiescent state is poised for activation, many of these H3K4me3-rich genes (most of which are expressed at low levels) are functionally important for activation and proliferation. Thus, gene expression and epigenetic profiling during stem cell lineage progression will provide important insights into genes and pathways that are programmed for activation in quiescent stem cells, rendering those cells poised for activation.

Perspective and concluding remarks

Advances in genetic approaches such as lineage tracing have now allowed the design of prospective isolation techniques for the purification of rare cell populations such as quiescent stem cells. Similarly, the use of conditional gene ablation approaches allows the functional analysis of individual genes and pathways in such rare populations in physiological settings. As demonstrated by studies of different stem cell compartments, one of the consequences of inhibiting essential signalling pathways that maintain the quiescent state is the premature activation or differentiation of stem cells. This is often followed by the exhaustion of the stem cell pool and results in impaired tissue homeo-stasis and regeneration, highlighting the importance of maintaining stem cell quiescence for tissue and organismal health. In some tissues, quiescent stem cells seem to serve as a reserve pool of stem cells and are only called into action upon tissue injury. A better understanding of stem cell quiescence and the intrinsic mechanisms by which such cells sense and respond to environmental signals will undoubtedly aid the design of new therapeutic approaches based on enhancing stem cell functionality.

Supplementary Material

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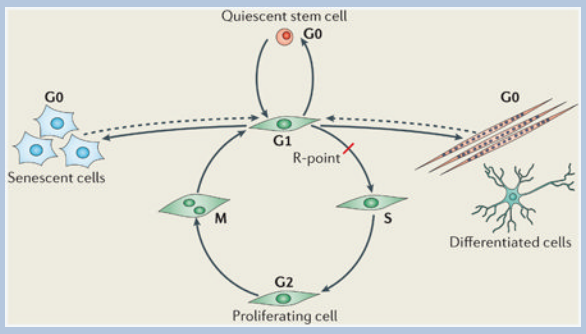
Glossary

Progenitor cell	Proliferating stem cell progeny that can differentiate into specific cell types
Heterochronic parabiosis	Whereby an old animal is surgically connected to a young animal to promote the establishment of a single, shared circulatory system between the two
Lineage tracing	The process of identifying all progeny of a single cell
Transit amplifying progenitors	Progenitor cells that replicate rapidly with very short cell cycle times for progenitor cell expansion

Box 1: Reversibility of the G0 state of the cell cycle



Somatic cells are able to enter reversible (quiescent) or irreversible (senescent and differentiated) G0 states from the G1 phase of the cell cycle before the restriction point (R-point). Once cells reach the R-point, they are committed to the next round of the cell cycle (see the figure). Subpopulations of stem cells reside in the quiescent state and enter the cell cycle when they become activated in response to extrinsic signals. The fate of a cell is determined during G1, and cells differentiate, become senescent or re-enter the quiescent state. Senescent cells are dysfunctional cells that have ceased proliferation and are permanently withdrawn from the cell cycle[148]. Increasing evidence suggests that senescence has a role in suppressing malignant tumour formation[148]. Moreover, the accumulation of senescent cells in aged tissues causes tissue damage due to factors that these cells secrete[149], and removal of these cells may delay tissue ageing[150]. Unravelling the mechanisms that regulate cellular senescence may provide clues as to how the relative reversibility of different G0 states is controlled and have broad implications for tissue regeneration, ageing and cancer.

Analogous to differentiated, non-cycling cells in mammals, some types of amphibians possess mature differentiated cells that are able to dedifferentiate and proliferate to regenerate lost tissues and even entire appendages[151]. In these amphibians, such as newts, differentiated multinucleate myotubes are able to undergo cellularization to generate mononucleated cells[152]. Surprisingly, intracellular pathways that mediate the remarkable regenerative capacity of these organisms seem to be intact in mammals. For example, myonuclei in terminally differentiated mammalian myotubes have been reported to exhibit cell cycle re-entry when exposed to an extract derived from regenerating newt limbs[153]. Also, overexpression of the homeobox-containing transcriptional repressor MSX1 in mammalian myotubes, a protein that is specifically expressed in undifferentiated cells in developing limb buds[154], has been reported to cellularize differentiated myotubes into proliferating mononucleated cells[155]. Furthermore, recent studies suggest that both terminally differentiated cells and senescent cells are able to re-enter the cell cycle by inhibiting tumour suppressors such as p53 and RB[156,157]. Together, these results indicate that intrinsic mechanisms inducing ‘irreversibly’ arrested cell types to enter the cell cycle (dotted arrows) are intact, although repressed, in mammalian cells.

Box 2: Quiescent stem cells and the stem cell niche

In addition to the intrinsic mechanisms that regulate stem cell quiescence, the stem cell niche (that is, a specific microenvironment that surrounds stem cells and has important regulatory functions) is essential for stem cell maintenance, including the maintenance of quiescence[158]. First described by Schofield in 1978 (REF. 159), stem cell niches have been identified for all types of adult stem cells in mammalian tissues. In malignant tissues, cancer stem cells are thought to take advantage of the niche that supports normal stem cell behaviours[158,160]. To understand the role of the stem cell niche, it is necessary to determine the composition of the niche (for a review, see REF 158). Local cellular stem cell niche components include other cell types such as those of the vasculature and interstitium, as well as matrix proteins and constituents. Soluble factors, either secreted from nearby cells or from distant sources, can influence stem cell function, resulting in alternative stem cell fates[161]. The recent development of new genetic tools has provided insights into the interaction between the niche and the stem cell. Using heterochronic parabiosis to study muscle stem cell (MuSC) ageing, it has been demonstrated that systemic niche factors are crucial regulators of quiescent stem cell function that change with age[162]. Another study, using a transgenic reporter of the regulatory factor SCF (stem cell factor), identified the major sources of SCF in the haematopoietic niche in bone marrow[163]. The interaction between the niche and quiescent stem cells is also relevant in stem cell ageing. Intriguingly, the disruption of the niche has been linked to the decline of stem cell function during the process of ageing[24]. Analysis of injured muscle has revealed an age–dependent decrease in the expression of the Notch ligand Delta-like 1, resulting in decreased Notch signalling and impaired MuSC proliferation[164]. By contrast, aged muscle fibres secrete fibroblast growth factor 2 (FGF2), which induces MuSCs to divide more frequently, resulting in the disruption of stem cell quiescence[24]. Thus, a better understanding of how quiescent stem cells interact with the niche will provide important insights into the components that regulate stem cell quiescence.

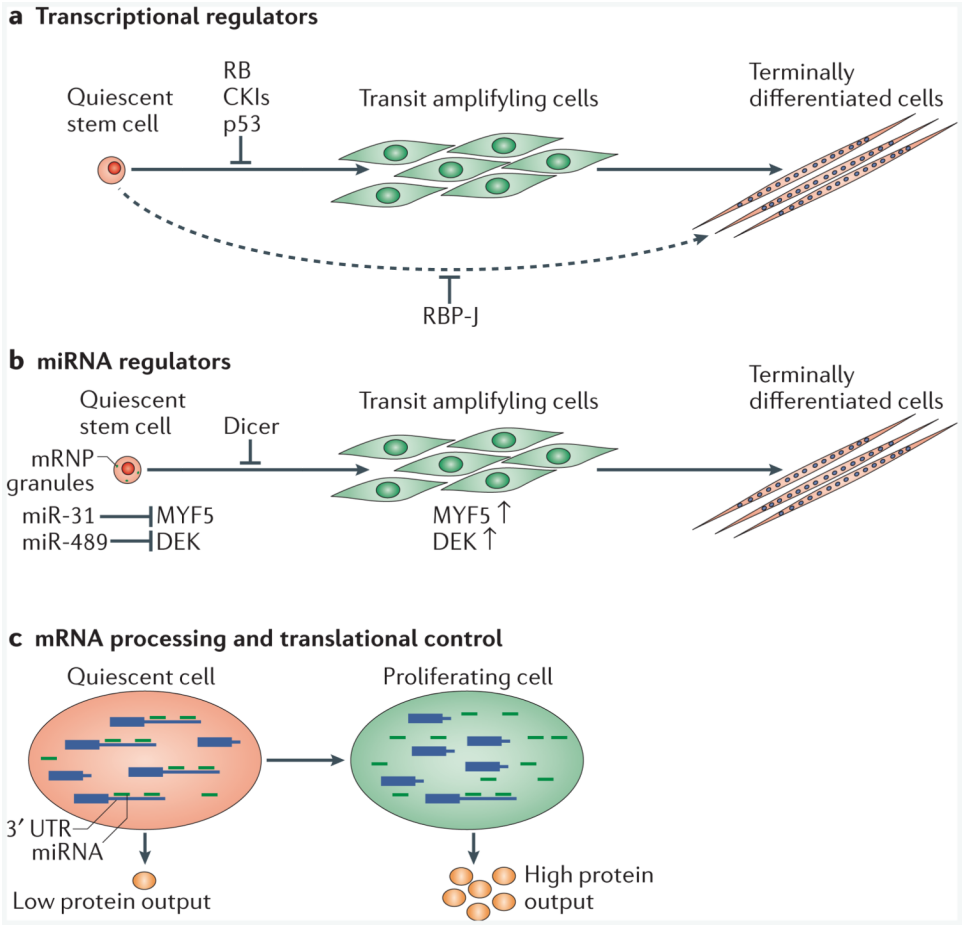


Figure 1. Molecular regulation of stem cell quiescence

Recent data suggest that the state of quiescence is actively regulated by different molecular mechanisms. **a** | Quiescence regulators such as RB, cyclin-dependent kinase inhibitors (CKIs) and p53 negatively regulate the activation of quiescent stem cells. In quiescent muscle stem cells, the loss of the downstream effector of the Notch signalling pathway, RBP-J, promotes spontaneous activation and terminal differentiation, in some cases without cell division. **b** | Quiescent stem cells are actively regulated by post-transcriptional mechanisms. The loss of the microRNA (miRNA)-processing enzyme Dicer or specific miRNAs promotes quiescent stem cell activation. **c** | Differential mRNA processing alters the susceptibility of mRNAs to miRNA regulation. In quiescent cells, distal polyadenylation signals (PASs) are used to generate mRNA transcripts with long 3′ untranslated regions (3′ UTRs). In proliferating cells, proximal PASs are used, which decrease the number of miRNA target sites on the transcripts, allowing some transcripts to escape miRNA-mediated inhibition and leading to increased protein expression.

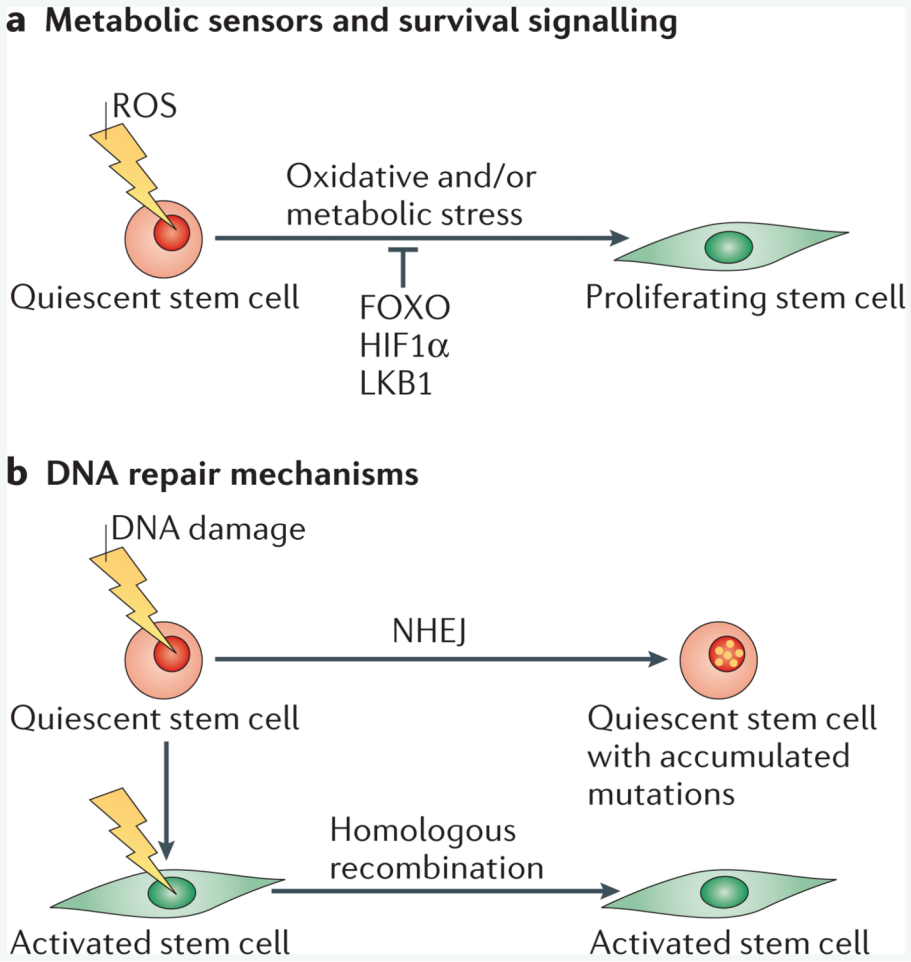


Figure 2. Survival mechanisms of quiescent and activated stem cells

a| Metabolic sensors an d effectors such as forkhead box O (FOXO), hypoxia-inducible factor 1α (HIF1α) and liver kinase B1 (LKB1) are expressed in quiescent stem cells. Expression of these molecules is essential for metabolic functions and survival of quiescent stem cells in adverse environments. These factors protect quiescent stem cells from oxidative stress caused by the accumulation of reactive oxygen species (ROS). Quiescent stem cells devoid of these pathways have an increased propensity to become activated and fail to maintain the stem cell pool. **b** | Non-homologous end-joining (NHEJ) and homologous recombination are pathways that repair DNA double-strand breaks (DSBs). Homologous recombination is a high-fidelity mechanism that uses homologous templates as guides for DSB repair, whereas NHEJ directly ligates the ends of the DSBs. DSBs that have been repaired by NHEJ can be imprecise when the overhangs at the DSBs are not compatible. In quiescent stem cells, the error-prone NHEJ mechanism is used for DSB repair, which suggests that DNA mutations accumulate in these cells throughout their life. MYF5, myogenic factor 5.

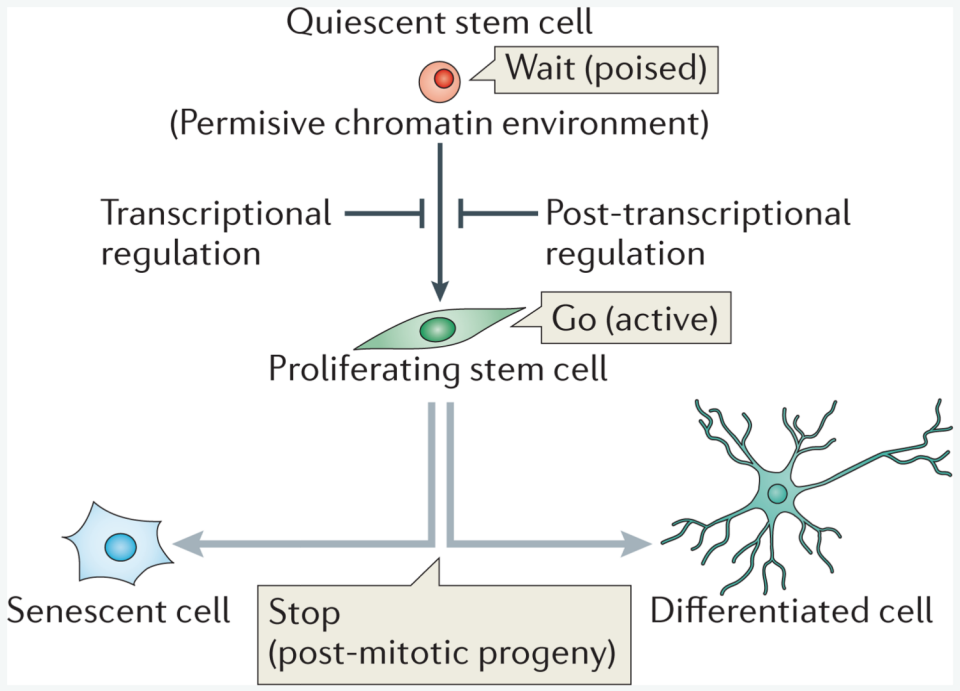


Figure 3. Quiescent stem cells are poised for activation

A proposed model of how the quiescent state of a stem cell constitutes a poised state for activation. Quiescent stem cells are actively regulated at the epigenetic, transcriptional and post-transcriptional level. The epigenetic landscape keeps the chromatin in a permissive state, which allows rapid transcriptional activation. Additional layers of transcriptional and post-transcriptional control safeguard quiescent stem cells to enable precise stem cell activation when necessary.

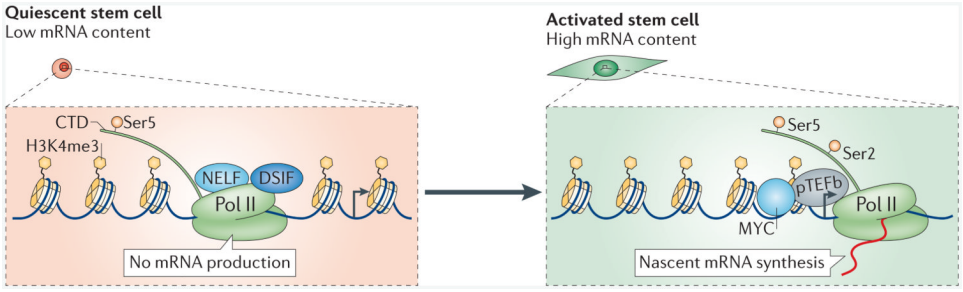


Figure 4. Transcriptional control of stem cell quiescence

Current data suggest that RNA polymerase II (Pol II) is paused at transcription start sites where histone H3 is trimethylated at Lys 4 (H3K4me3; which is a permissive histone mark). Quiescent stem cells have low mRNA content. The carboxy-terminal domain (CTD) of PolII is phosphorylated at Ser5 but not the Ser2, which indicates transcriptional initiation but not transcriptional elongation. Many types of quiescent stem cells lack Ser2 phosphorylation at the Pol II CTD, which suggests that transcriptional elongation does not occur in these cells, and hence the mRNA levels are low. In quiescent stem cells, Pol II is associated with the negative elongation factors DSIF (DRB sensitivity inducing factor) and NELF. Upon stem cell activation, phosphorylation of DSIF, of NELF (negative elongation factor) and of Ser2 of the Pol II CTD, in combination with the expression of MYC, which is recruited to promoters together with transcription elongation factors (such as pTEFb (positive transcription elongation factor b)), transform the state of promoter proximal pausing into productive elongation and lead to mRNA synthesis.

Table 1

Quiescent stem cell gene signature*

Function	Downregulated genes‡	Upregulated genes‡
Cell cycle progression and checkpoint control	*ANLN, BIRC5, CCNA2, CCNB1, CCNE2, SGOL1*	*CCND3, PDK1*
DNA replication and chromosome segregation	*MCM4, PCNA, RRM2, TOP2A*	
Mitochondrial function	*CYCS, MTCH2, SLC25A5*	
Chromatin and nucleosome assembly	*H2AFZ, HAT1*	*SMARCA2*
Regulation of transcription		*FOXO3, EZH1, PRDM5, PTOV1, ZFP30, ZBTB20, PHF1, CTDSP1, THRA, TEF*
RNA processing	*DDX39*	*DICER1*
Other	*2810417H13Rik, CAPZA1, HADHB, IDH3A, KPNA2, PGK1*	*A930001N09Rik, BCAS3, DDX3Y, GABARAPL1, GLTSCR2, ITM2A, IL18, ZYX, EPHX1, CLSTN1, GSTK1, 5730403B10Rik, DDT, IVD, FHL1, NDRG2, GRINA, PIK3R1, FYN, CHKB, PINK1, ULK2, DNAJB9, PFDN5, CTSF, CRIM1, SEPP1, GABBR1, GRB10, BBS2, RPS14, IGF2R, SELENBP1, RNF167, MAP1LC3A*

*Comparison of microarray data sets revealed a gene signature that is common to quiescent haematopoietic stem cells (HSCs), muscle stem cells (MuSCs) and hair follicle stem cells (HFSCs). Selected genes (30 out of 71 genes) are shown and grouped on the basis of pathways in which they are presumed to function. Genes exhibiting expression level changes that are shared among the stem cell compartments are listed under ‘other’. Consistent with the dormant phenotype of a quiescent stem cell, genes that are involved in cell cycle progression, DNA replication or mitochondrial functions are mostly downregulated in quiescent stem cells.

‡For a full list of gene definitions see Supplementary information S1 (table)).