Cellular quiescence is a reversible non-proliferating state. The reactivation of ‘sleep-like’ quiescent cells (e.g. fibroblasts, lymphocytes and stem cells) into proliferation is crucial for tissue repair and regeneration and a key to the growth, development and health of higher multicellular organisms, such as mammals. Quiescence has been a primarily phenotypic description (i.e. non-permanent cell cycle arrest) and poorly studied. However, contrary to the earlier thinking that quiescence is simply a passive and dormant state lacking proliferating activities, recent studies have revealed that cellular quiescence is actively maintained in the cell and that it corresponds to a collection of heterogeneous states. Recent modelling and experimental work have suggested that an Rb-E2F bistable switch plays a pivotal role in controlling the quiescence–proliferation balance and the heterogeneous quiescent states. Other quiescence regulatory activities may crosstalk with and impinge upon the Rb-E2F bistable switch, forming a gene network that controls the cells’ quiescent states and their dynamic transitions to proliferation in response to noisy environmental signals. Elucidating the dynamic control mechanisms underlying quiescence may lead to novel therapeutic strategies that re-establish normal quiescent states, in a variety of hyper- and hypo-proliferative diseases, including cancer and ageing.

1. Introduction

There are $10^{13}–10^{14}$ cells in our human body. At any given time, the vast majority of these cells are non-dividing and outside of an active cell cycle. Some of these non-dividing cells (e.g. senescent or terminally differentiated cells) are irreversibly arrested; they can no longer re-enter the cell cycle to proliferate under normal physiological conditions. By contrast, a subset of non-dividing cells is ‘reactivatable’ and can enter the proliferative cell cycle in response to physiological growth signals; these cells are called quiescent cells (figure 1). Examples of quiescent cells include many adult stem cells, progenitor cells, fibroblasts, lymphocytes, hepatocytes and some epithelial cells. The exact number of quiescent cells in the body is not well characterized.

Unlike many unicellular organisms in which the cell can reside in either a quiescent dormant state or a growth state depending on the environment, in adult multicellular organisms the quiescent cellular state is unique to higher organisms. In lower multicellular organisms, such as Caenorhabditis elegans, all cells in the adult body are ‘post-mitotic’ and no longer able to proliferate. However, in higher multicellular organisms with prolonged lifespans, such as mammals, reversible quiescent cells are fundamental to forming tissues capable of renewal and regeneration, which is critical for the tissue homeostasis of the adult body. Cellular quiescence also provides protection against stress and toxicities, which is especially important for long-lived cells, such as stem cells [1,2]. On the other hand, the balance between cellular quiescence and proliferation needs to be carefully regulated in tissues of higher organisms; misregulation of the quiescence–proliferation balance can lead to a wide range of hypo- and hyper-proliferative diseases, such as fibrosis, autoimmune diseases, cancer and ageing.

Regarding the two sides of the quiescence–proliferation balance, while cell proliferation and its regulation have been well studied, cellular quiescence is poorly understood. One recent PubMed search with ‘cell proliferation’ returned over 190 000 articles, while searches for ‘cell quiescence’ and ‘cellular quiescence’ returned about 200 hits each. The lack of study of cellular quiescence...
is at least partially owing to the fact that, for a long time, quiescence had been simply regarded as a passive and dormant cellular state lacking proliferation activities.

Studies in the past few years have revealed that, instead of being a passive state, quiescence is actively maintained in the cell. Quiescent cells are transcriptionally active: in addition to the downregulation of proliferation-related genes, quiescent cells actively express a group of genes that are distinct from those in proliferating cells or in cell cycle arrested cells forced by the overexpression of cyclin-dependent kinase inhibitors (CKIs) p21 or p27 [3–6]. At least some of the actively expressed genes in quiescent cells contribute to the reversibility of quiescence (e.g. by suppressing terminal differentiation and apoptosis) [3]. As a result, quiescent cells, but not forced cell cycle arrested cells, are resistant to the inductions of differentiation and senescence [3,7]. In addition, while many quiescent cell types exhibit reduced metabolic activities, quiescent fibroblasts maintain comparable metabolic rates to those in proliferating counterparts [8,9].

Recent studies including ours have further shown that quiescence is not a unique state but rather a collection of heterogeneous states. Quiescence has traditionally been considered a single non-proliferating state (also called ‘G0’) [10–13]) outside of the cell cycle. However, cells induced to quiescence by different signals (e.g. serum starvation, loss of adhesion and cell contact inhibition at high cell density) exhibit overlapping yet distinct gene expression profiles, suggesting that cells may enter different quiescent states depending on the initiating signals [3]. We found that fibroblasts that are serum starved for longer periods of time appear to access ‘deeper’ states of quiescence: the longer serum-starved cells (e.g. those starved for 6 versus 2 days) were less likely to exit quiescence in response to growth signals at non-saturating levels (e.g. 1% serum; Kwon & Yao 2014, unpublished data); furthermore, among those cells that exited quiescence, longer serum-starved cells did so at a slower speed than shorter serum-starved cells (similar to earlier observations [14,15]). Yet, the longer starved cells could still re-enter the cell cycle to proliferate under normal growth conditions (e.g. 10% serum), demonstrating that they reside in a reversible quiescent state, not an irreversibly arrested state, such as senescence. Together, accumulating evidence indicates that quiescence goes beyond a single homogeneous state.

What mechanisms underlie the actively maintained, heterogeneous quiescent states? Answers to this question will help in uncovering a currently underappreciated layer of complexity in growth control and provide novel insights into the cause and treatment of hyper- and hypo-proliferative diseases. High-throughput studies in the past few years have suggested a long list of cellular activities associated with quiescence, particularly in fibroblasts and adult stem cells, such as haematopoietic stem cells (HSCs) [3–6]. Meanwhile, recent computational modelling studies have begun to provide an effective framework to integrate descriptive ‘parts lists’ into a mechanistic understanding of the basic controls of cellular quiescence, which will be reviewed below.

2. Phenotypic quiescence – proliferation transition

2.1. Restriction point

The proliferation of normal, untransformed mammalian cells in culture requires serum, which contains a combination of growth factors. Specifically, cells are sensitive to extracellular serum signals in the G1 phase of the cell cycle prior to a critical time point, namely the ‘restriction point’ or ‘R-point’ [16]. If serum starvation occurs in the G1 phase before the R-point, cells will withdraw from the cell cycle and enter quiescence. After passing the R-point, however, the cell cycle becomes autonomous—even when serum is removed, the cell cycle will continue until it is completed. Therefore, it appears that the R-point is a ‘point of no return’ at which a cell commits to proliferation. In actively growing Swiss 3T3 cells, whose average cycle time is 16 h with an average 7 h G1 phase, the R-point is approximately 3.5 h after cell birth [17].

There are two key characteristics of the R-point: first, it sets a high threshold that separates quiescence from proliferation, serving as a noise filter against uncontrolled and accidental cell growth; second, it provides a low-maintenance mechanism to ensure that, once initiated, the cell cycle will be completed regardless of later fluctuations in the extracellular environment, which is fundamental to maintaining genome integrity.

2.2. G0 versus G1: two non-proliferating phases before DNA replication

There is an R-point in every cell cycle before the start of cell proliferation. However, the first R-point that cells encounter when they exit quiescence is distinct from the other R-points in actively growing cells. Despite a historical debate regarding whether quiescent cells reside in an extended G1 phase at positions where they last see growth signals [18–20], quiescence is typically considered a distinct G0 state outside of the cell cycle. Quiescent G0 cells are similar to the cells in the G1 phase of a proliferative cell cycle (G1 cells): they both reside between M- and S-phases and contain non-replicated 2n DNA content. However, compared with G1 cells, G0 cells experience a significant delay in re-entering S-phase. In Swiss 3T3 cells, such a delay lasts about 8 h [17]. The reasons behind this delay in quiescent cells are not entirely clear, but may be owing to the fact that proteins (e.g. CDC6) required for creating the DNA replication origins are removed from chromatin in G0 (but not G1) cells [21].

2.3. Historical models on the quiescence – proliferation transition

In the 1970s and 1980s, a number of mathematical models were proposed to describe the transition between cellular quiescence and proliferation and the apparent cell-to-cell variations in this
process within a mammalian cell population. One class of models, ‘transition probability’ (TP) models [22–25], assume that a cell has two distinct phases: non-replication (NR-phase) and replication (R-phase). Cells leave the NR-phase randomly but with a constant probability; they then enter the R-phase in which replicative activities are deterministic. In TP models, it is the random transition from the NR-phase to the R-phase that is thought to create the variations in growth rates of cells in a population. The NR-phase in original TP models referred primarily to the G1 phase of actively proliferating cells; it was soon extended to the quiescent state (the G0 state) in serum-starved cells (and, correspondingly, an additional random transition from the G0 to G1 phase was proposed) [26].

Another class of models, ‘growth controlled’ (GC) models [27,28] or ‘continuum’ models [18,29], proposed that the different growth rates (i.e. different cell cycle durations) of cells in a population do not result from the probabilistic transition from the NR-phase to the R-phase, but rather from the cell-to-cell variations in biomass and cell metabolism as well as the related time required to complete essential steps in the cell cycle. Integrating TP and GC models, the hybrid ‘sloppy size control’ (SSC) model [30,31] proposed that the quiescence–proliferation transition is a random process with cell-size-dependent probability. Interestingly, all of these different models (TP, GC and SSC) fit well with various types of experimental data; however, they are all descriptive only at the population distribution level. Interest in these models gradually faded but was later reinvigorated by findings in molecular and cell biology, particularly in the genes that regulate the quiescence–proliferation transition.

3. Rb-E2F bistable switch: a mechanistic framework underlying quiescence

3.1. Rb-E2F pathway

Among cellular activities that regulate the quiescence–proliferation transition in mammalian cells, the Rb-E2F pathway plays a pivotal role (figure 2). Rb (retinoblastoma) was the first identified tumour suppressor gene [32]. The Rb protein family also contains p130 and p107; these so-called pocket proteins (pRb, p107 and p130) regulate proliferation [70]. Very recently, it was shown that a low versus continual proliferating cells [56].

Quiescent cells typically feature lower levels of Rb-E2F pathway activators (e.g. CycD [53–55], Cdk2 [56]) and high levels of pathway repressors, such as Cdk inhibitors (e.g. p21 [57], p27 [58,59]), Rb family proteins (especially p130 [43,60,61]) and E2F repressors that are either dependent (e.g. E2F4 [43,62]) or independent (e.g. E2F6 [63]) of Rb family proteins to silence E2F target genes. Downregulation of Cdk inhibitor activities (e.g. p27 [59,64], p21 [55,57,65,66]) can lead to quiescence exit and cell cycle re-entry. Similarly, the disruption of all three Rb family proteins [67–69] and the acute loss of the Rb function in quiescent cells lead to cell proliferation [70]. Very recently, it was shown that a low versus increasing Cdk2 activity (as directly controlled by p21) at mitotic exit marks the bifurcation of a cell population into quiescent versus continual proliferating cells [56].

3.2. Rb-E2F bistable switch

The ‘high-threshold, low-maintenance’ transition between quiescence and proliferation at the R-point is reminiscent of a bistable switch, as seen in several decision-making processes [71–74]. Regulation by positive feedback is a hallmark of a bistable switch [75,76]. Despite the fact that positive-feedback loops are under-represented in biological networks [77], they are enriched in the Rb-E2F pathway, suggesting functional non-randomness (e.g. the mutual inhibition between Rb and E2F and the E2F self-activation mentioned above and others,
including the mutual inhibition between CycE/Cdk2 and p27 [78]). Several mathematical models of the Rb-E2F-cyclin/Cdk network were proposed and they suggested the theoretical possibility that the mammalian R-point is controlled by a bistable mechanism [79–83].

By coupling single-cell experiments (measuring E2F expression dynamics in individual cells using a destabilized GFP reporter) with a simplified Rb-E2F model, we have recently shown that the Rb-E2F pathway indeed functions as a bistable switch [84]. This Rb-E2F bistable switch converts graded and transient serum growth signals into a binary (Off and On) and hysteretic E2F activity in individual cells (figure 2). Once turned ‘On’ by strong growth stimulation, E2F remains On even when the serum simulation is diminished [84]. The all-or-none E2F activation directly underlies the all-or-none transition between quiescence and proliferation. At the single-cell level, the very cells that re-entered the cell cycle in response to serum growth signals were those that switched On E2F [84]. Importantly, the Rb-E2F bistable switch is resettable: if the serum concentration drops below a ‘deactivation threshold’ (corresponding to a serum concentration that is lower than the switch’s activation threshold; see fig. 1 in [84]), the final E2F level eventually returns to the monostable Off state after a time delay [84,85]. By creating a wide hysteresis loop, the resettable Rb-E2F switch (in the steady-state domain) drives the irreversible cell cycle progression after the R-point (in the temporal domain) [85].

Using a computational search of all possible topologies derived from a simplified Rb-E2F circuit, a 3-node module (containing coarse-grained nodes EE, RP and MD, which correspond to the E2F activators, the Rb family proteins and the linear signalling cascade from Myc to CyclD, respectively; figs 1 and 2 in [85]) has been identified as the minimal module responsible for creating the Rb-E2F bistable switch. This core module contains a mutual inhibition loop between Rb and E2F and a feed-forward motif from Myc to E2F (figure 2). Experimental disruption of this core motif (by a small chemical inhibitor of Cdk2) abolishes the bistability in the Rb-E2F circuit [85].

The Rb-E2F bistable switch model provides a mechanistic explanation for the R-point transition between quiescence and proliferation. Interestingly, the historical TP and GC models can be reconciled into the same Rb-E2F bistable switch model: the simulated stochastic E2F activation based on a stochastic Rb-E2F model with a given set of parameters (e.g. parameters related to protein synthesis, turnover and modification as well as the degree of stochasticity) can be uniquely mapped to coarse-grained parameters defining the TP and GC models, respectively [86]. Therefore, the stochastic Rb-E2F model can be considered as a common mechanistic basis for the seemingly different TP and GC models, each of which holds true in describing particular aspects of the cell cycle transition.

4. Activation threshold of the Rb-E2F switch defines heterogeneous quiescent states

4.1. An analogue-to-digital convertor

Extracellular quiescence and growth signals are ‘analogue’ as they can vary continuously in degrees and durations. By contrast, the final quiescence–proliferation decision of each individual cell is ‘digital’ (all or none) because otherwise incomplete cell proliferation would disrupt genome integrity. A bistable system provides a necessary analogue-to-digital conversion mechanism allowing cells to respond to a complex environment yet still have a discrete cell-fate output. Along these lines, the Rb-E2F bistable switch is the only experimentally verified bistable system in mammalian cell cycle entry; it serves as a ‘core’ control system enabling the necessary analogue-to-digital conversion in the cell. Consistent with this notion, the few genes whose exogenous expression alone is sufficient to drive quiescent cells into proliferation (e.g. Myc, E2F, CycE) [51,87,88] are all part of the Rb-E2F bistable switch (figure 2).

4.2. E2F activation threshold underlies heterogeneous quiescent states

The E2F-Off state of the Rb-E2F bistable switch defines cellular quiescence versus proliferation. Between the different quiescent states induced by different durations of serum starvation (e.g. 2 versus 6 days), there is little difference in the E2F-Off state (i.e. the basal E2F expression level); yet, the serum strength required to turn On the Rb-E2F bistable switch varies (Kwon & Yao 2014, unpublished data). We define the minimum serum concentration required to turn On the Rb-E2F bistable switch in over half of a cell population within a time frame (e.g. 48 h) as the ‘E2F activation threshold’. A deeper quiescent state (e.g. in longer starved cells) features a higher E2F activation threshold (figure 3a), which can also be illustrated from computer simulations based on the Rb-E2F bistable switch model (figure 3b,c). Therefore, an emerging model is that a unique E2F activation threshold (in terms of minimal serum stimulation) defines each quiescent state. This model also predicts that, given the same growth stimulation, cells at a deeper quiescent state will exhibit a slower speed of switching E2F from the Off to On state (figure 3a), consistent with earlier observations that longer serum-starved cells are slower to re-enter the cell cycle [14,15].

In our Rb-E2F bistable switch model, the E2F activation threshold and the switch’s resettable property (discussed in the previous section) are dynamic properties intrinsic to the Rb-E2F pathway, determined by the balance between the pathway’s activators (e.g. cyclin/Cdkks) and inhibitors (e.g. Cdk inhibitors and Rb family proteins). Cells under different environmental conditions or with different cell types are likely to display different profiles of these activators and inhibitors (e.g. in terms of their abundance as well as their synthesis and turnover rates), which will affect the parameter values in the Rb-E2F model that determine the final E2F activation threshold. Future studies are needed to further test the multi-state quiescence model and its underlying control mechanisms (e.g. potentially on the gene regulatory, epigenetic or metabolic level), as well as to characterize the Rb-E2F switch behaviours that are likely to be qualitatively identical but quantitatively different in different settings (e.g. in stem cells versus fibroblasts, in tissues in vivo versus under culture conditions in vitro).

4.3. E2F activation threshold may define the reversibility of quiescence

Under senescence and terminal differentiation, the expression of Cdk inhibitors (e.g. p16 and p21) is greatly increased [88–92], which raises the E2F activation threshold. When the
E2F activation threshold increases above physiological serum levels, the Rb-E2F switch becomes irreversible under normal growth conditions. However, such ‘irreversible’ states can be reverted by decreasing the E2F activation threshold, which can be accomplished by reducing the high level of Cdk inhibitors [55] or their activators, such as p53 and oncogenic Ras [93], or by the forced expression of exogenous cyclin/Cdks [94]. Therefore, an E2F activation threshold within the physiological range is likely to distinguish reversible quiescent states from irreversibly arrested non-proliferating states, such as senescence and terminal differentiation.

5. Other quiescence regulators

Besides the Rb-E2F pathway, several other cellular pathways have been reported to regulate cellular quiescence and the quiescence–proliferation transition. Such quiescence regulators include Notch-HES1, p53, and stress and metabolic response pathways, as well as autophagy, microRNA (miRNA) and epigenetic mechanisms. These quiescence regulators crosstalk with the Rb-E2F pathway: some directly impinge on the Rb-E2F pathway and affect its bistable switch dynamics (and thus the final E2F activation threshold); some are regulated by the Rb-E2F bistable switch and serve as its ‘effectors’ to regulate cellular quiescence.

5.1. Notch-HES1 pathway

The Notch signalling pathway is an important regulator in tissue maintenance and regeneration during development [95]. Notch is also involved in the regulation of cellular quiescence through its effector proteins, transcriptional regulators RBPJ and HES1. The deletion of RBPJ leads to spontaneous cell cycle re-entry and eventually the depletion of quiescent muscle stem cells [96,97]. The expression of HES1 is upregulated in quiescent fibroblasts, but not in the same cell type that undergoes cell cycle arrest by ectopic p21 [3]. HES1 has been shown to be required for maintaining the reversible quiescent states by preventing premature senescence and differentiation [7,98]. Interestingly, HES1 is a transcriptional repressor of several key players in the Rb-E2F pathway, including E2F1 [99], p21 [100] and p27 [101].

5.2. p53 pathway

p53 is a master regulator of many cellular responses, most notably DNA damage response and apoptosis. p53 is
important for quiescence maintenance of human fibroblasts and HSCs; p53 disruption can lead to quiescence exit in these cells [102,103]. The role of p53 in quiescence maintenance may involve its target genes, Gfi-1 and Necdin [103,104], and such a role is negatively regulated by the transcription factor MEF/ELF4 [105]. Notably, p53 activates p21, a Cdk inhibitor and critical component of the Rb-E2F pathway.

5.3. Stress and metabolic response pathways
As quiescent cells may stay in the non-proliferating state for a prolonged time under suboptimal conditions, they need to cope with accumulated cellular stresses and metabolic changes. Cellular activities such as those of the FOXO [106,107] and ATM-BID [108] pathways protect quiescent cells from the oxidative stress caused by the accumulation of reactive oxygen species (ROS); depletion of these cellular activities leads to quiescence exit in HSCs and an increase in their proliferation and apoptosis. Quiescent cells often exhibit low metabolic activities characterized by a decrease in glucose uptake and glycolysis, reduced translation rates, as well as reduced PI3K and mTOR pathway activities [9]. The PI3K-Akt pathway crosstalks with the Rb-E2F pathway by inhibiting p21 activity through phosphorylation [109,110]. Similarly, the deletion of HIF1α [111] (involved in hypoxia regulation), LKB1 [112–114] (a regulator of AMPK that is an mTOR and FOXO pathway target) and negative regulators of mTOR (such as Fbxw7 [115], PTEN [116,117], PML [118] and TSC [119]) leads to the depletion of HSCs, suggesting the importance of metabolic response pathways in maintaining cell quiescence. Interestingly, E2F was recently found acting as a regulatory switch coordinating proliferation and metabolic pathways by repressing key genes involved in oxidative metabolism [120].

5.4. Autophagy
Quiescent cells are associated with activation of autophagy pathways [121]. Autophagy is a process of ‘self-eating’ that recycles organelles and removes damaged components through a lysosomal degradation pathway. Autophagy provides nutrients for cell survival under suboptimal growth conditions [122–124] and is essential for maintaining HSC quiescence [125]. Autophagy is actively repressed by mTOR [126–128] and is regulated by the Rb-E2F pathway [129,130].

5.5. MicroRNAs
miRNAs have also been implicated in the regulation of quiescence at the post-translational level. The miRNA profiles of quiescent stem cells and fibroblasts are different from their proliferative or differentiated progenies [131–134]. Several miRNAs, such as miR-126 [135] and miR-489 [131], are important for maintaining quiescent stem cells, while let-7, miR-125 and miR-29 regulate key aspects of fibroblast quiescence [134]. Knockout of Dicer, the miRNA processing factor, leads to quiescence exit of muscle stem cells [131]. There is also recent evidence showing that cellular quiescence is regulated by mechanisms that alter the length of the 3' untranslated region (UTR) of mRNA and thus the potential miRNA susceptibility [1,136–138]. The miRNA pathways crosstalk with the Rb-E2F pathway: for example, miR-221 and miR-222 that regulate the quiescence–proliferation transition are known to target Cdk inhibitors p27 and p57 [139,140]; the let-7 family of miRNAs antagonizes Myc [141]; the miR-17–92 cluster that is induced by Myc negatively regulates E2F1 [142–145].

5.6. Epigenetic regulators
Epigenetic regulations including DNA methylation and histone modifications modify the chromatin states (e.g. accessible or repressed) and the corresponding cellular gene expressions [146–148]. Quiescence may be associated with a unique epigenetic state and chromatin structure that help in maintaining the reversibility of quiescence against terminal differentiation or senescence [98,149]. For example, polycomb group genes EZH1 and EZH2—methyltransferases for histone H3 lysine 27 (H3K27)—are essential for the maintenance of quiescent haematopoietic and hair follicle stem cells [150–152]; specific methylation states of histone H4 lysine 20 (H4K20me2 and H4K20me3) are found to promote chromatin compaction and quiescence entry in primary human fibroblasts [153]. The Rb-E2F pathway also plays an important role in epigenetic modifications [154]. For example, the RB/E2F family proteins interact with various chromatin-modifying complexes that are involved in stable transcription repression [36,39–42]. The E2F proteins also interact with HCF-1, which recruits the MLL family of histone H3 lysine 4 (H3K4) methyltransferases to induce transcriptional activation [155,156].

6. Conclusion
Cellular quiescence and its reversible transition into proliferation in response to environmental signals are critical to the physiological functions of many important cell types in our body (e.g. stem and progenitor cells). A model is emerging from recent studies that the Rb-E2F bistable switch serves as a core analogue-to-digital converter between environmental signals and quiescence–proliferation decisions. Meanwhile, other quiescence regulatory pathways crosstalk with the Rb-E2F bistable switch: some modulate the activation/inhibition balance of the Rb-E2F switch and thus the final E2F activation threshold, and others are regulated by the Rb-E2F switch and serve as its ‘effectors’ to regulate cellular quiescence. The cross-talk between the Rb-E2F bistable switch and quiescence regulatory pathways (and possibly between these pathways themselves) forms a gene network. This gene network integrates environmental signals into the final control of cellular quiescence and its heterogeneous, reversible state. Integrating quantitative modelling and detailed experimentation (e.g. single-cell measurements) holds the promise to dissect and reconstruct the quiescence regulatory gene network. Elucidating this quiescence regulatory network is fundamental to understanding the accurate control of cellular quiescence. This new knowledge could profoundly impact therapeutic strategies by suggesting how to re-establish normal quiescent states in a variety of hyper- and hypo-proliferative diseases, such as cancer and ageing.

Acknowledgements. I thank Dr Lingchong You and Dr Hilary Coller and two anonymous reviewers for critical readings and comments on the manuscript. This work was partially supported by the American Cancer Society (G.Y.; IRC7400134) and a Faculty Seed Grant from the University of Arizona Foundation (G.Y.).


