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Greg H. Enders
Editor

Cell Cycle Deregulation in Cancer

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Part I
Starting the Cell Division Cycle

Chapter 1
Escape from Cellular Quiescence

Elena Sotillo and Xavier Graña

Abstract Quiescent: From Latin *quies*, referring to a state of being at rest, dormant, inactive, quiet, still (Merriam-Webster, 2009, Online Dictionary: http://www.merriam-webster.com/dictionary/quiescent). This term refers to a state of dormancy as opposed to a proliferative state. However, quiescent cells are in any other regard metabolically active. In many tissues with relative fast cell renewal rates the primary function of a small group of undifferentiated cells is limited to self-renewal (stem cells). These cells remain quiescent most of the time dividing only occasionally. In other tissues, key cell types perform fundamental tissue functions while remaining quiescent. Both stem cells and cells from tissues that renew via simple duplication can remain quiescent for long periods of time while retaining the capacity to re-enter the cell cycle. This chapter will discuss the mechanisms emerging as responsible for the maintenance of quiescence as well as those pathways that mediate quiescence entry and exit. We will also review signaling pathways deregulated during infection by Simian Virus 40 (SV40) and oncogenic transformation, which result in unscheduled exit from quiescence into the cell cycle, with focus on SV40 small t antigen.

1.1 Quiescence: The Reversible State

Eukaryotic cells can be in a dividing proliferative state or they can enter non-dividing states. There are four possible non-dividing states: quiescence (G0), senescence, differentiation, and apoptosis. Importantly, only quiescent cells can reversibly re-enter the cell cycle upon appropriate stimuli, whereas terminally differentiated (for the most part) and senescent cells, which can also survive for long periods of time, have permanently withdrawn the cell cycle (Fig. 1.1). In multicellular organisms, commitment to a round of DNA replication and cell division

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requires adequate concentration of mitogens in the environment, space, and for adherent cells, a substrate to attach to. Thus, deprivation of mitogens, lack of adhesion, or growth to high density drive normal cells into quiescence (Fig. 1.1). Recent studies have uncovered that each of these cell cycle exit-initiating signals elicits a distinct gene expression signature (Coller et al., 2006). However, to preserve the reversibility of the quiescent state, a shared "quiescent gene expression program" that includes genes that suppress differentiation and apoptosis is implemented in all instances.

It is well established that the quiescent state is associated with an increase in the expression of the CDK inhibitor p27 (Sherr and Roberts, 1999). Unexpectedly, the study of the gene expression fingerprints that characterize quiescence has also revealed that quiescence is not equivalent to growth arrest induced via inhibition of CDKs. Cells ectopically expressing the p21/p27 CDK inhibitors exhibit a distinctive program of gene expression that includes a portion of the genes found

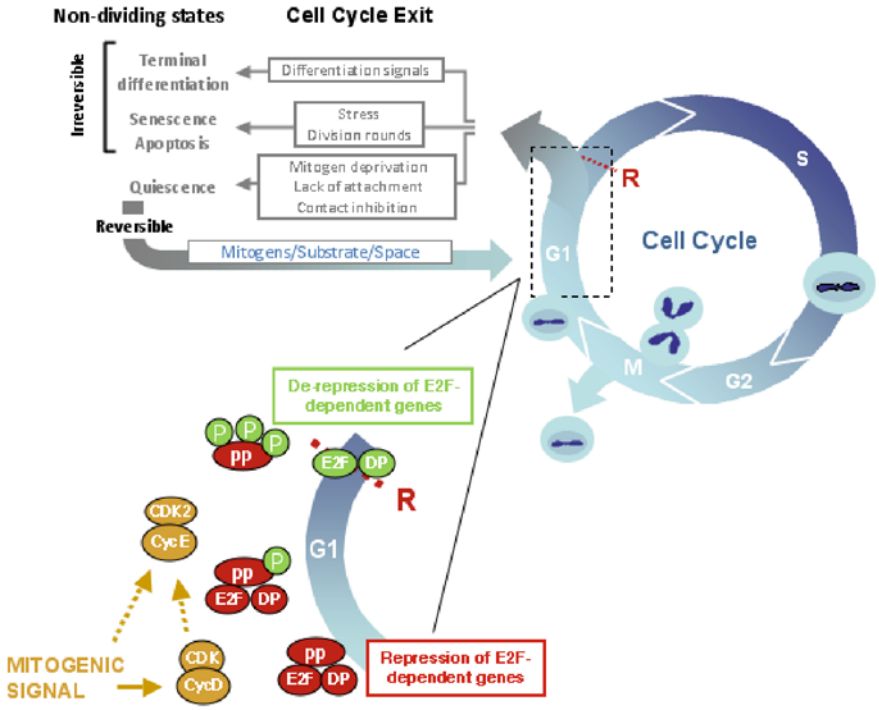

Fig. 1.1 Fate of proliferating normal cells upon cell cycle exit. Upon cell cycle exit, cells can enter three non-dividing stable states: terminal differentiation, senescence, and quiescence. Of these, only cellular quiescence is reversible. Cellular quiescence can be triggered by mitogenic starvation, growth to high density, and lack of attachment to substratum. The restriction point (R) is the point in G1 phase where cells commit to a round of DNA replication and cell division. Cells require mitogens in the post-mitotic G1 prior to the R. Mitogens activate G1 CDKs, which cooperatively inactivate pocket proteins and activate the E2F program of gene expression required for cell cycle progression (see text)

downregulated by all quiescent signals mentioned above, but it does not induce upregulation of genes that suppress differentiation or inhibit apoptosis (Coller et al., 2006). In agreement with the observation that CKI inhibitors are upregulated during differentiation along particular lineages, overexpression of p21 in dermal fibroblasts induced growth arrest but did not prevent MyoD-induced differentiation. In contrast, fibroblasts forced into quiescence by contact inhibition or mitogenic withdrawal are resistant to differentiation signals (Coller et al., 2006). These results show that cellular quiescence is not a mere consequence of cell cycle exit but rather a unique resting state that preserves cells in environments that are not suitable for proliferation.

More recently, the mechanisms that control the reversibility of cellular quiescence have started to be unveiled. Because the transcriptional repressor Hairy and Enhancer of Split1 (HES1) is induced by signals that force fibroblasts into quiescence but is not regulated when cell cycle exit is induced by overexpression of CKIs (Coller et al., 2006), Sang et al. tested whether HES1 modulates the reversibility of cellular quiescence (Sang et al., 2008). Remarkably, it was found that ectopic expression of HES1 in dermal fibroblasts prevents p21-induced irreversible senescence, although it cannot reverse this phenotype if senescence is attained prior to HES1 expression. More importantly, their work also demonstrated that MyoD-induced differentiation of proliferating fibroblasts is prevented by ectopic expression of HES1 and that inactivation of HES1 in quiescent fibroblasts is sufficient to induce spontaneous senescence or trigger myogenic differentiation in response to MyoD activation. Thus, HES1 emerges as a pivotal candidate to control the reversibility of the quiescent state.

1.2 Overcoming the Restriction Point

1.2.1 The Restriction Point

In unicellularorganisms such as yeast, the availability of nutrients in the environment primarily determines their proliferation rate. In contrast, nutrients in the environment of cells in multicellular organisms are not typically limiting, and thus proliferation rates are determined by mitogens produced by other cells or by genetic developmental programs. The cell cycle can be subdivided in two functionally distinct parts based on their dependency on mitogens for cell cycle progression (Fig. 1.1). The mitogen-dependent phase spans the period of the cell cycle beginning with initiation of post-mitotic G1 to the Restriction point (R), which was first defined by Arthur Pardee (Pardee, 1974). Once cells surpass R, they are committed to a round of DNA replication and cell division, and the progression and continuity from one phase to the next depend solely on the cell's efficiency to faithfully complete DNA replication, chromosomal segregation, cytokinesis, and other required intermediate steps. On the other hand, normal post-mitotic early G1 cells that encounter an environment with limiting mitogens, extracellular substrate attachment, or space, enter a reversible quiescence state.

The main challenge faced by a cell exiting quiescence is to synthesize de novo all the gene products required for successful cell cycle entry and passage through R. E2F transcription factors control the expression of many genes whose products are essential, or at least important, for cell cycle progression. In quiescent cells, repressor E2Fs (E2Fs 4 and 5) form complexes with pocket proteins (typically p130 and the retinoblastoma protein, pRB) which silence E2F-dependent gene expression (reviewed in Graña et al., 1998; Mulligan and Jacks, 1998; Blais and Dynlacht, 2004; Rowland and Bernards, 2006). Mitogens activate intracellular signaling pathways that trigger activation of G1 cyclin/CDK complexes, which in turn disrupt E2F/pocket protein complexes via phosphorylation of the pocket protein. It is thought that once the E2F-gene expression program is set in motion to warrant the expression of sufficient levels of DNA replication enzymes and other cell cycle proteins and regulators, cell cycle progression becomes insensitive to both positive and negative external mitogenic stimuli (Fig. 1.1).

1.2.2 G1-Cyclins/CDK, pRB, and E2F Transcription Factors

Since this book contains a chapter devoted to the interplay between CDKs and E2F-dependent transcription, the focus of this section will be restricted to the events important for quiescence exit/entry.

G1-cyclins, together with their catalytic partners, the CDKs, are the key effectors of mitogenic signaling that drive cells out of quiescence in propitious environmental conditions. There are three mammalian isoforms of cyclin D (D1, D2, and D3) that exhibit tissue-specific expression. D-type cyclins bind to CDK4 or CDK6 (CDK4-6) and are activated in mid-G1. E-type cyclins, E1 and E2, bind to CDK2 leading to its activation later in G1.

Mitogenic stimulation activates RAS, which induces cyclin D1 transcription (Albanese et al., 1995) and stabilization through RAF/MAPK and PI3K/AKT mitogenic pathways (Diehl et al., 1998; Henry et al., 2000). Cyclin D/CDK4-6 complexes promote activation of cyclin E/CDK2 complexes through sequestration of CDK inhibitors (CKIs) from the CIP/KIP family (p21, p27, and p57). The trimeric complex cyclin D/CDK4-6/CKI shuttles into the nucleus, where it phosphorylates multiple sites on p130/pRB, relieving repressor E2Fs from pocket protein inhibition to initiate expression of early E2F-dependent genes, which in turn will generate more cyclin E (Fig. 1.2). The increase in cyclin E expression and the sequestration of CKIs by cyclin D/CDK4-6 complexes ensure accumulation of CKI-free cyclin E/CDK2 complexes that can be phosphorylated on the activating T-loop of CDK2 by the CDK-activating kinase (CAK) (Kato et al., 1994; Kaldis et al., 1998; Sherr and Roberts, 2004). As cyclin E/CDK2 active complexes emerge, a positive feedback loop ensures rapid activation of CDK2 through direct phosphorylation of CKIs, triggering their degradation, and hyperphosphorylation of pocket proteins facilitating additional accumulation of cyclin E and CDK2. CDK2 completes the inactivation of pocket proteins initiated by CDK4-6, which results in forceful elimination of repressor E2F complexes at the promoters and the expression of activator

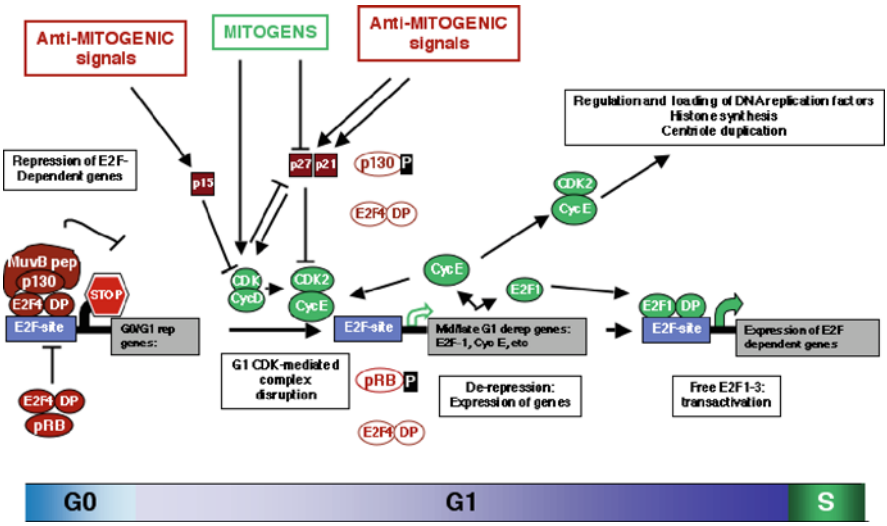

Fig. 1.2 Mitogens stimulate cell cycle re-entry via activation of the E2F-program of gene expression. Transition into the G1 phase of the cell cycle from quiescence requires activation of E2F-dependent gene expression. Expression of E2F-dependent genes is silent in quiescent cells. Promoters of E2F-dependent genes are occupied by E2F complexes containing repressors E2Fs and p130, as well as homologs of *C. elegans* synthetic multivulva class B gene products (MuvB pep). Mitogenic stimulation results in activation of CDKs by inducing G1 cyclin accumulation and inactivation of CKIs through various mechanisms. G1 CDKs phosphorylate pocket proteins disrupting their interaction with repressor E2Fs coinciding with the expression of gene products. Among the upregulated proteins are activator E2Fs (E2F1-3) that are recruited to promoters coinciding with recruitment of HATs and promoter activity. Cyclin E is an E2F-regulated gene product that helps inactivate pocket proteins, but also targets other substrates for phosphorylation that are important for DNA replication and centriole duplication. Antimitogenic signaling negatively regulates CDKs through upregulation of CKIs

E2Fs (E2F1-3), which are subsequently recruited to multiple E2F-dependent promoters coinciding with expression of E2F-dependent genes. Obviously, there are other players that participate in the activation of these CDKs and E2F-dependent gene expression, so readers are directed to more comprehensive reviews (Blais and Dynlacht, 2004; Rowland and Bernards, 2006; Blais and Dynlacht, 2007). It is important to note at this time that whereas cyclin D/CDK4-6 primary substrates are pocket proteins and Smad3 (Liu and Matsuura, 2005), both involved in repression of cell cycle-dependent gene expression, cyclin E/CDK2 functions are not limited to pocket protein inactivation in G1. Cyclin E/CDK2 phosphorylates multiple factors involved in centrosomal duplication, replication origin licensing and firing, and control of histone synthesis (Moroy and Geisen, 2004) (Fig. 1.2).

Overexpression of G1 cyclins is common in primary tumors and derived tumor cell lines (Malumbres and Barbacid, 2001). Considering that mitogenic signaling converges in the activation of G1 cyclin/CDK complexes, deregulation of G1 cyclins in tumor cells may reduce the threshold of mitogenic stimulation required for passage through the R or for escaping quiescence. In this regard, early studies showed

that overexpression of either D1 or E shortens G1 phase upon mitogenic stimulation. However, quiescent primary non-transformed fibroblasts that ectopically express cyclin D1 and/or E do not exit quiescence if the environment is deprived of mitogens or if the cells are arrested by growth to high density (Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994; Sotillo et al., 2008, 2009). In contrast, similar expression of cyclin D1 and E in certain tumor cell lines is sufficient to trigger exit from quiescence in the absence of any mitogenic stimulation (Calbó et al., 2002). Experiments performed in our laboratory have shown that in quiescent tumor-derived T98G cells forced expression of cyclin E leads to formation of active CDK2 complexes, pocket protein phosphorylation, and activation of the E2F program concomitantly with cell cycle entry. Under the same conditions expression of cyclin E in quiescent normal human fibroblasts (NHF) leads to formation of inactive complexes failing to trigger cell cycle entry. Concentrations of serum as low as 0.1% make quiescent NHF responsive to deregulated cyclin E expression, suggesting that other mitogen-dependent events, besides cyclin E accumulation, are required to fully activate CDK2 and exit G0. This is consistent with previous work showing that microinjection of active G1 cyclin/CDK complexes into the nucleus of primary human WI38 fibroblasts is sufficient to induce DNA synthesis (Connell-Crowley et al., 1998).

Despite the clear important role of G1 CDKs in mediating passage through R and triggering E2F-dependent gene expression, ablation of G1 CDKs and cyclins in mice has evidenced a high level of functional redundancy and compensation among these G1 cyclin/CDK complexes in triggering inactivation of pocket proteins and other essential events during the cell cycle (Malumbres and Barbacid, 2009). Targeted disruption of D-type cyclins, E-type cyclins, CDK4-6, or CDK2 reduces inactivation of pocket proteins but not below a threshold that could prevent E2F-dependent gene expression in both proliferating and serum starved and re-stimulated MEFs (Lee and Sicinski, 2006; Berthet and Kaldis, 2007; Malumbres and Barbacid, 2009). Indeed, even fibroblasts obtained from mouse embryos that simultaneously lack expression of CDK2, CDK4, and CDK6 proliferate and exit quiescence in response to serum stimulation (Santamaria et al., 2007). Thus, CDK1 via its binding with cyclin E appears sufficient to inactivate pocket proteins and induce passage through R. Of note, serum-starved cyclin E1–/–; E2–/– double knock-out MEFs are unable to re-enter cell cycle when stimulated with mitogens. However, this is due to a defect in loading of MCM2 onto chromatin, as pocket proteins are inactivated and E2F-dependent genes expressed (Geng et al., 2003). These results indicate great plasticity and compensation among cyclins and CDKs in many cell types, with function of some of them only essential in particular cell types.

1.2.3 Is Inactivation of Pocket Proteins Beyond a Certain Threshold Sufficient for Passage Through R?

Ablation of the three pocket proteins in MEFs makes these cells bypass cell cycle exit signals induced by mitogen withdrawal, contact inhibition, and loss of attachment, but cells become apoptotic (Dannenberg et al., 2000; Sage et al., 2000). Pocket