

Chapter 2

Cell-Cycle Signaling, Epigenetics, and Nuclear Function

Abstract Like many other cells, mammalian cardiac cells are able to divide and proliferate during development and during a short time after birth. However, the ability to divide decreases dramatically in the neonatal period, and adult cardiomyocytes are unable to proliferate. In contrast, postnatal cardiomyocytes from some lower vertebrates (e.g., zebrafish, newt) maintain the capability to divide, although the mechanisms underlying these species differences are unknown.

As a result of the inability of adult cardiac cells to proliferate, the heart is unable to regenerate new functional tissue following injury, which can cause myocardium dysfunction and even death. Recently, the dogma that the heart is a terminally differentiated nonproliferating organ has been challenged as cardiac stem cells capable of converting to cardiomyocyte-like cells were identified. Thus, in the last several years, two strategies have been used for cardiac repair: induction of endogenous cardiomyocyte proliferation and cell replacement therapy.

Our understanding of the mechanisms that control proliferation of cardiovascular cells has increased significantly in recent years. Studies in proliferating cells and animal models have identified groups of genes and proteins that control cell division; cyclins, cyclin-dependent kinases, and their inhibitors are essential for cell cycle progression, and the retinoblastoma protein and transcription factors (i.e., E2F) modulate the activities of cell cycle-regulators.

In this chapter, we review the cell cycle machinery and discuss how this controls the proliferation of cardiomyocytes. In addition, we analyze the role of sirtuin-dependent deacetylation in cell cycle progression and proliferation, the functioning and regulation of telomere/telomerase system, and the integration of reactive oxygen species into cell proliferation. New insights into the epigenetic components of cell inheritance, the stable transmission of cellular information beyond just DNA, highlighting DNA methylation, and chromatin organization as major candidates for carriers of epigenetic information are also presented.

Keywords Cell cycle • Epigenetics • Telomerase • IGF-1 • Redox signaling

Introduction

Cell proliferation is a sequence of events in which cells duplicate their contents and then divide. The cell cycle encompasses series of events that takes place in a cell leading to its division and duplication (replication). Before a cell can enter cell division, it needs to take up nutrients. All of the preparations are done during the interphase. Interphase proceeds in three stages (phases), G_1 , S, and G_2 . Cell division operates in a cycle. Therefore, interphase is preceded by the previous cycle of mitosis and cytokinesis. Progression of the cell through the different phases is highly regulated.

Proliferation of cardiac cells mediates mammalian heart growth during fetal development, and in some species heart regeneration as well. During the first weeks after birth, mammalian cardiomyocytes *in vivo* lose their ability to proliferate and exit the cell cycle. Thus, the vast majority of adult cardiomyocytes that have stopped proliferating enter a state of quiescence called G_0 . Recently, it has been reported that the mammalian heart contains minor populations of resident cardiac stem cells (CSCs) retaining the ability to proliferate.

The natural compensatory processes of the injured heart are limited to hypertrophy of the remaining cardiomyocytes and replacement of necrotic regions with fibrotic scar tissue. To correct the loss of cardiomyocytes, two strategies have been employed in recent years: cell replacement through cell therapy and induction of endogenous cardiomyocyte proliferation.

In this chapter, we discuss how the cell cycle machinery controls the growth of cardiac myocytes, and other cellular components of the cardiovascular system.

Regulators of Cell Cycle: Cyclin-Related Mechanism

Quiescent cells are usually found in the G_0 phase, state where mRNA and protein syntheses are minimal. They reenter the cycle at the G_1 phase following binding of a growth factor to its extracellular receptor. For instance, mitotically competent

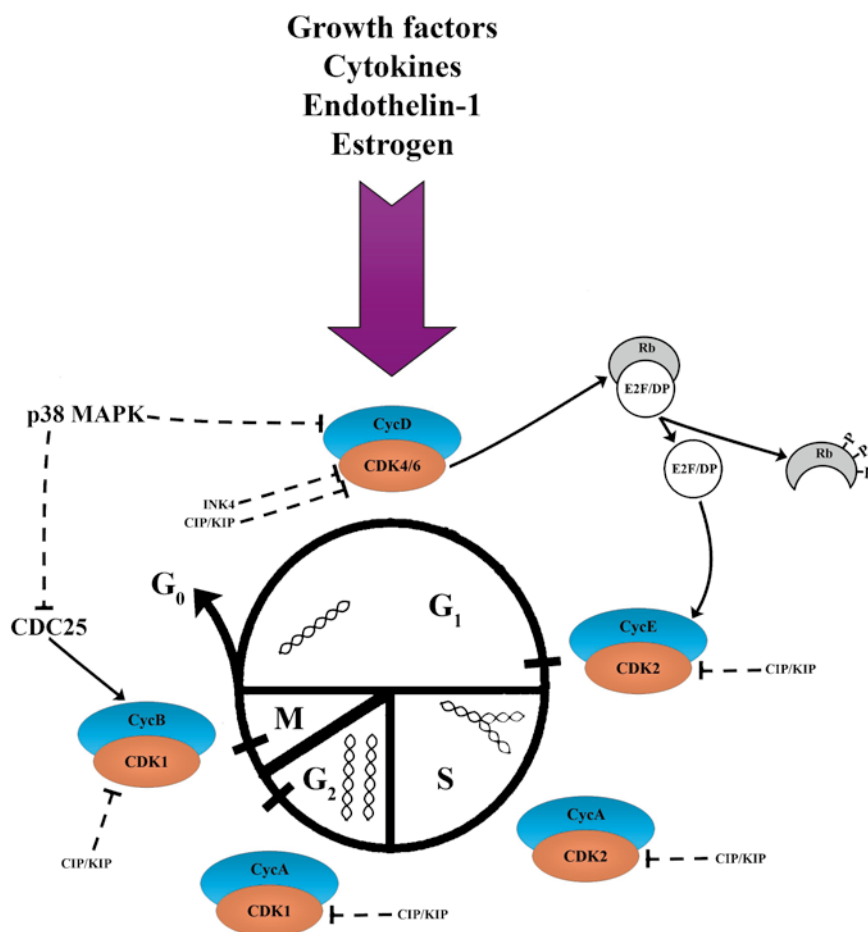
adult mouse CSCs express stem cell antigen-1 (Sca-1). Sca-1 is the phosphatidylinositol (PtdIns)-anchored protein that stimulates CSC cell cycle progression via Akt. Knockdown of Sca-1 stops proliferation of CSCs *ex vivo* and during regeneration of the ischemic myocardium [1]. Similarly, EGF and bFGF stimulate the neural stem cell cycle progression through the phosphorylation of Akt [2]. A very unique mechanism of cell growth negative regulation was described by Kang et al. [3]. It involves G protein coupled receptor (GPCR) and β -arrestin1. In human neuroblastoma cell, activation of δ -opioid receptor (DOR) results in growth inhibition. This DOR-regulated epigenetic pathway is G protein-independent and begins with DOR-induced trafficking of β -arrestin1 to the nucleus. In the nucleus, β -arrestin1 targets the promoter region of p27 (see below), recruits histone acetyltransferase p300, resulting in enhanced local histone H4 acetylation and stimulated transcription of p27. p27 inhibits cyclin–cyclin-dependent kinase (CDK) complex, leading to cell cycle inhibition.

Regulation of mammalian cell cycling is achieved by the sequential formation, activation, and inactivation of a series of cell cycle regulatory molecules. Cyclins are a family of eight proteins (A–H), which are synthesized and destroyed

during each cell cycle. All cyclins contain a homologous nine residue sequence near the N terminus (“cyclin box”) that binds to specific CDKs. Several cyclins (C, D, E) are short-life proteins and function during the G_1 phase, being later ubiquitinated and degraded. Cyclins A and B remain stable in interphase, but degrade during mitosis also by a ubiquitin-dependent pathway (Fig. 2.1).

Cyclins are regulatory subunits for CDKs. The CDKs are a family of seven protein kinases (1–7) which bind to, and are activated by specific cyclins. CDKs are constitutively expressed in cells, but they have catalytic activity only in the complex with cyclins. As cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals, different cyclin–CDK combinations (active CDKs) operate at different phases of the cell cycle. For example, CDKs 4–6 complex with cyclin D and function during the G_0 / G_1 phases of the cell cycle; CDK2 associates with cyclins A and E and functions during the G_1 phase and during the G_1 /S transition; and CDK1 binds to cyclins A and B and functions in the S, G_2 , and M phases. CDK-dependent phosphorylations activate or inactivate target proteins to orchestrate a coordinated entry into the next phase of the cell cycle.

Fig. 2.1 Mammalian cell cycle. Distinct cyclin (Cyc)/cyclin-dependent kinase (CDK) complexes regulate different phases of cell cycle. Activities of CDKs are, in turn, inhibited by CDK inhibitors, INK4 and CIP/KIP. Diagram also shows the relationship between Cyc/CDKs and several major regulators of cell cycle. Abbreviations: CDC25 protein tyrosine phosphatase, CIP/KIP a family of CDK inhibitors (p21, p27, p57), E2F/DP a family of transcription factors regulating the transcription of the genes involved in the progression to the S phase from the G_1 (G_0) phase of cells, INK4 inhibitor of kinase 4, p38 MAPK p38 mitogen-activated protein kinase, Rb retinoblastoma susceptibility protein



Upon receiving a promitotic extracellular signal (growth factor), G_1 cyclin–CDK complex is activated to prepare the cell for S phase; cyclin D binds to CDK4, forming the active cyclin D–CDK4 complex. It promotes the expression of transcription factors that stimulate the expression of S cyclins and of enzymes required for DNA replication. In particular, active cyclin D–CDK4 complex phosphorylates the retinoblastoma susceptibility protein (Rb). The hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex (which was bound to the E2F responsive genes, effectively “blocking” them from transcription), activating E2F. Activation of E2F results in transcription of various genes such as cyclin E, cyclin A, DNA polymerase, and thymidine kinase. Thus, the cyclin E produced binds to CDK2, forming the cyclin E–CDK2 complex, which pushes the cell from G_1 to S phase (G_1/S transition). Also, during the G_1 phase a prereplication complex (pre-RC) is formed on DNA replication origins by the assembly of several factors: origin recognition complex, Cdc6, Cdt1, and minichromosome maintenance (MCM) 2–7 helicase complex.

There are several S phase inhibitory proteins which halt the cell cycle in G_1 phase. Some of them belong to the *CIP/KIP* family (p21, p27, and p57). p21 is activated by p53, and p27 is activated by transforming growth factor β (TGF β). *CIP/KIP* proteins inhibit cyclin–CDK complex.

Another family of inhibitory proteins, INK4a/ARF family, includes p16INK4a protein (binds to CDK4) and p14arf protein. The G_1 cyclin–CDK complexes promote the degradation of S phase inhibitors.

Several processes are responsible for activation of pre-RC in S phase. First, active S cyclin (cyclin A–CDK2 complex) phosphorylates Cdc6. Phosphorylation is an inhibitory modification of Cdc6, and inactivated Cdc6 gets ubiquitinated and degrades in proteasome. Second, Cdt1 becomes inhibited by geminin. With Cdc6 and Cdt1 no longer bound, MCM-proteins can unwind the double-stranded DNA, and DNA replication begins. Also during S and G_2 phases, mitotic cyclin–CDK complex (cyclin B) is synthesized but stays inactivated.

Cyclin B along with CDK1 forms active cyclin B–CDK1 complex, which initiates the G_2/M transition. Initiation of mitosis by active mitotic cyclin B–CDK1 complex happens via stimulation of downstream proteins involved in chromosome condensation and mitotic spindle assembly. One of them is the anaphase-promoting complex (APC) with a ubiquitin ligase activity. APC promotes a cascade of events leading to degradation of structural proteins associated with chromosomes; initially, APC targets securin for degradation. In the absence of this inhibitory protein, protease separase cleaves cohesin allowing separation of sister chromatids and the beginning of anaphase. At the end of anaphase, APC triggers ubiquitination/degradation of mitotic cyclins, so telophase and cytokinesis can proceed (Fig. 2.1).

Phosphatases Cdc25

Prior to prophase, CDK1 stays in the cytosol in a phosphorylated (phospho-threonine-14 and phospho-tyrosine-15), inactive form. During prophase, CDK1/cyclin B1 complexes accumulate in the nucleus and are activated through the phosphatase Cdc25C by dephosphorylation of threonine 14 and tyrosine 15 of CDK1.

Proliferation of Embryonic Myocytes

There is now extensive knowledge about the genetic and molecular regulation of myocardial cellular development and cardiac morphogenesis. Cardiac muscle is derived from cells in the anterior lateral plate mesoderm. Myoblasts, also known as the embryonic progenitor cells that gives rise to myocytes undergo a series of proliferation to form the primitive straight heart tube. Multiple agents are involved in the regulation of cardiac myocyte proliferation including, insulin-like growth factor (IGF-1) [4], fibroblast growth factors (FGF) [5], neuregulin and receptors (erbB3, erbB4) [6], erythropoietin and erythropoietin receptor [7], retinoic acid and retinoic acid receptor, and cytokines, including cardiotropin-1 and interleukin-6. Cardiac muscle cells remain mitotically active and proliferate through the fetal and early perinatal period, but shortly after birth, mitotic division of cardiac myocytes becomes routinely undetectable.

Nonproliferating Adult Cardiomyocytes

During postnatal maturation of the mammalian heart, proliferation of cardiomyocytes essentially ceases. One interpretation is that cardiomyocytes of the maturing mammalian heart irreversibly exit from the cell cycle and become terminally differentiated. An alternative interpretation is that cardiomyocytes withdraw from the cell cycle and develop blocks at the G_0/G_1 and G_2/M transition phases of the cell cycle with the potential for reactivation.

Little is known about factors that induce cardiomyocytes to withdraw from the cell cycle. The majority of mature cardiomyocytes (85%) are growth-arrested at the G_0 or G_1 phase, suggesting that factors such as Rb protein, which blocks the G_1/S transition, become constitutively active in cardiomyocytes. Active Rb or related factors preclude E2F transcription factors from activating genes required for DNA synthesis. Rb is present in an active (hypophosphorylated) form because in adult cardiomyocytes there are no CDK-1 and CDK-2 which inactivate Rb by phosphorylation.

It is apparent that the absence of CDK-1 and CDK-2 is not enough to stop proliferation because (1) even under these conditions adult cardiomyocytes are able to synthesize DNA which leads to polyploidization of nuclei or multinucleation [8]. (2) Although IGF-1 induced upregulation of cyclins and cell cycle proteins, it was unable to cause proliferation of adult cardiomyocytes [9]. (3) Gene targeting studies show that overexpression of G_1 and S phase-promoting cell cycle regulators are not sufficient to override the mechanisms that limit cell division in mature cardiomyocytes [10]. There is some evidence that active mitotic cyclin B1/CDK1 complex enables adult cardiomyocytes to overcome the G_2/M block [11].

Persistent stress leads to ongoing remodeling in which cardiomyocyte death exceeds cardiomyocyte renewal, resulting in progressive heart failure. Cell-based therapies capable to reactivate the cell cycle in adult cardiomyocytes will promote myocardial regeneration. Recently, a number of molecular strategies showed new targets for such therapies. For example, Tseng et al. [12] reported that inhibition of glycogen synthase kinase-3 (GSK-3) reactivates the cell cycle in cardiomyocytes via increasing the expressions of G_1 - and S-phase-acting cyclins D1 and A and decreasing the expression of the CDK inhibitor, p27. Other studies have shown that cell cycle reactivation in adult cardiomyocytes can be initiated by extracellular matrix protein, periostin. This effect, at least in part, is realized via integrin-mediated signal transduction pathways [13]. It seems important to note that induction of cardiomyocyte proliferation in the damaged areas of myocardium might be not enough for heart regeneration and has only a transient healing effect if other cells [vascular smooth muscle cells (VSMCs), fibroblasts, endothelial cells] are not restored.

Another possibility is that of cardiomyocyte renewal mediated by endogenous CSCs. In 2003, a seminal discovery documented that the myocardium contains endogenous CSCs and cardiomyocyte progenitor cells [14, 15].

Finally, it is worth to mention that in contrast to mammalian cells, adult myocardial cells from newt and zebrafish preserve the ability to proliferate [16, 17]. In adult newt, for instance, approximately one-third of the initial cardiomyocyte population grown in culture progress through mitosis and enter successive cell divisions [18].

Proliferating Vascular Cells

Proliferation of VSMCs is a main contributor to vein bypass graft failure and restenosis, thus the targeting of VSMC proliferation is of great significance. One approach is to inhibit VSMC cycle. For example, sirolimus (rapamycin) arrests G_1 phase via the upregulation of the CDK inhibitor, p27 [19]. Another agent, paclitaxel, stabilizes microtubules and leads

to a G_2/M phase arrest [19]. Also, it is possible to reduce VSMC proliferation by selective inhibition of transcription factor E2F3 (promotor of G_1 -to-S phase transition) [20] or by overexpression of transcription factor FoxO3 (inductor of p27) [21].

Regulators of Cell Cycle: Sirtuins

Histone acetylation is the main type of covalent histone modification. Histone acetyltransferases (HATs) acetylate histones on lysines, whereas histone deacetylation involves histone deacetylases (HDACs). HATs and HDACs act in an opposing manner to control the acetylation state of histones and other proteins. Three mammalian coactivators, p300/CBP, P/CAF, and TAFII 250, have been identified as HATs. HDACs are grouped into four classes: class I HDACs (1, 2, 3, and 8) are related to yeast RPD3, class II HDACs (4, 5, 6, 7, 9, and 10) are related to yeast HDA1, and class III HDACs (SIRT1–7) are related to yeast Sir2. HDAC11 falls into a fourth class. Class I and II HDACs are zinc-dependent enzymes.

Gathered observations have indicated that HDACs, which belong to class III (sirtuins), induce cell cycle progression and proliferation of many cell types. Sirtuins deacetylate substrates range from histones to transcriptional regulators. They are not inhibited by trichostatin and require NAD^+ as a cofactor for their activity. These enzymes have been found to regulate apoptosis and muscle differentiation. In the heart, SIRT2–6 are expressed at high levels, whereas SIRT7 have low abundance.

Some experimental data suggest that the regulatory effect of SIRT1 is realized via modification of certain major players in cell cycle G_1 to S transition control, such as Rb and E2F1. As described above, hypophosphorylated Rb in resting and early G_1 cells represses transcription of E2F-regulated genes necessary for S-phase entry. As an additional regulatory mechanism, Rb (and its family members p107 and p130) can be acetylated by HATs, like p300 or Tip 60 [22, 23]. Acetylation obstructs efficient phosphorylation of Rb, and cells are maintained in a growth-arrested state. SIRT1 deacetylates Rb-family proteins [24]. Deacetylated Rb can now be phosphorylated to relieve repression of E2F-dependent cycle genes. Another important mechanism leading to increased proliferation was described by Rathbone et al. [25]. In rat muscle precursor cells, SIRT1-dependent cell proliferation was accompanied by decreased level of p21, an inhibitor of cyclin D–CDK4/6 activity. In this case, it is likely that SIRT1 deacetylated p53 [26], which lead to suppression of p21 synthesis and activation of CDK4/6. Thus, SIRT1 in combination with CDK4/6 activities restarts the cell cycle.

SIRT1 is highly expressed in vascular endothelial cells during blood vessel growth and controls their angiogenic activity. Endothelial-restricted SIRT1-deficient mice develop normally in the absence of ischemic stress but are unable to produce ischemia-induced neovascularization, indicating that endothelial SIRT1 is a mediator of stress-induced sprouting angiogenesis signaling. Gain- and loss-of-function approaches undertaken by Potente et al. [27] showed that under ischemic stress conditions SIRT1 interacts, deacetylates, and represses the transcriptional activity of the forkhead transcription factor Foxo1, an essential negative regulator of blood vessel development. Cardiac defects observed in SIRT1-deficient mice indicate that SIRT1 likely plays an important role in the development of heart [28].

In contrast to SIRT1, SIRT2 participates in the cell cycle at the G₂ to M checkpoint and negatively regulates cellular proliferation. Also, SIRT2 causes extension of mitosis in the normal cell cycle and prevents cells progression into mitosis in response to mitotic stress (by blocking chromatin condensation) [29–31].

The levels, activity, and localization of SIRT2 are tightly regulated by phosphorylation/dephosphorylation. SIRT2 is phosphorylated both *in vitro* and *in vivo* on serine 368 by the cell-cycle regulator cyclin B1–CDK1, and phosphorylation at this site is required for SIRT2-mediated delay in cell cycle progression [29, 31]. Phosphorylation of SIRT2 can be reversed by the phosphatase Cdc14. Apparently, dephosphorylation provokes degradation of SIRT2 via ubiquitination/26S proteasome-dependent pathway [29]. Thus, Cdc14 controls the cell cycle-dependent abundance of SIRT2, and mitotic exit.

Many aspects of SIRT2 function remain unknown. So far, two major potential targets have been described for this deacetylase: chromatin and the microtubules. A direct influence of chromatin condensation during the G₂ to M transition is possible due to SIRT2-catalyzed histone H4 lysine 16 deacetylation and deacetylation of this lysine during mitosis (G₂/M transition) [32]. Notwithstanding, further research is needed to clarify the role of histone acetylation for mitosis progression. The other known SIRT2 substrate is α -tubulin [33]. Since acetylation of tubulin seems to stabilize microtubules, SIRT2-catalyzed deacetylation might disrupt them and inhibit cell proliferation.

Regulators of Cell Cycle: Telomerase

Telomerase and its target, telomeres, play very important role as “mitotic clock” that regulates a number of cell divisions and the time when cell has to cease proliferation (replicative senescence). A number of signaling pathways are involved in the regulation of telomerase activity (see below).

To define the functional importance of telomerase/telomeres “axle,” we will start with a description of some aspects of DNA replication and explanation of telomere/telomerase function. Subsequently, we will highlight the function and regulation of telomerase/telomere system in the cardiovascular system.

Eukaryotic genomic DNAs are linear molecules and exist as highly organized complexes with nuclear proteins and RNAs, the chromosomes. At their ends, the chromosomal DNA is composed of an array of guanine-rich, 6–8 base-pair-long repeats that terminates with a 3′ single-stranded-DNA, and specifically binds a number of proteins. These DNA–protein structures at the ends of chromosome, the telomeres, are very important structures to control cell genomic stability and cell replicative senescence.

The major function of telomeres is to compensate for incomplete DNA replication at chromosomal ends. The problem of incomplete DNA replication arises from the nature of DNA polymerase which replicates DNA during the S phase of cell cycle. This enzyme can only synthesize DNA in a 5′–3′ direction. At the replication fork, there is no abnormalities for DNA polymerase to synthesize *de novo* strands on the basis of parental leading strand (strand oriented 3′–5′ direction), as the enzyme just follows moving the replication fork and keeps adding nucleotides complementary to the unzipped leading strand in a 5′–3′ direction.

A completely different mechanism takes place at the lagging parental template strand (strand oriented in the 5′–3′ direction). In this strand, DNA polymerase has to start the replication in the nearby fork, which requires a short RNA primer attached to lagging DNA strand – double-stranded initiation point. From this initiation point, the enzyme moves apart from the replication fork and synthesizes a new strand until it reaches a double-stranded DNA, created previously (previous initiation point). As a result of every single action of DNA polymerase, a short DNA fragment (100–200 nucleotides) is formed on the basis of lagging parental template strand. While replication continues, the lagging parental template strand contains a series of these short fragments of DNA called Okazaki fragments that initially are separated by RNA-primers. Later RNA bases are replaced with DNA bases by the endonuclease/DNA polymerase, and DNA fragments are “glued” together by the DNA ligase to create a continuous strand of daughter DNA strand.

When the replication fork reaches the very end of the chromosome, the RNA primer binds to the 5′-end of the lagging template strand, and the replication of the most-5′-piece of template strand proceeds as described. However, after removal of RNA template bases from this fragment it is impossible for DNA polymerase to substitute them with DNA bases because there is no 5′-upstream double-stranded primer. Finally, the single-stranded 5′-ends of replicated chromosomes (approximately 20 bases initially paired with

RNA primers) get degraded. As a result, if there is no protective mechanism, chromosome would lose genetic material and would grow shorter and shorter in subsequent replications.

Fortunately, eukaryotic cells do have telomeres which prevent losses of genetic information and slow down replication-related chromosomal fraying. In addition, telomeres protect chromosomes from being fused. As mentioned previously, telomeres are 6–8 base-pair-long repeats (in vertebrates, the repeat is 5'-TTAGGG-3'), and the total length of these repeats reaches several kilobases in humans. In most somatic cells, the telomeres serve as disposable DNA sequences that shorten during replication instead of meaningful genetic information-carrying DNA sequences. According to some hypotheses, telomeres determine certain number of cell divisions; eventually after a number of divisions, telomeric sequences are running out, and this induces replicative senescence of the cell followed by blockage of cell division.

Most of somatic cells lose telomeric sequences as a result of a series of replications, because they are unable to restore them. At the same time, germ cells, stem cells, and certain leukocytes express active enzyme telomerase, responsible for the synthesis of telomeres. Telomerase is a “ribonucleoprotein complex” composed of a protein component, the telomerase reverse transcriptase (TERT) and an RNA primer sequence, the telomerase RNA component (TERC). TERC is several hundred bases in length noncoding RNA with a template region, 3'-CAAUCCCAAUC-5' (telomere repeat – see above). Telomerase can base pair the first few nucleotides of the TERC template to the last telomere sequence on the chromosome, and then the TERT-component adds new telomere repeat using TERC as a template. This process of telomere elongation repeats several times.

As mentioned earlier, cardiomyocytes remain mitotically active and proliferate through the fetal period, and exit cell cycle during the early perinatal period. In mice genetic models, when TERT is transgenically expressed, it maintains telomere length and delays cardiomyocyte cell cycle exit. Proliferative signaling by TERT happens under mitogenic conditions, but is insufficient to control exiting cardiac cells from proliferation. This is why in TERT-overexpressing mice, cardiac proliferation is also subsided eventually (by 3 months of age) [34]. In a number of noncardiac cells (human fibroblasts, keratinocytes), forced expression of functional TERT also prevents the loss of telomeric DNA and prolongs the cellular life span. Conversely, the overexpression of a catalytically inactive form of TERT causes premature cell senescence and apoptosis [35].

The vast majority of adult cardiomyocytes are of the postmitotic nature, and the mitotic clock could not be applied to them. However, c-kit-positive undifferentiated resident cardiac progenitor cells (CPCs) have also been detected in the adult heart, and they give rise to new myocytes in the adult myocardium.

When a progenitor cell divides, two daughter cells are formed; they may maintain parental cell properties or become amplifying cells. Amplifying cells divide rapidly and simultaneously differentiate. Amplifying cardiac cells have a limited number of population doublings. Such a restriction in cell division correlates with the progressive downregulation of telomerase during differentiation. It has been assumed that aging effects on CPCs lead to an imbalance between telomerase activity and length of telomeres, resulting in critical telomeric shortening, permanent withdrawal from the cell cycle, and CPC senescence [36]. There is evidence that in mouse cardiac CPCs, IGF-1 activates telomerase activity and induces CPC division. This mechanism involves PI3K-Akt pathway and leads to the phosphorylation/activation of TERT [37].

Another potential target for telomerase in myocardium is the endothelium. Upregulation of endogenous telomerase by fibroblast growth factor-2 (FGF-2) [38] or overexpression of TERT [39] promotes endothelial cell proliferative capacity and function. Conversely, atherogenic factors suppress telomerase activity and accelerate endothelial cell senescence [40]. Similar to IGF-1 on cardiomyocytes, estrogens can increase telomerase activity in endothelial cells via PI3K-Akt pathway and subsequent phosphorylation of TERT [41]. Furthermore, critically short telomeres in mice markedly impair angiogenesis [42].

In conclusion, the modulation of telomerase activity and the control of telomeric length may represent an important therapeutic tool in regenerative medicine, in particular, when regeneration of cardiac tissue after injury is required. Restoring telomerase activity may be of benefit to the native vasculature and to angiogenic therapies for ischemia.

Regulators of Cell Cycle: Redox Signaling

Several lines of evidence suggest that redox signaling exists and plays a role in the regulation of cell cycle and proliferation. For instance, nuclear levels of the major redox indicator, thiol tripeptide glutathione (GSH), change during the cell cycle, with the highest levels found in the S and G₂/M phases, and depletion of GSH leads to reduced proliferation [43, 44]. Also, the majority of oxidant-sensitive proteins conduct transcription, nucleotide metabolism, (de)phosphorylation, and (de)ubiquitinylation during the G₂/M phase indicating that oscillations of the intracellular redox environment may regulate oxidant-sensitive proteins.

A number of proteins, which belong to the classes of transcription factors, chromatin-modifying enzymes, kinases, and phosphatases, are regulated by oxidative stress (OS). Thus, Src family kinases (Src, Yes) are activated under OS and tyrosine phosphorylate TERT. Phospho-Tyr₇₀₇-TERT

forms a complex with export receptor CRM-1 and the nuclear GTPase Ran, which leads to translocation of TERT out of the nucleus [45, 46]. Tyrosine phosphorylation of TERT under OS conditions is further accelerated by oxidation/inhibition of protein tyrosine phosphatase Shp-2, so it is unable to maintain unphosphorylated TERT within the nucleus [46]. Therefore, an imbalance in the redox status seems to enhance the activity of the nuclear Src family of tyrosine kinases and to inhibit the nuclear tyrosine phosphatase, which contributes to diminished telomerase activity and accelerated replicative senescence.

Another phosphatase, T-cell protein tyrosine phosphatase (TC-PTP), seems to be responsible for OS-caused reduction of sprouting angiogenesis. In endothelial cell, OS induces translocation of the 45 kDa TC-PTP from the nucleus to the cytosol, where it dephosphorylates specific tyrosine residues on vascular endothelial growth factor receptor 2 (VEGFR2). Dephosphorylation delays vascular endothelial growth factor (VEGF)-induced VEGFR2 internalization and thus inhibits growth of endothelial cells and reduces angiogenesis [47]. Oxidation of another phosphatase, Cdc25C, also influences cell cycle progression. In the latter case, oxidation-enhanced formation of the disulfide bond between cysteine-330 and -377 in Cdc25C promotes phosphorylation of serine-216. This phosphorylation leads to binding of Cdc25C to 14-3-3 protein and to the nuclear export of Cdc25C [48]. As a result, Cdk1 cannot be activated during prophase, and cells do not proliferate staying arrested at G₂/M-checkpoint. Moreover, the association of OS with telomere shortening and senescence has been suggested by observations on vascular endothelial cells [38], VSMCs [49], and cardiomyocytes. For example, elevated production of ROS in mice with experimentally developed diabetes leads to a significant decrease of telomeric length in cardiomyocytes and CPCs. Deletion of p66^{shc} (adaptor protein which enhances ROS-mediated cell injury) prevents OS in transgenic animals; in the diabetic heart, the CPC pool may be protected and, thereby, myocyte regeneration and vessel formation can occur [50].

Regulators of Cell Cycle: MicroRNAs

Recently, a novel mechanism involving posttranscriptional regulation by small microRNAs (miRNAs) has emerged as a regulator of cell proliferation. In cultured myoblasts and cardiac progenitor cells, for example, *miR-133* stimulates proliferation by targeting serum response factor (SRF), which is important in muscle proliferation [51, 52]. Surprisingly, transgenic mice lacking *miR-133a-1* and *miR-133a-2* (double-mutant) have excessive cardiac proliferation [53]. It is possible that dysregulation of the cell cycle control observed in

double-mutant mice, is related to the upregulation of the *miR-133a* mRNA target, cyclin D2.

As mentioned earlier, postnatal mouse cardiomyocytes terminally exit the cell cycle after the first 10 days of life. However, adult mice have an increase in mitotic cardiac myocytes along with cardiac hyperplasia when they lack another microRNA, *miR-1-2*. Genome-wide profiling of *miR-1-2*-deficient adult hearts reveals a broad upregulation of positive regulators of the cell cycle and downregulation of tumor suppressors, indicating cell reentering the cell cycle [54].

MicroRNA was shown to be involved also in angiogenic signaling in endothelial cells. Normally endothelial cells express *miR-126*. This microRNA represses Sprouty-related protein 1 (SPRED-1) and the p85- β regulatory subunit of phosphatidylinositol 3-kinase (PI3KR2), negative regulators of VEGF signaling. Endothelial cells from *miR-126*-deficient mice show diminished angiogenesis because they have elevated levels of inhibitors of VEGF/Erk/Akt-dependent angiogenic cascade [55, 56].

Epigenetic Component of Cell Inheritance

Cell genomic DNA is organized into chromatin – complex combination of DNA, RNA, and proteins (mainly histones, but also many other proteins), which maintains DNA structure and orchestrates the pattern of active/silent genes unique for each cell type. During replication, chromatin undergoes genome-wide alterations in structure, which have to be restored in the daughter cells to maintain cell identity and the physiological status that existed in the former parental cell. At the same time, there must be some flexibility in the genome regulatory machinery to allow programmed changes in the identity of the daughter cells when needed (differentiation). Recently, several regulatory systems have been discovered that recover chromatin structure and function after DNA replication during phase S of cell cycle. They can be referred as mechanisms of epigenetic inheritance, stable transmission of information about regulation of genome function that occurs without alterations in the DNA sequence [57].

Epigenetic transfer of chromatin structure from parental to daughter cell through S phase/mitosis is possible due to a number of epigenetic marks – heritable instructions, that determine whether, when, and how particular genetic information will be read. These marks function during DNA replication and cell division, and include DNA methylation, histone modifications, histone variants, histone modifiers, other nonhistone chromatin proteins, and nuclear RNA. Thanks to epigenetic marks, the cell type specific-state of chromatin can survive through perturbations that occur during the replication fork in the S phase (Fig. 2.2).

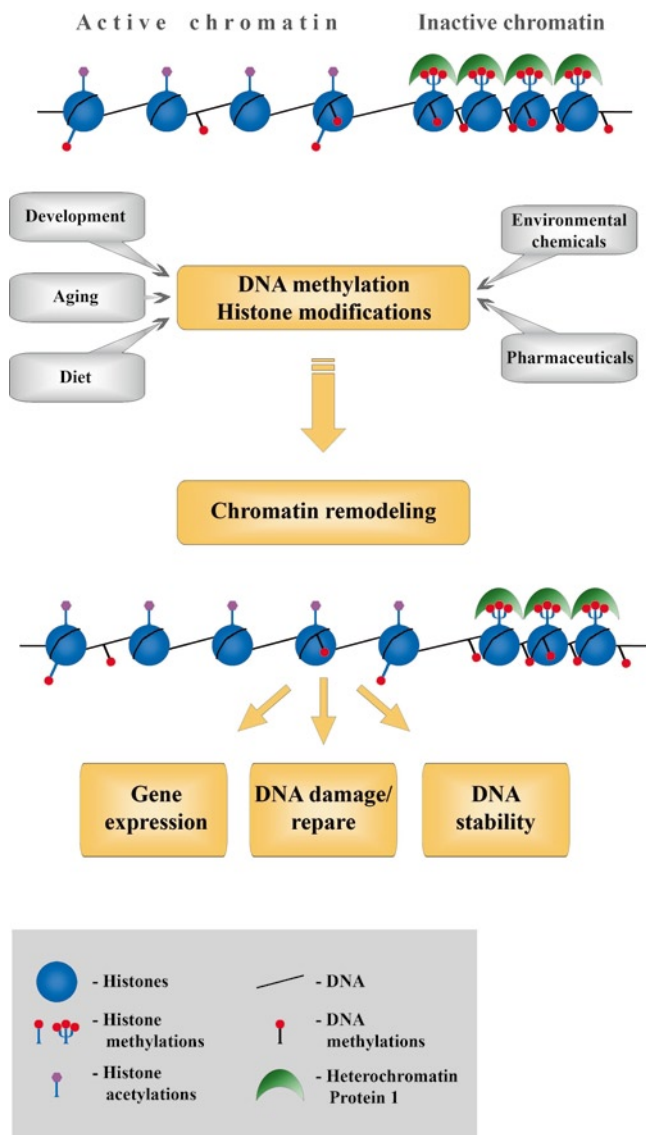


Fig. 2.2 Epigenetic factors mediating gene expression, DNA damage/repair and DNA stability

When DNA replicates, chromatin undergoes a wave of disruption and subsequent epigenetic marks-regulated restoration in the wake of the passage of a replication fork. Also, DNA replication is a stage where epigenetic changes lead to cell differentiation and development. DNA methylation of newly replicated DNA at the fork is ensured by NP95/DNA methyltransferase 1 using hemimethylated DNA as a template. The resulting methylamines in the daughter strand represent parental DNA. This newly synthesized methylated DNA is a template for several histone modifiers (HDAC, Lys methyltransferases G9a and SETDB). Also, in the replication fork area one or several mechanisms are believed to be available to restore the histone-based nucleosomal pattern of newly synthesized strands, similar to parental DNA. Although the processes that underlie this phenomenon are not well understood, histones H3 and H4 are likely candidates to transmit

information about chromatin organization from one cell cycle to the next. Several hypotheses on the mechanism of histone epigenetic inheritance have been recently proposed [58].

Some carriers of epigenetic information function independently from DNA replication. Examples include inheritance of transcriptionally active regions of genomic DNA and several types of condensed chromatin, known as heterochromatin (mainly centromere). The types of cell genomic material mentioned above are characterized by enrichment in special kind of histones, histone H3.3 and centromere-specific histone H3 (CenH3), respectively. These histones seem to serve as epigenetic marks via incorporation into particular regions of DNA in a replication-independent manner (late telophase–early G_1 phase) [33]. It is worth to note that many DNA- and histone-modifying and remodeling proteins contribute to the mitotic inheritance of nuclear DNA organization, such as HDACs, Lys methyltransferases, and chaperones.

Conclusions

Scientific data accumulated in the last several years support the concept that under normal conditions there is a low level of cardiomyocyte death and turnover in the heart, based on the new cardiomyocytes originating from endogenous cardiac precursor (stem) cells. A challenge for research is to create strategies to optimize and enhance CSC response to myocardial stresses in order to rescue myocardial remodeling effectively.

Targeting the cell cycle has many important implications in cardiovascular medicine. For instance, changes in the proliferative activity of the vasculature may prevent vein bypass graft failure and restenosis of vessels after angioplasty interventions. Recent observations suggest that reprogramming of the cardiomyocyte cell cycle is also possible although the precise mechanism(s) by which cell cycle-regulating molecules withdraw cardiomyocytes from the cell cycle remains to be determined. The potential for re-initiation of cardiac cell division is to provide a powerful approach for repairing damaged areas of cardiac tissue following an injury (infarct).

Further studies are needed to understand the regulation of the cardiac cell cycle in order to therapeutically reactivate cell cycle in existing cardiomyocytes, and/or to design methodologies which use differentiated CSCs.

Summary

- Quiescent cells are usually found in the G_0 phase. They reenter the cycle at the G_1 phase following binding of a growth factor to its extracellular receptor. Regulation of mammalian cell cycling is achieved by cyclins which are

- synthesized at specific stages of the cell cycle, in response to various molecular signals. Cyclins are regulatory subunits for protein kinases called CDKs.
- G_1 active cyclin D-CDK4 complex phosphorylates the retinoblastoma susceptibility protein, which leads to the activation of transcription factor E2F and transcription of various genes necessary for cell cycle.
 - There are several proteins that regulate CDK activity: two families of protein inhibitors (CIP/KIP family and INK4a/ARF family) and the activatory Cdc25 phosphatases.
 - Multiple agents are involved in the regulation of fetal cardiomyocyte proliferation (IGF-1, FGF, neuregulin, erbB, etc.). Shortly after birth, mitotic division of cardiac cells becomes routinely undetectable.
 - The majority of mature cardiomyocytes (85%) are growth arrested at the G_0 or G_1 phase. Little is known about the factors that induce cardiomyocytes to withdraw from the cell cycle, although there is some evidence that active Rb protein, in the absence of several CDKs, is involved in cardiac cell cycle arrest.
 - The seminal discovery that the myocardium contains endogenous CSCs and cardiomyocyte progenitor cells has been documented.
 - In contrast to mammalian cells, adult myocardial cells from newt and zebrafish preserve the ability to proliferate.
 - Proliferation of VSMCs is a main contributor to vein bypass graft failure and restenosis. Thus, the targeting of VSMC proliferation is of great interest.
 - Sirtuins (SIRT) are class III HDACs which regulate cell cycle progression and proliferation of many cell types. The regulatory effect of SIRT1 is carried out via modification of Rb and E2F1 and leads to increased proliferation.
 - SIRT1 is highly expressed in the vascular endothelial cells during blood vessel growth and controls their angiogenic activity.
 - SIRT2 participates in the cell cycle at the G_2 to M checkpoint and negatively regulates cellular proliferation. Many aspects of SIRT2 function remain unknown. Two major potential targets described for SIRT2 are chromatin and the microtubules.
 - Telomerase and its target telomeres play a very important role of "mitotic clock" that regulates the number of cell divisions and the time when cell has to cease proliferation (replicative senescence). Major function of telomeres is to compensate for incomplete DNA replication at chromosomal ends.
 - Telomerase is an enzyme responsible for synthesis of telomeres. Most somatic cells do not express telomerase, and they lose telomeric sequences as a result of a series of replications because they are unable to restore them.
 - In mouse CPCs, IGF-1 activates telomerase activity and induces CPC division. This mechanism involves the PI3K-Akt pathway and leads to the phosphorylation/activation of TERT.
 - In endothelium, FGF-2 and estrogens activate telomerase and promote endothelial cell proliferative capacity.
 - Several lines of evidence have established that redox signaling exists and plays a role in the regulation of the cell cycle and proliferation. Oscillations of the intracellular redox environment may regulate oxidant-sensitive proteins during the G_2/M phase.
 - Src family tyrosine kinases are activated under oxidative stress and phosphorylate/inactivate telomerase.
 - Oxidative stress affects several phosphatases (Shp-2, TC-PTP, Cdc25C), which results in accelerated replicative senescence, reduction of angiogenesis, and the arrest of proliferation.
 - A novel mechanism of cell proliferation regulation involves posttranscriptional regulation by microRNAs.
 - Chromatin orchestrates the pattern of active/silent genes unique for each cell type. Epigenetic transfer of chromatin structure from parental to daughter cell through S phase/mitosis is possible due to a number of epigenetic marks. Major marks include DNA methylation and histone modification patterns. Some carriers of epigenetic information function independently from DNA replication.

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