Cellular models for fundamental and applied biomedical research

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Chapter 1

General introduction and outline of thesis

Background

Cell culture models play an important role in biomedical research and will continue to do so given the growing opposition to vivisection and the limited predictive value of animal models for human disease. Moreover, cell culture models can be easily established to mimic physiological or pathological processes, which is difficult to accomplish using *in silico* models. While non-cellular *in vitro* models are highly suitable for studying simple biochemical processes, cell culture models recapitulate many of the complex regulatory circuits governing protein activity *in vivo* and hence allow investigation of diverse physiological processes. Also, cell culture models offer the possibility to address fundamental research questions in a much more simplified, specific and controllable manner than can be achieved using *in vivo* models.

Aging is a complex and multifactorial process driven by genetic and environmental factors and govern by various interacting molecular pathways that lead to the physiological decline of biological systems. A variety of experimentally tractable cellular models were employed in recent years to study the basic mechanisms of aging, knowledge of which is essential to the development of effective therapeutic interventions against age-related diseases[1]. Due to the complexity of striated muscle structure and function, standardized cell culture models are a real asset for studying skeletal and cardiac muscle biology and disease. Cultures of skeletal myoblasts or cardiomyocytes (CMCs) can mimic physiological or pathological conditions, making them well-suited for proof-of-concept studies and for developing novel therapeutic interventions for specific diseases[2,3]. Especially, cultured cardiac cell monolayers have become a popular model system for electrophysiological studies[4] into the mechanisms of cardiac arrhythmias[5-7] as well as drug-induced ventricular pro-arrhythmic effects[8]. Brown adipose tissue (BAT) represents a potential therapeutic target to treat obesity and associated metabolic disorders because of its capacity to ingest and combust glucose and fatty acids for thermoregulation[9]. The properties of BAT has been investigated using cultures of primary brown preadipocytes (BPAs). Although these studies have yielded new insights into adipocyte biology[10-12], the full potential of BAT culture models has not been utilized due to the scarcity of starting material and the limited proliferation capability of primary BPAs. A potential solution to this problem would be to establish lines of BPAs by cellular immortalization. Although cell immortalization offers the possibility to produce virtually unlimited cell sources by inducing cell cycle reentry and by bypassing cell senescence, generation of robust cell lines that can recapitulate (most of) the properties of the primary cells from which they were derived requires a thorough understanding of the mechanisms of cell quiescence, proliferation, differentiation, senescence and apoptosis.

Cell cycle

To divide, a cell must grow, copy its genetic material (DNA), and physically split into two daughter cells. Cells perform these tasks in an orderly and tightly regulated series of events known as the cell cycle, which is divided into two major phases, the interphase and the mitotic phase. During the interphase, the cell grows and duplicates its nuclear DNA. The interphase is divided in three subphases. During gap phase 1 (G1 phase), the cell grows physically larger, copies organelles except for the nucleus

and synthesizes the molecular building blocks needed for the next subphase. In the synthesis (S) phase, the cell copies its chromosomal DNA and duplicates a microtubule-organizing structure called the centrosome, which helps to separate the DNA during the mitotic phase. During gap phase 2 (G2 phase), the cell grows more, makes additional macromolecules and organelles, and begins to reorganize its contents in preparation for mitosis. In the mitotic phase, the cell's chromosomal DNA is equally separated into two new daughter nuclei and the cell's cytoplasm and organelles are split after the formation of a cleavage furrow producing two new daughter cells. The segregation of duplicated chromosomes into daughter nuclei is called karyokinesis; the separation of cytoplasm and organelles is called cytokinesis.

The cell cycle is controlled by the activity of cyclin-CDK (cyclin-dependent kinase) complexes. CDKs are always present but need to be activated by phase-specific cyclins. Mitogens induce the synthesis of cyclin D early in the G1 phase. Cyclin D associates with CDK4 and CDK6 resulting in the hyperphosphorylation of the retinoblastoma protein pRB and the disruption of complexes between this pocket protein and several members of the E2F family of transcription factors. This allows these EF2 family members to form a complex with the in transcription factor DP (dimerization partner) and to induce the expression of various genes involved in cell cycle progression including the gene encoding cyclin E which is necessary for G1/S transition. CDKs are further activated during cell cycle by cyclin A and B to drive transition from S phase to G2 phase and from G2 phase to M phase, respectively. The activities of cyclin-CDK complexes are regulated by CKD inhibitors including the INK4 family (p15, p16, p18 and p19) and CIP/KIP family (p21, p27 and p57) which bind to CDKs and inactive the complexes (**Figure 1**).

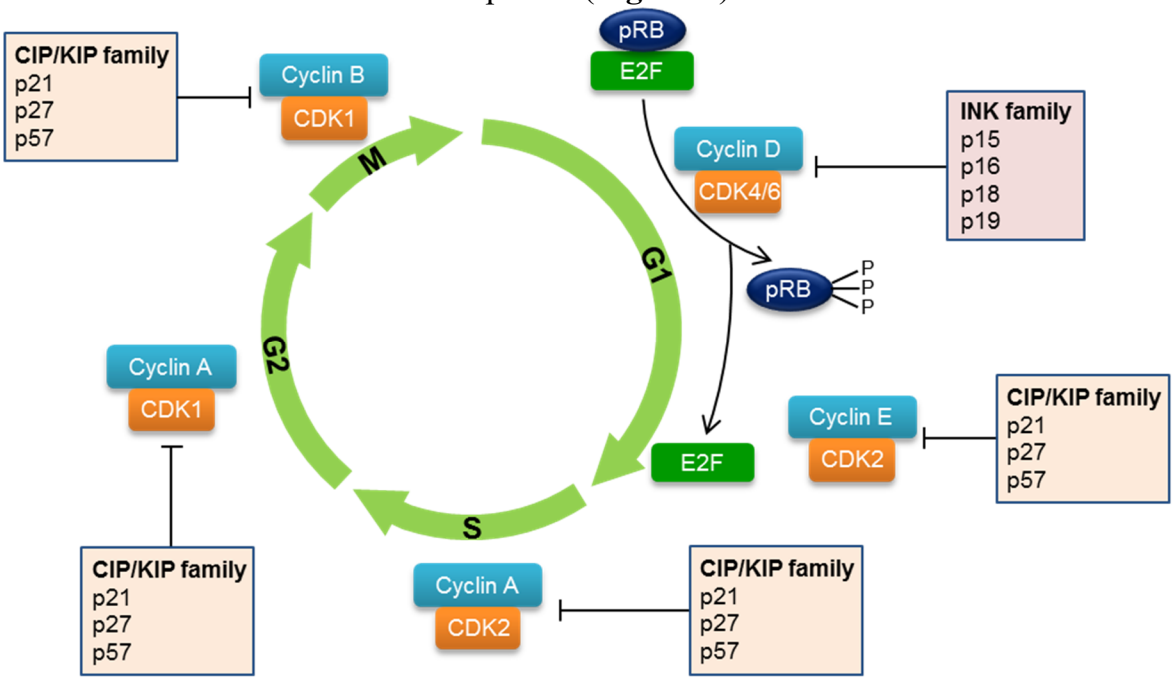

Figure 1. Cell cycle regulation.
The CDK-cyclin complexes promote cell cycle progression, while the CDK inhibitors stop it. CDK4/6-cyclin D is activated in phase G1, CDK2-cyclin E in phase G1/S, CDK2/1-cyclin A in phase S/G2 and CDK1-cyclin B in phase M. The CIP/KIP family (p21, p27 and p57) inhibit a wide range of cyclin-CDK complexes, yet the INK4 family (p15, p16, p18 and p19) specifically diminish CDK4/6 activity.

Cell fates

The fate of a cell is determined by the changes in its gene expression pattern and associated activities, which will lead to cell proliferation, quiescence, differentiation, senescence or death (**Figure 2**). Mitotic cells such as cells in the skin and gut repetitively progress through phases G1, S, G2 and M of the cell cycle. Mitotic cells can also enter the G0 phase of the cell cycle. This state of cell cycle arrest is observed in quiescent, (terminally) differentiated and senescent cells. Quiescent cells such as adult stem cells, fibroblasts and lymphocytes are temporarily arrested in G0 phase and readily reenter the cell cycle when exposed to mitogens. Dormant cells are quiescent cells showing very low metabolic activity, which is frequently interpreted as being in a deeply quiescent state. Cells like adipocytes, cardiomyocytes and neurons are believed to lose the capacity to enter the cell cycle and to permanently reside in the G0 phase of the cell cycle once they are fully differentiated. However, recent studies have shown that both self-renewal of cardiac tissue and regeneration upon damage mainly results from the cell cycle reentry and proliferation of existing cardiomyocytes[13-18]. In general mitotic cells will stop dividing and become senescent after a limited number of divisions. Senescent cells cannot replicate anymore because of telomeres shorting, irreparable DNA damage, nutritional deficiency, harmful chemicals, oxidative stress or physiologic stress. Except for the stem, progenitor and precursor cells that escape from death by being converted in more differentiated cell types, all other cells finally die because of natural turnover or due to injury, including the death of the organism. Cell deaths can occur by various different mechanisms. Two of the major types of cell death are apoptosis and necrosis[19]. Apoptosis, also known as programmed cell death, is a normal physiological, non-inflammatory process to remove unwanted cells. In this process, apoptotic bodies are formed to confine cell organelles and other parts, which will be phagocytosed by macrophages. Necrosis, also known as unprogrammed cell death, is a less orderly process than apoptosis, in which cells and organelles swells resulting in membrane rupture. As a consequence, the cellular content is released in the extracellular space triggering an inflammatory response and possible causing further damage to adjacent cells[20]. A major difference between normal somatic cells and transformed cells is their proliferation potential. Normal somatic cells in culture have a limited ability to multiply themselves known as the Hayflick limit (see below), while transformed cells have evaded normal cellular senescence due to mutations resulting in acquisition of unlimited proliferation ability, evasion of apoptosis, growth factor independency, non-responsiveness to growth inhibitors, promotion of angiogenesis and invasive capacity. Immortalized cells have not necessarily undergone neoplastic transformation, but have accumulated sufficient mutations to proliferate unlimitedly. Upon transplantation immunocompromised mice, transformed cells will form tumors in contrast to immortalized cells. More details about cellular senescence, quiescence and immortalization are described below.

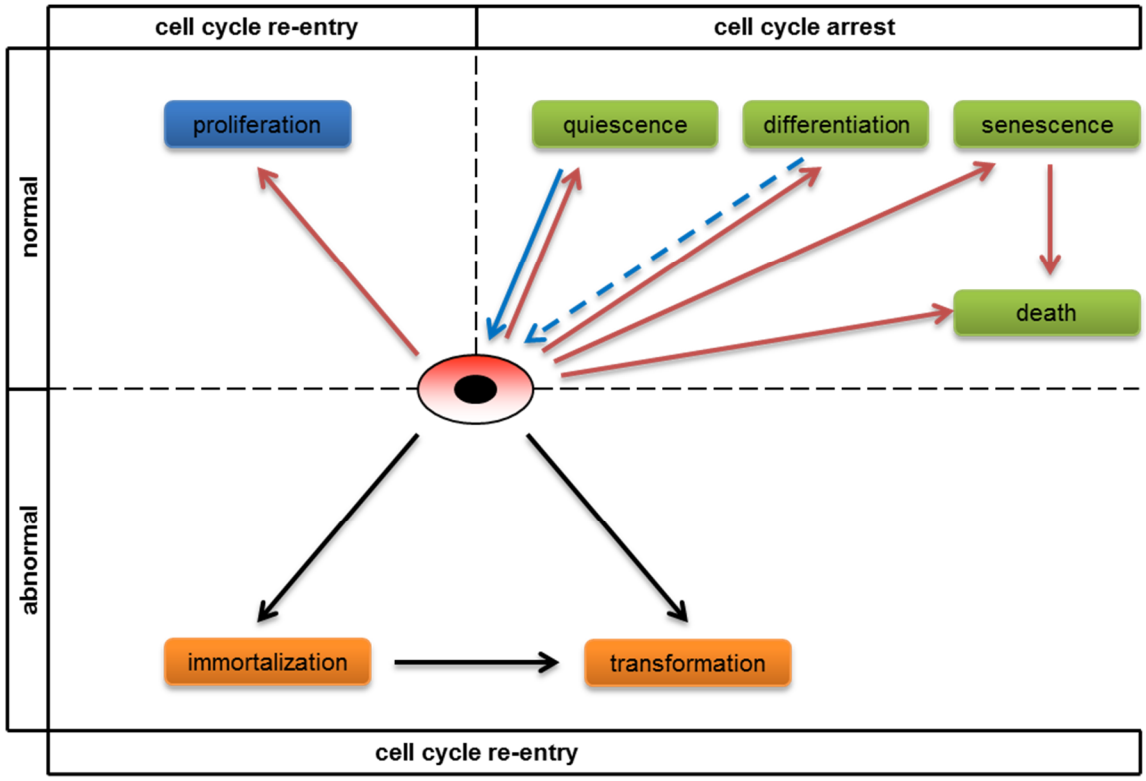

Figure 2. Possible cell fates.
Under normal conditions, cells adopt one of five distinct cell fates: they can remain in the cell cycle and continue to proliferate or leave the cell cycle and become quiescent, differentiated or senescent or undergo apoptotic or necrotic cell death. Under pathological conditions, cells can escape from senescence or apoptosis and become immortalized or transformed following the activation of endogenous oncogenes and/or the inactivation of endogenous tumor suppressor genes. Cell immortalization can also be brought about by oncogenic viruses or via genetic engineering.

Cellular senescence

Senescence as a common stress response

Normal mammalian somatic cells such as fibroblasts, myoblasts and preadipocytes, grown in culture fail to proliferate after a finite number of divisions and ultimately encounter senescence. This phenomenon was first found in human fibroblast cultures by Hayflick[21] and later referred to as replicative senescence[22-24]. Replicative senescence is thought to be the result of progressive telomere shortening as a cell-intrinsic mechanism[25,26]. Cellular senescence can also be stimulated by various types of cell stress like lack of nutrients, harmful chemicals (e.g. cytostatic drugs), oxidative stress or physiologic stress (e.g. caused by aberrant expression of tumor suppressor or oncogenes and excessive growth factor stimulation), which block the proliferation of cells[27-29]. This type of senescence is referred to as premature or induced senescence[30]. These findings have led to a distinction between "replicative senescence" which is induced by a cell-intrinsic mechanism, and "stress-induced premature senescence" which is triggered by extrinsic factors[31]. In many cases, replicative and premature senescence are interconnected. Telomere shortening is, for instance, accelerated by oxidative stress. Actually, cells may suffer cumulative damage from multiple stresses during culture. Senescence will be triggered when cellular damage has reached a

certain threshold, whatever has caused the damage. Senescent cells undergo dramatic changes in morphology and function. They generally become enlarged and flattened, have an increased lysosomal content, suffer from mitochondrial dysfunction and secrete increased amounts of inflammatory, growth-promoting, and remodeling factors. They may also form senescence-associated heterochromatin foci, often display a chronic DNA damage response, are incapable of synthesizing DNA for mitosis, show high resistance to apoptosis and are unresponsive to mitogenic stimuli. However, senescent cells are still metabolically active for months or even years[27,32-35].

p53 and pRB as key regulators of senescence

Molecular pathways of senescence are complex involving multiple layers of regulation with cooperative changes. The p53 tumour suppressor protein and the pocket proteins (*i.e.* pRB, p107 and p130) have been shown to play critical roles in the induction of senescence. The p53 protein is an important cell cycle regulator of both the G1/S and G2/M checkpoints by activating CDKN1A transcription. CDKN1A codes for p21 protein, which is a member of the CIP/KIP family of tumor suppressor proteins. These proteins inhibit various cyclin-CDK complexes required for promotion of the cell cycle (**Figure 3**). Hypophosphorylated pRB controls the G1/S checkpoint by directly binding to and inhibiting various E2F family members thereby repress gene transcription required for transition from the G1 to S phase of the cell cycle. In actively dividing cells, the suppressive effect of pRB on E2Fs is lost due to hyperphosphorylation of pRB by cyclin D-CDK4 and cyclin D-CDK6 complexes. In senescent cells the activity of these complexes is inhibited by members of the INK4 family of tumor suppressor proteins[36]. (**Figure 3**).

Numerous studies have explored the roles of p53 and pocket proteins in the induction and maintenance of senescence in mouse embryo fibroblasts (MEFs). Suppression of p53 expression allows cells to escape from senescence and leads to their indefinite expansion[37]. Inactivation of pRB alone is not enough to ease senescence of MEFs, while accompanying ablation of other pocket proteins including p107 and p130 strongly increased their proliferation capacity[38,39]. This demonstrates that the inactivation of either p53 or all three pocket proteins is sufficient to block the initiation of senescence. Suppression of either p53 or pRB in senescent MEFs is not enough to maintain senescence but leads to reactivation of the cell cycle[37,40]. Collectively, these findings suggest that p53 and the pocket proteins control cellular senescence in a hierarchical manner, in which the activation of pocket proteins is downstream of that of p53. Indeed, as mentioned before, p53 actives p21, which in turn inhibit cyclin-CDK complexes involved in the phosphorylation and inactivation of the pocket proteins. However, MEFs lacking p21 still undergo senescence and have a lifespan similar to wild-type cells[41], which shows that p21 is not the only link between p53 and pocket protein activity in the regulation of senescence (**Figure 3**).

Cellular quiescence

As indicated above, cells can enter into either of three different G0 states. While senescent and (terminally) differentiated cells have permanently excited the cell cycle (irreversible G0 arrest), quiescent cells only temporarily reside in the G0 phase

(reversible G0 arrest) and reenter the cell cycle in response to normal physiological stimuli[42]. The notion that terminally differentiated cells are irreversibly arrested in G0 has been challenged by the finding that mature hepatocytes dedifferentiate and reenter the cell cycle after injury of the liver and contribute to its regeneration[43-46]. Similar observation have recently been made for terminally differentiated cardiomyocytes[13-18]. Hence, not only quiescent cells but also terminally differentiated cells can exit the G0 phase and resume cell division.

In quiescent cells, p53 has been considered as main molecular regulator of cell cycle arrest[47]. Similar to senescence, the mechanism by which p53 mediates quiescence is dependent on the activation of p21, a CDK inhibitor and critical component of the pRB-E2F pathway[48]. In addition to p53, quiescent cells typically feature lower levels of pRB-E2F pathway activators, such as cyclin D[49-51] and high levels of pathway repressors, such as p21[48]. The disruption of all three pocket proteins and the acute loss of pocket protein function in quiescent cells lead to cell proliferation[38-40,52]. pRB deficiency in quiescent skeletal stem cells leads to an increase of proliferation and a loss of terminal differentiation capacity[38,53]. All of the above suggest that the RB-E2F pathway plays a pivotal role in quiescence. Among the E2F family of transcription factors, E2F1, -2 and -3 are considered "E2F transcriptional activators" and E2F4 and -5 are deemed "E2F transcriptional repressors"[54]. In cell cycle-arrested cells, hypophosphorylated pRB prevents cell cycle reentry by binding and repressing E2F1, -2 and -3, which activate genes required for the transition from G1 into S phase. In addition, hypophosphorylated p130 and p107 form complexes with E2F4 and -5. The complexes bind to the promotors of E2F target genes and recruit chromatin remodeling factors that block transcription[55,56]. In quiescent cells that are stimulated with mitogens, cyclin-CDK complexes phosphorylate pRB thereby releasing E2F1, -2 and -3. Similarly, phosphorylation of p130 and p107 abolishes their interaction with E2F4 and -5 repressors[57,58]. Especially E2F4-p130 complexes are very prominent in resting (*i.e.* G0-arrested) cells suggesting that they play a pivotal role in the maintenance of cell quiescence[59-61] (**Figure 3**).

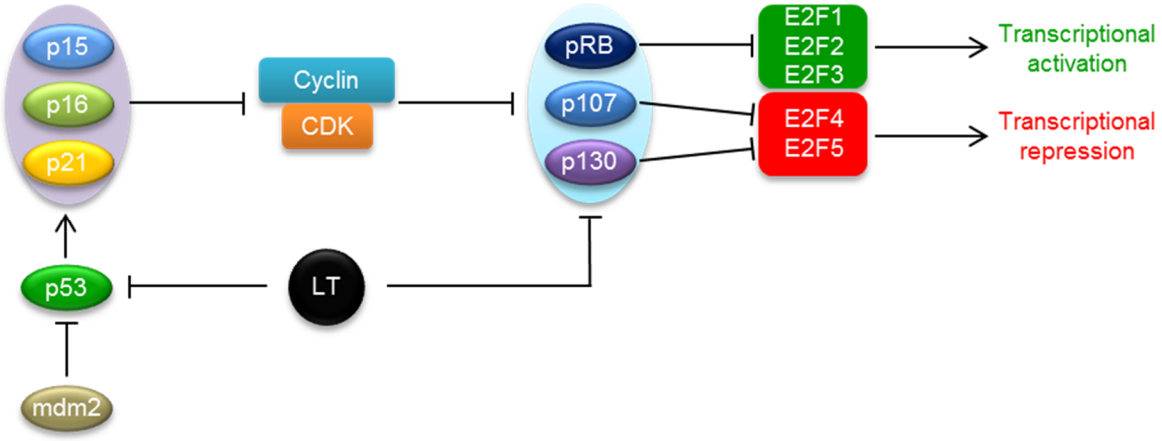

Figure 3. Participation of cell cycle regulators in cell cycle arrest.
p53 activates CDK inhibitors (e.g. p15, p16 and p21), which in turn disrupts cyclin-CKD complexes involved in the inactivation of the pocket proteins (pRB, p107 and p130). This allows the pocket proteins to arrest cell in a non-proliferative state by blocking the transcription of E2F target genes. LT, large tumor antigen. mdm2, mouse double minute 2.

Simian virus 40 LT antigens & cell immortalization

Simian virus 40 (SV40) is a small double-stranded non-enveloped DNA virus with a circular genome and an icosahedral capsid in the family of Polyomaviridae. The SV40 genome is composed of three main regions: (i) an early region encoding large tumor antigen (LT), 17kT and small tumor antigen (ST), (ii) a late region encoding the viral coat proteins (VP1, VP2, VP3 and VP4) and the agnoprotein (Agno) and (iii) the regulatory region containing the early and late promotor and the origin (Ori) of replication (**Figure 4A**). LT, 17kT and ST are produced from alternative splice products of the early viral pre-mRNA and are co-expressed in infected cells. The amino-terminus of LT contains a J domain which contributes to viral DNA replication[62]. Downstream of the the J domain, there is a LXCXE motif that can directly bind to the pocket proteins (*i.e.* pRB, p107 and p130)[63,64]. The J domain cooperates with the LXCXE motif to disrupt the interaction between E2F family members and the pocket proteins[63]. The DNA-binding domain (DBD) of LT recognizes Ori sequences and this interaction is essential for the initiation of viral DNA replication. The helicase domain works as DNA helicase to unwind the dsDNA template for viral replication and it also interacts with p53[65]. ST contains a J domain followed by a cellular protein phosphatase 2A (PP2A) binding region which contains two zinc fingers. ST binds the A and C subunits of PP2A and displaces the B subunit, thereby inhibiting the pro-apoptotic activity of PP2A[66,67.] 17kT shares several regulatory domains with LT that mediate some of its most important functions including stimulation of cell proliferation by binding to pocket proteins[68] (**Figure 4B**).

LT plays an essential role in multiple steps of the viral life cycle. After infection, LT induces host cells to enter the S phase thereby creating optimal conditions for the replication of viral DNA. Meanwhile, LT works as the DNA helicase and then utilizes the host's replication machinery to replicate the viral DNA. Besides, LT is also involved in transcription and virion assembly. Given the capability of LT to incite cell proliferation, LT has been successfully used to immortalize multiple primary cell types with no/limited proliferative capacity *in vitro*[69-71]. Our current understanding indicates LT's ability to drive cell proliferation is mainly due to its interaction with the pocket proteins and p53[72,73]. Quiescent cells including differentiated cells are trapped in G0 by the pocket proteins[42]. Hypophosphorylated pocket proteins bind to E2Fs and thereby block gene expression required for nucleotide synthesis, DNA replication, cell cycle progression and apoptosis. When cells are mitogenically stimulated, pocket proteins become phosphorylated allowing E2F-mediated S phase gene transcription. Instead of disrupting E2F-pocket protein complexes by pocket protein hyperphosphorylation, LT binds to hypophosphorylated pocket proteins setting free the pocket protein-bound E2Fs resulting in E2F-dependent gene expression and cell cycle reentry[74]. The p53 level in normal cells remains very low because it binds to Mdm2 which induces ubiquitination and subsequent degradation of p53[72,75]. Besides Mdm2, many other proteins affect the biological activity and stability of p53 through various types of post-translational modifications. Different cellular stress conditions lead to stabilization of p53 allowing it to act as a transcriptional activator of genes mediating apoptosis or cell cycle arrest[76,77]. LT inactivates p53 by covering its DNA-binding domain and thus block the expression of

p53-dependent genes, which leads to cell cycle reentry and avoids cell cycle arrest as well as apoptosis[78] (**Figure 3**).

Exposure of SV40 particles to chemical mutagens has resulted in the recovery of several temperature-sensitive LT mutants (ts-LTs) that can cause cell immortalization/transformation at permissive temperatures (≤ 34°C) but fail to do so at nonpermissive temperatures (≥ 37°C)[79,80]. These ts-LTs have been used to control the activity of LT allowing conditional immortalization of primary mammalian cells.

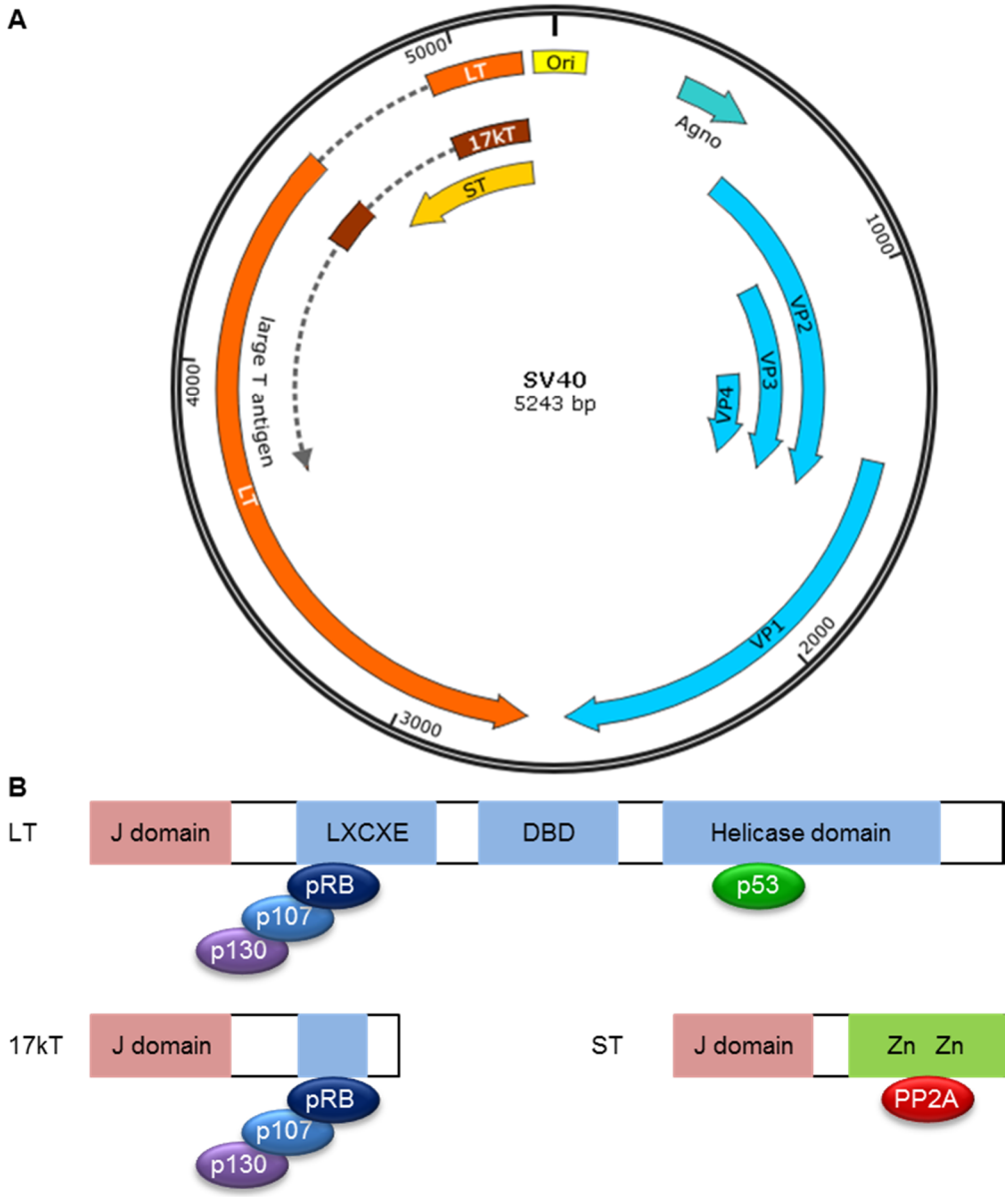

Figure 4. Structure of SV40
(A) Genomic structure of SV40. The circular double-stranded DNA genome contains three main regions: the regulatory region, an early region encoding LT, 17kT and ST, and a late region encoding the viral coat proteins VP1, VP2, VP3 and VP4 as well as the agnoprotein. (B) The functional domains of SV40 T antigens. The J domain is shared by all three antigens. LT also contains a pocket protein-binding LXCXE motif, a DNA-binding domain (DBD), a p53-binding helicase domain. 17kT shares several regulatory domains with LT including a pocket protein binding domain. ST contains a J domain followed by a PP2A-binding region with two zinc-fingers.

For instance, ts-LT tsA58, which carries an alanine to valine substitution at amino acid position 438 that abolishes its binding to and inhibition of p53 at nonpermissive temperatures[81] was expressed in transgenic mice to generate conditional immortalized cell lines from different tissues[82,83].

Cell cycle regulation in cardiomyocytes

In the embryonic and fetal hearts of vertebrates, including mammals, cardiomyocytes have high cell cycle activity and are capable of DNA synthesis, karyokinesis and cytokinesis resulting in cardiomyocyte division and an increase of cardiomyocyte number. However, the proliferative capacity of postnatal hearts differs from various species. Zebrafish have cardiomyocyte proliferative capacity throughout life. A complete regeneration of heart tissue lost from the resection of the ventricular apex can be managed in adult zebrafish through the proliferation of pre-existing cardiomyocytes[15,84]. In contrast, mammalian cardiomyocytes lose their proliferative capacity shortly after birth and the expansion of postnatal mammal heart driven by non-mitotic growth produces multinucleated or polyploid cells and hypertrophic cells[85-88]. In mice, during the first week of postnatal period, the majority of cardiomyocytes undergo karyokinesis in the absence of cytokinesis, which generates a population of binucleated cardiomyocytes[85]. In this event, cardiomyocytes enter the M phase of the cell cycle and perform nuclear division, but they do not undergo cytokinesis[86,88]. In contrast, the timeline of human cardiomyocyte proliferative capacity is not clear, however, more than half of all adult human cardiomyocytes are polyploid as a result of DNA duplication without nuclear division[87]. The polyploidazation results from shuttling between G1, S and G2 phase without entering into the M phase. Although damage-induced cardiomyocyte proliferation in adult mammals has been observed[13,89,90], the growth of stressed and injured mammalian hearts is largely caused by hypertrophy. Under physiological and pathological hypertrophy-stimulating conditions, cardiomyocytes enter the G1 phase of cell cycle where the grow in size by synthesis of extra RNAs and proteins, but they fail to undergo S phase[91-93] (**Figure 5**).

This different behavior of antenatal and postnatal cardiomyocytes is due to the developmental changes in cell cycle activity[86,94]. The cell cycle of cardiomyocyte is tightly controlled by transcription factors and regulators thereof like the pocket proteins, which play an essential role in cell cycle exit and differentiation of cardiomyocytse[95]. For example, p107 and p130 interact with E2F family members to inhibit DNA synthesis in the fetal and neonatal cardiomyocytes, respectively[96]. Moreover, pRB silences multiple proliferation-promoting genes in adult cardiomyocyte to maintain their post-mitotic state by interaction with heterochromatin protein 1γ[97,98]. Apart from the pocket proteins, transcription factors such as Meis1, MEF2D, FoxO1, and FoxO3, inhibit cell cycle progression by increasing the expression of p21, p27, p15, and p16[99-102]. In contrast, transcription factors, such as E2F1, E2F2, E2F4, CASZ1, GATA4, Tbx20, and FoxM1, stimulate cell cycling by increasing the expression of cyclins[99,101,103-106]. Besides, PI3K-AKT, Wnt/β-catenin and YAP signaling pathways play important roles in cardiomyocyte proliferation[107-111]. Manipulation of the cell cycle in post-mitotic cardiomyocytes may be an attractive strategy to obtain large numbers of cardiomyocytes for *in vitro* studies, cardiac cell

therapy and the regeneration of injured hearts from inside by re-awaking cell cycling in cardiomyocytes surrounding the site(s) of myocardial damage.

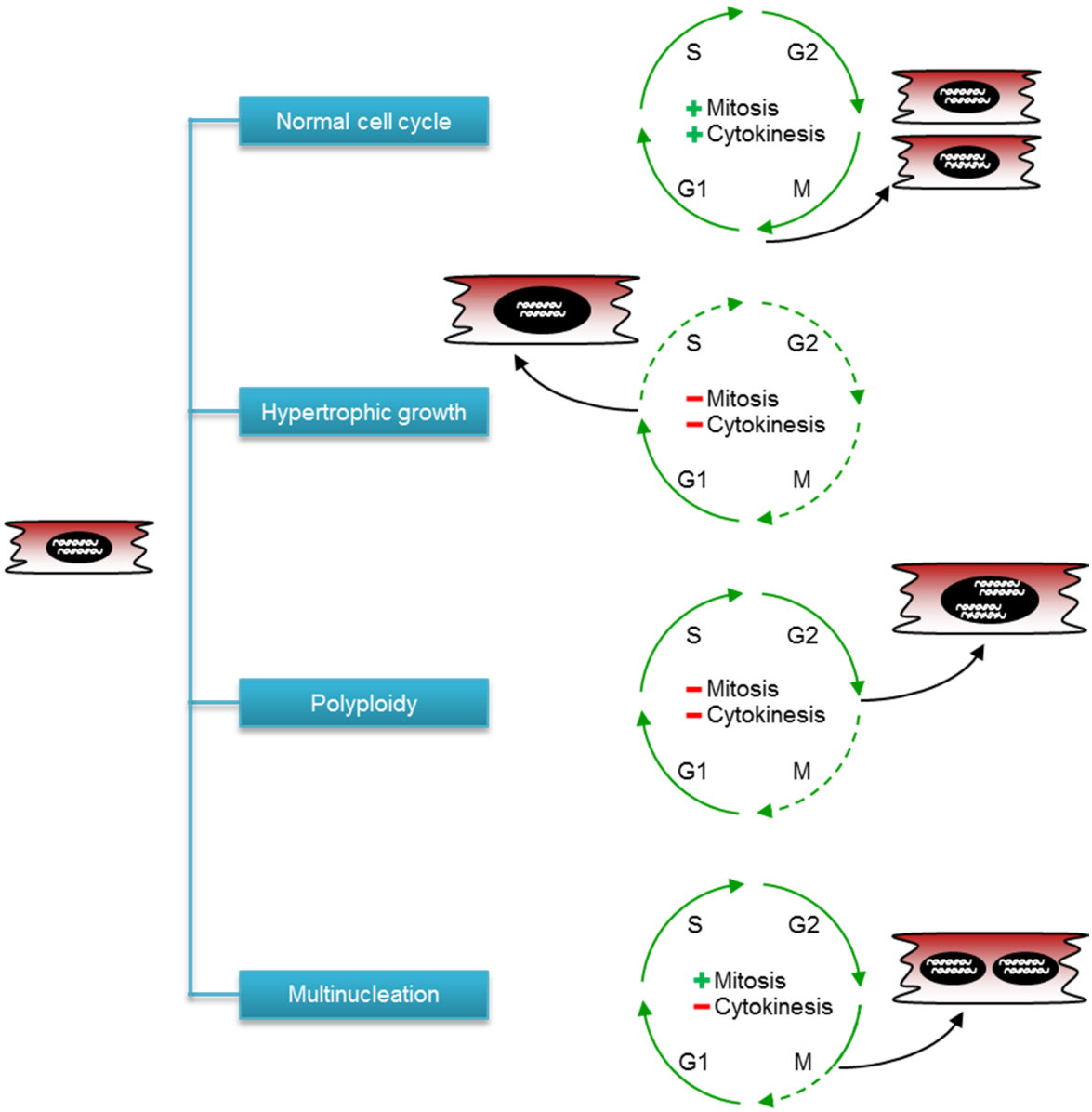

Figure 5. Cell cycle activities in cardiomyocytes.
In a typical cell cycle, cell successively enters the G1 phase (for synthesis of RNAs and proteins), S phase (for DNA synthesis), G2 phase (for additional growth) and M phase (for karyokinesis and cytokinesis) resulting in two individual cells. In the presence of hypertrophic stimuli, cardiomyocytes enter the G1 phase and grow in size by synthesis of RNAs and proteins, but they fail to enter the S phase. Polyploidazation results from shuttling between G1, S and G2 phase without entering into the M phase. Multinucleation occurs when cardiomyocytes enter the M phase and perform karyokinesis, but are withdraw from the cell cycle before cytokinesis.

Aim and outline of thesis

The aim of this thesis is to develop and use cell culture models for fundamental and applied biomedical research. Cell immortalization is a promising approach to produce large number of cells from same amounts/scarce primary staring material by inducing cell cycle reentry or bypassing cell senescence. Accordingly, a major part of this thesis was dedicated to the generation of robust cellular model systems by cell immortalization for addressing fundamental and applied research questions in a reproducible, flexible and standardized way.

Bypassing cellular senescence is a prerequisite for cell immortalization. It is therefore important to have a better understanding of the mechanism of senescence before immortalizing cells. A variety of proteins and pathways were found to be involved in senescence. However, the role of heterochromatin proteins and their contribution to abnormal chromatin organization in cell senescence is largely unknown. **Chapter II** of this thesis uses premature MEFs as a senescence model system which is derived from progeria disorder mice and studies the role of heterochromatin proteins and the associated chromatin organization in DNA damage response (DDR)-induced senescence.

In order to get familiar with the properties of immortalized cells including their culture conditions, proliferation and differentiation, an existing line of immortalized human myoblast is cultured and its differentiation is investigated in **Chapter III**. With the aid of this cell line, a novel non-destructive quantitative assay to monitor cell fusion is developed.

Cardiac cell therapy, tissue engineering and *in vitro* studies into cardiac arrhythmias or for testing the potential cardiotoxicity of drugs require large numbers of functional and homogeneous cardiomyocytes. Progress in these areas of research is impeded by the limited availability of heart tissue and the very low mitotic activity of cardiomyocytes highlight the urge to develop new/rich source of cadiomyocytes for both basic and translational research. **Chapter IV** describes the generation and characterization of a immortalized atrial myocyte cell line with preserved cardiomyogenic differentiation capacity. Through controlled expansion and differentiation of this cell line, large numbers of functional cardiomyocytes are generated, hence providing an attractive cell model for cardiac research.

BAT is regarded as a potential target to treat obesity and associated metabolic disorders because of its capacity to take up and combust glucose and fatty acids for thermoregulation. However, its cellular and molecular investigation has been hampered due to high cellular heterogeneity and a limited availability of cell material. In **Chapter V,** monoclonal lines of immortalized BPAs are generated by using mouse BAT as starting material. The long-term proliferation and high adipogenic capacity of the cell lines provide an attractive model system for fundamental and applied research into BAT.

Besides immortalized cell lines, primary cardiomyocytes were also used to solve research questions. In **Chapter VI**, neonatal rat ventricular myocyte cultures are established as an *in vitro* model for investigating the action potential-prolonging and associated proarrhythmic effects of Kv11.1 blockers. In this study, a newly designed

and synthesized compound LUF7244 is shown to prevent drug-induced proarrhythmic effects as by an allosteric mechanism.

Finally, **Chapter VII** provides the summary and conclusions drawn from each study of this thesis, as well as future perspectives related to the application of immortalized cells.

References

1. Ludovico P, Osiewacz HD, Costa V, Burhans WC. Cellular models of aging. *Oxid Med Cell Longev* 2012;**2012**.
2. Goetsch K, Myburgh K, Niesler CU. In vitro myoblast motility models: investigating migration dynamics for the study of skeletal muscle repair. *J Muscle Res Cell Motil* 2013;**34**:333-347.
3. Parameswaran S, Kumar S, Verma RS, Sharma RK. Cardiomyocyte culture—an update on the in vitro cardiovascular model and future challenges. *Can J Physiol Pharmacol* 2013;**91**:985-998.
4. Herron TJ, Lee P, Jalife J. Optical imaging of voltage and calcium in cardiac cells & tissues. *Circ Res* 2012;**110**:609-623.
5. Iravanian S, Nabutovsky Y, Kong C-R, Saha S, Bursac N, Tung L. Functional reentry in cultured monolayers of neonatal rat cardiac cells. *American Journal of Physiology-Heart and Circulatory Physiology* 2003;**285**:H449-H456.
6. Bub G, Glass L, Publicover NG, Shrier A. Bursting calcium rotors in cultured cardiac myocyte monolayers. *Proceedings of the National Academy of Sciences* 1998;**95**:10283-10287.
7. Bursac N, Tung L. Acceleration of functional reentry by rapid pacing in anisotropic cardiac monolayers: formation of multi-wave functional reentries. *Cardiovasc Res* 2006;**69**:381-390.
8. Yu Z, Liu J, van Veldhoven JP, IJzerman AP, Schalij MJ, Pijnappels DA, Heitman LH, de Vries AA. Allosteric modulation of Kv11. 1 (hERG) channels protects against drug-induced ventricular arrhythmias. *Circulation: Arrhythmia and Electrophysiology* 2016;**9**:e003439.
9. Townsend K, Tseng Y-H. Brown adipose tissue: recent insights into development, metabolic function and therapeutic potential. *Adipocyte* 2012;**1**:13-24.
10. Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, Lo JC, Zeng X, Ye L, Khandekar MJ, Wu J, Gunawardana SC, Banks AS, Camporez JP, Jurczak MJ, Kajimura S, Piston DW, Mathis D, Cinti S, Shulman GI, Seale P, Spiegelman BM. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* 2014;**156**:304-316.
11. Tseng YH, Cypess AM, Kahn CR. Cellular bioenergetics as a target for obesity therapy. *Nat Rev Drug Discov* 2010;**9**:465-482.
12. Stanford KI, Goodyear LJ. The therapeutic potential of brown adipose tissue. *Hepatobiliary Surg Nutr* 2013;**2**:286-287.
13. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* 2001;**344**:1750-1757.
14. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. *Science* 2009;**324**:98-102.
15. Jopling C, Sleep E, Raya M, Marti M, Raya A, Izpisua Belmonte JC. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation.

Nature 2010;**464**:606-609.
16. Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* 2010;**464**:601-605.
17. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S, Kuhn B. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci U S A* 2013;**110**:1446-1451.
18. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* 2013;**493**:433-436.
19. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 2013;**1833**:3448-3459.
20. Lauber K, Ernst A, Orth M, Herrmann M, Belka C. Dying cell clearance and its impact on the outcome of tumor radiotherapy. *Front Oncol* 2012;**2**:116.
21. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961;**25**:585-621.
22. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences* 1995;**92**:9363-9367.
23. Coates PJ. Markers of senescence? *The Journal of pathology* 2002;**196**:371-373.
24. Cong Y-S, Wright WE, Shay JW. Human telomerase and its regulation. *Microbiol Mol Biol Rev* 2002;**66**:407-425.
25. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. *science* 1998;**279**:349-352.
26. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;**345**:458-460.
27. Serrano M, Blasco MaA. Putting the stress on senescence. *Curr Opin Cell Biol* 2001;**13**:748-753.
28. Lloyd AC. Limits to lifespan. *Nat Cell Biol* 2002;**4**:E25-E27.
29. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16 INK4a. *Cell* 1997;**88**:593-602.
30. Drayton S, Peters G. Immortalisation and transformation revisited. *Curr Opin Genet Dev* 2002;**12**:98-104.
31. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. *The international journal of biochemistry & cell biology* 2005;**37**:961-976.
32. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. *Curr Biol* 1999;**9**:939-945.
33. Narita M, Nuñez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. Rb-mediated heterochromatin formation and silencing

of E2F target genes during cellular senescence. *Cell* 2003;**113**:703-716.
34. Bérubé NG, Smith JR, Pereira-Smith OM. The genetics of cellular senescence. *The American Journal of Human Genetics* 1998;**62**:1015-1019.
35. Gorbunova V, Seluanov A, Mao Z, Hine C. Changes in DNA repair during aging. *Nucleic Acids Res* 2007;**35**:7466-7474.
36. Lanigan F, Geraghty J, Bracken A. Transcriptional regulation of cellular senescence. *Oncogene* 2011;**30**:2901.
37. Dirac AM, Bernards R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *Journal of biological chemistry* 2003;**278**:11731-11734.
38. Dannenberg J-H, van Rossum A, Schuijff L, te Riele H. Ablation of the retinoblastoma gene family deregulates G1 control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes & development* 2000;**14**:3051-3064.
39. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T. Targeted disruption of the three Rb-related genes leads to loss of G1 control and immortalization. *Genes & development* 2000;**14**:3037-3050.
40. Sage J, Miller AL, Pérez-Mancera PA, Wysocki JM, Jacks T. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* 2003;**424**:223-228.
41. Pantoja C, Serrano M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene* 1999;**18**.
42. Yao G. Modelling mammalian cellular quiescence. *Interface Focus* 2014;**4**:20130074.
43. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 2004;**39**:1477-1487.
44. Fausto N. Liver regeneration. *J Hepatol* 2000;**32**:19-31.
45. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science* 1997;**276**:60-66.
46. Bucher NL, Farmer SR. Liver regeneration following partial hepatectomy: genes and metabolism. Liver growth and repair: Springer, 1998:3-27.
47. Liu Y, Elf SE, Miyata Y, Sashida G, Liu Y, Huang G, Di Giandomenico S, Lee JM, Deblasio A, Menendez S. p53 regulates hematopoietic stem cell quiescence. *Cell stem cell* 2009;**4**:37-48.
48. Cheng T, Rodrigues N, Shen H, Yang Y-g, Dombkowski D, Sykes M, Scadden DT. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 2000;**287**:1804-1808.
49. Ladha MH, Lee KY, Upton TM, Reed MF, Ewen ME. Regulation of exit from quiescence by p27 and cyclin D1-CDK4. *Molecular and cellular biology* 1998;**18**:6605-6615.
50. Boonstra J. Progression through the G1-phase of the on-going cell cycle. *J Cell Biochem* 2003;**90**:244-252.
51. Pajalunga D, Mazzola A, Salzano AM, Biferi MG, De Luca G, Crescenzi M. Critical requirement for cell cycle inhibitors in sustaining nonproliferative states. *The Journal of cell biology* 2007;**176**:807-818.
52. Viatour P, Somervaille TC, Venkatasubrahmanyam S, Kogan S, McLaughlin

ME, Weissman IL, Butte AJ, Passegué E, Sage J. Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. *Cell stem cell* 2008;**3**:416-428.
53. Hosoyama T, Nishijo K, Prajapati SI, Li G, Keller C. Rb1 gene inactivation expands satellite cell and postnatal myoblast pools. *Journal of Biological Chemistry* 2011;**286**:19556-19564.
54. Attwooll C, Denchi EL, Helin K. The E2F family: specific functions and overlapping interests. *The EMBO journal* 2004;**23**:4709-4716.
55. Vandel L, Nicolas E, Vaute O, Ferreira R, Ait-Si-Ali S, Trouche D. Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. *Molecular and Cellular Biology* 2001;**21**:6484-6494.
56. Nielsen SJ, Schneider R, Bauer U-M, Bannister AJ, Morrison A, O'carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE. Rb targets histone H3 methylation and HP1 to promoters. *Nature* 2001;**412**:561.
57. Lavoie JN, L'Allemain G, Brunet A, Müller R, Pouysségur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *Journal of Biological Chemistry* 1996;**271**:20608-20616.
58. Cheng M, Sexl V, Sherr CJ, Roussel MF. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proceedings of the National Academy of Sciences* 1998;**95**:1091-1096.
59. Ikeda M-A, Jakoi L, Nevins JR. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. *Proceedings of the National Academy of Sciences* 1996;**93**:3215-3220.
60. Shin EK, Shin A, Paulding C, Schaffhausen B, Yee AS. Multiple change in E2F function and regulation occur upon muscle differentiation. *Molecular and Cellular Biology* 1995;**15**:2252-2262.
61. Vairo G, Livingston DM, Ginsberg D. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes & Development* 1995;**9**:869-881.
62. Campbell KS, Mullane KP, Aksoy IA, Stubdal H, Zalvide J, Pipas JM, Silver PA, Roberts TM, Schaffhausen BS, DeCaprio JA. DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication. *Genes & development* 1997;**11**:1098-1110.
63. Stubdal H, Zalvide J, Campbell KS, Schweitzer C, Roberts TM, DeCaprio JA. Inactivation of pRB-related proteins p130 and p107 mediated by the J domain of simian virus 40 large T antigen. *Molecular and cellular biology* 1997;**17**:4979-4990.
64. DeCaprio JA, Ludlow JW, Figge J, Shew J-Y, Huang C-M, Lee W-H, Marsilio E, Paucha E, Livingston DM. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* 1988;**54**:275-283.
65. Lilyestrom W, Klein MG, Zhang R, Joachimiak A, Chen XS. Crystal structure

of SV40 large T-antigen bound to p53: interplay between a viral oncoprotein and a cellular tumor suppressor. *Genes & development* 2006;**20**:2373-2382.
66. Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell* 1990;**60**:167-176.
67. Pallas DC, Weller W, Jaspers S, Miller T, Lane W, Roberts T. The third subunit of protein phosphatase 2A (PP2A), a 55-kilodalton protein which is apparently substituted for by T antigens in complexes with the 36-and 63-kilodalton PP2A subunits, bears little resemblance to T antigens. *J Virol* 1992;**66**:886-893.
68. Comerford S, Schultz N, Hinnant E, Klapproth S, Hammer R. Comparative analysis of SV40 17kT and LT function in vivo demonstrates that LT's C-terminus re-programs hepatic gene expression and is necessary for tumorigenesis in the liver. *Oncogenesis* 2012;**1**:e28.
69. Ageberg M, Gullberg U, Lindmark A. The involvement of cellular proliferation status in the expression of the human proto-oncogene DEK. *Haematologica* 2006;**91**:268-269.
70. Lamplot JD, Liu B, Yin L, Zhang W, Wang Z, Luther G, Wagner E, Li R, Nan G, Shui W. Reversibly Immortalized Mouse Articular Chondrocytes Acquire Long-Term Proliferative Capability while Retaining Chondrogenic Phenotype. *Cell transplantation* 2014.
71. Richter C, Thieme S, Bandoła J, Laugsch M, Anastassiadis K, Brenner S. Generation of inducible immortalized dendritic cells with proper immune function in vitro and in vivo. *PloS one* 2013;**8**:e62621.
72. Bond GL, Hu W, Levine AJ. MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr Cancer Drug Targets* 2005;**5**:3-8.
73. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 1979;**17**:43-52.
74. Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y, Whyte P, Rundell K, Brodsky JL, Pipas JM. The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Molecular and cellular biology* 1997;**17**:4761-4773.
75. Coutts AS, La Thangue NB. The p53 response: emerging levels of co-factor complexity. *Biochem Biophys Res Commun* 2005;**331**:778-785.
76. Brady CA, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, Broz DK, Basak S, Park EJ, McLaughlin ME. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* 2011;**145**:571-583.
77. Beckerman R, Prives C. Transcriptional regulation by p53. *Cold Spring Harb Perspect Biol* 2010;**2**:a000935.
78. Bargonetti J, Reynisdottir I, Friedman PN, Prives C. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. *Genes & development* 1992;**6**:1886-1898.
79. Kimura G, Dulbecco R. Isolation and characterization of temperature-sensitive mutants of simian virus 40. *Virology* 1972;**49**:394-403.
80. Yamaguchi N, Kuchino T. Temperature-sensitive mutants of simian virus 40

selected by transforming ability. *J Virol* 1975;**15**:1297-1301.
81. Ray S, Anderson ME, Tegtmeyer P. Differential interaction of temperature-sensitive simian virus 40 T antigens with tumor suppressors pRb and p53. *J Virol* 1996;**70**:7224-7227.
82. Jat PS, Noble MD, Ataliotis P, Tanaka Y, Yannoutsos N, Larsen L, Kioussis D. Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proceedings of the National Academy of Sciences* 1991;**88**:5096-5100.
83. Yanai N, Suzuki M, Obinata M. Hepatocyte cell lines established from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene. *Exp Cell Res* 1991;**197**:50-56.
84. Mercola M, Ruiz-Lozano P, Schneider MD. Cardiac muscle regeneration: lessons from development. *Genes & development* 2011;**25**:299-309.
85. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. *American Journal of Physiology-Heart and Circulatory Physiology* 1996;**271**:H2183-H2189.
86. Ikenishi A, Okayama H, Iwamoto N, Yoshitome S, Tane S, Nakamura K, Obayashi T, Hayashi T, Takeuchi T. Cell cycle regulation in mouse heart during embryonic and postnatal stages. *Development, growth & differentiation* 2012;**54**:731-738.
87. Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C. Dynamics of cell generation and turnover in the human heart. *Cell* 2015;**161**:1566-1575.
88. Chattergoon NN, Louey S, Stork P, Giraud GD, Thornburg KL. Unexpected maturation of PI3K and MAPK-ERK signaling in fetal ovine cardiomyocytes. *American Journal of Physiology-Heart and Circulatory Physiology* 2014;**307**:H1216-H1225.
89. Kajstura J, Zhang X, Reiss K, Szoke E, Li P, Lagrasta C, Cheng W, Darzynkiewicz Z, Olivetti G, Anversa P. Myocyte cellular hyperplasia and myocyte cellular hypertrophy contribute to chronic ventricular remodeling in coronary artery narrowing-induced cardiomyopathy in rats. *Circ Res* 1994;**74**:383-400.
90. Quaini F, Cigola E, Lagrasta C, Saccani G, Quaini E, Rossi C, Olivetti G, Anversa P. End-stage cardiac failure in humans is coupled with the induction of proliferating cell nuclear antigen and nuclear mitotic division in ventricular myocytes. *Circ Res* 1994;**75**:1050-1063.
91. Huang S, Zou X, Zhu JN, Fu YH, Lin QX, Liang YY, Deng CY, Kuang SJ, Zhang MZ, Liao YL. Attenuation of microRNA-16 derepresses the cyclins D1, D2 and E1 to provoke cardiomyocyte hypertrophy. *J Cell Mol Med* 2015;**19**:608-619.
92. Hinrichsen R, Hansen A, Haunsø S, Busk P. Phosphorylation of pRb by cyclin D kinase is necessary for development of cardiac hypertrophy. *Cell Prolif* 2008;**41**:813-829.
93. Busk PK, Bartkova J, Strøm CC, Wulf-Andersen L, Hinrichsen R, Christoffersen TE, Latella L, Bartek J, Haunsø S, Sheikh SP. Involvement of cyclin D activity in left ventricle hypertrophy in vivo and in vitro. *Cardiovasc*

Res 2002;**56**:64-75.
94. Tane S, Okayama H, Ikenishi A, Amemiya Y, Nakayama KI, Takeuchi T. Two inhibitory systems and CKIs regulate cell cycle exit of mammalian cardiomyocytes after birth. *Biochem Biophys Res Commun* 2015;**466**:147-154.
95. MacLellan W, Garcia A, Oh H, Frenkel P, Jordan M, Roos K, Schneider M. Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. *Molecular and Cellular Biology* 2005;**25**:2486-2497.
96. Flink IL, Oana S, Maitra N, Bahl JJ, Morkin E. Changes in E2F complexes containing retinoblastoma protein family members and increased cyclin-dependent kinase inhibitor activities during terminal differentiation of cardiomyocytes. *J Mol Cell Cardiol* 1998;**30**:563-578.
97. Sdek P, Zhao P, Wang Y, Huang C-j, Ko CY, Butler PC, Weiss JN, MacLellan WR. Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. *The Journal of cell biology* 2011;**194**:407-423.
98. Tamamori-Adachi M, Goto I, Yamada K, Kitajima S. Differential regulation of cyclin D1 and D2 in protecting against cardiomyocyte proliferation. *Cell Cycle* 2008;**7**:3768-3774.
99. Sengupta A, Kalinichenko VV, Yutzey KE. FoxO1 and FoxM1 Transcription Factors Have Antagonistic Functions in Neonatal Cardiomyocyte Cell-Cycle Withdrawal and IGF1 Gene RegulationNovelty and Significance. *Circ Res* 2013;**112**:267-277.
100. Estrella NL, Clark AL, Desjardins CA, Nocco SE, Naya FJ. MEF2D deficiency in neonatal cardiomyocytes triggers cell cycle re-entry and programmed cell death in vitro. *Journal of Biological Chemistry* 2015;**290**:24367-24380.
101. Xiang F-l, Guo M, Yutzey KE. Overexpression of Tbx20 in adult cardiomyocytes promotes proliferation and improves cardiac function after myocardial infarction. *Circulation* 2016:CIRCULATIONAHA. 115.019357.
102. Mahmoud AI, Kocabas F, Muralidhar SA, Kimura W, Koura AS, Thet S, Porrello ER, Sadek HA. Meis1 regulates postnatal cardiomyocyte cell cycle arrest. *Nature* 2013;**497**:249-253.
103. Ebelt H, Hufnagel N, Neuhaus P, Neuhaus H, Gajawada P, Simm A, Müller-Werdan U, Werdan K, Braun T. Divergent Siblings. *Circ Res* 2005;**96**:509-517.
104. Dingar D, Konecny F, Zou J, Sun X, von Harsdorf R. Anti-apoptotic function of the E2F transcription factor 4 (E2F4)/p130, a member of retinoblastoma gene family in cardiac myocytes. *J Mol Cell Cardiol* 2012;**53**:820-828.
105. Dorr KM, Amin NM, Kuchenbrod LM, Labiner H, Charpentier MS, Pevny LH, Wessels A, Conlon FL. Casz1 is required for cardiomyocyte G1-to-S phase progression during mammalian cardiac development. *Development* 2015;**142**:2037-2047.
106. Rojas A, Kong SW, Agarwal P, Gilliss B, Pu WT, Black BL. GATA4 is a direct transcriptional activator of cyclin D2 and Cdk4 and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. *Molecular and cellular biology* 2008;**28**:5420-5431.
107. Bersell K, Arab S, Haring B, Kühn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 2009;**138**:257-270.

108. Novoyatleva T, Diehl F, van Amerongen MJ, Patra C, Ferrazzi F, Bellazzi R, Engel FB. TWEAK is a positive regulator of cardiomyocyte proliferation. *Cardiovasc Res* 2009;**85**:681-690.
109. Novoyatleva T, Sajjad A, Pogoryelov D, Patra C, Schermuly RT, Engel FB. FGF1-mediated cardiomyocyte cell cycle reentry depends on the interaction of FGFR-1 and Fn14. *The FASEB Journal* 2014;**28**:2492-2503.
110. Buikema JW, Mady AS, Mittal NV, Atmanli A, Caron L, Doevendans PA, Sluijter JP, Domian IJ. Wnt/β-catenin signaling directs the regional expansion of first and second heart field-derived ventricular cardiomyocytes. *Development* 2013;**140**:4165-4176.
111. Lin Z, Zhou P, von Gise A, Gu F, Ma Q, Chen J, Guo H, van Gorp PR, Wang D-Z, Pu WT. Pi3kcb Links Hippo-YAP and PI3K-AKT Signaling Pathways to Promote Cardiomyocyte Proliferation and SurvivalNovelty and Significance. *Circ Res* 2015;**116**:35-45.