Cell Cycle Regulation and Differentiation in Cardiovascular and Neural Systems
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The brain and heart have been used as metaphors for thinking and feeling, for cognition and emotion. These ideas have been demonstrated to have a high scientific value. In fact, complex physiopathological relationships exist between these two organs. This book will deal with several aspects of the cardiovascular and nervous systems from a new viewpoint. It will describe the differences and similarities in their differentiation pathways with a peculiar emphasis on the role of cell cycle regulation and cell differentiation.

Cell cycle exit represents the fundamental step to trigger differentiation of cells and induction of a novel program of gene expression leading to the elaboration of a specialized phenotype. Moreover, there is evidence demonstrating that several components of the cell cycle machinery play a major role also in cell specification and differentiation both in neural and cardiovascular systems.

The differentiation process will be evaluated starting from the most early cell precursor, i.e., stem cells. The attention of readers will be focused also on transcription factors with differentiating properties and on their relationship with cell cycle regulators.

In summary, this book will offer an in-depth analysis of the differentiation process in two systems that have profound relationships with one another and, therefore, will help us to better understand their biology by providing the tools to dissect the molecular basis of pathological conditions.

The book will prompt the scientific community to perceive cell cycle regulation and differentiation under a novel and more comprehensive light.
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Part I
Introduction to Cell Cycle
and Differentiation
Chapter 1
Short Introduction to the Cell Cycle

Antonio Giordano and Umberto Galderisi

Abstract  Molecular pathways that regulate cell growth and differentiation are now beginning to be understood. This is mainly due to the identification of molecules that orchestrate the cell cycle. Cyclins and the cyclin-dependent kinases (CDKs) are the major players in the control of cell cycle progression. Cyclins do not have enzymatic activity and CDKs are inactive without a partner cyclin. The CDKs regulate the function of multiple proteins involved in DNA replication and mitosis by phosphorylating them at specific regulatory sites, activating some and inhibiting others to coordinate their activities. In this way cyclin/CDK complexes coordinate an ordered passage from a cell cycle phase to the next one. Multiple levels of regulation of cyclin/CDK complexes are present in “cell machinery” to obtain a tight control of cell cycle progression. In this chapter we will address these items.

Keywords  Cell cycle · Cyclins · Cyclin-dependent kinases (CDKs) · Cyclin kinase inhibitors (CKIs) · Retinoblastoma proteins

1 Introduction

In order for an organism to develop, two kinds of processes are fundamental: cell division and cell differentiation. Both of these formative pathways must be carefully regulated and coordinated for normal growth to occur. The mechanisms that control cell growth and differentiation are now beginning to be understood. This is mainly due to the identification of molecules that orchestrate the cell cycle.

What is a “cell cycle”? It is the series of ordered events in a eukaryotic cell between one cell division and the next. During this time period a cell duplicates its chromosomes and then gives rise to two daughter cells, each with the same DNA
content. Cell cycle can be described in terms of four distinct phases: G1 phase, S phase, G2 phase, and M phase. During G1 phase a strong increase in RNA and protein synthesis occurs before cell duplicates its DNA (S phase). During G2 phase DNA replication is completed and cell increases in size in order to give rise to the two daughter cells. Following G2 phase, cell enters into mitosis (M phase), during which nuclear division occurs. At the end of this process cell splits into two new cells (cytokinesis). Cells can also enter in a quiescent state (G0 phase) where they arrest cellular division for long periods of times even indefinitely. This exit from cell cycle represents the fundamental step to trigger differentiation of cells and induction of a novel program of gene expression leading to the elaboration of a specialized phenotype [1, 2].

On the other hand, cells can permanently arrest division due to age or accumulated DNA damage and enter the “senescent state.” Senescence is the alternative to apoptosis, the programmed cell death, that eliminates “damaged” cells [3, 4].

Key molecules controlling cell cycle progression are the cyclins and the cyclin-dependent kinases (CDKs). Cyclins are the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer. Cyclins do not have enzymatic activity and CDKs are inactive without a partner cyclin. The CDKs regulate the function of multiple proteins involved in DNA replication and mitosis by phosphorylating them at specific regulatory sites, activating some and inhibiting others to coordinate their activities. In this way cyclin/CDK complexes coordinate an ordered passage from a cell cycle phase to the next one [5].

Multiple levels of regulation of cyclin/CDK complexes are present in “cell machinery” to obtain a tight control of cell cycle progression. Such regulation relies on (1) controlled expression and degradation of cyclins, (2) activating and inhibitory phosphorylation and dephosphorylation of the CDKs, and (3) expression and destruction of inhibitory proteins, the CDK inhibitors (CKIs), that associate with CDK/cyclin complexes [5–11].

Cell concentration of cyclins changes during cell cycle since they are produced and degraded as needed. For example, growth factors can promote G1 cyclin production, then to pass to the next phase of cycle the cyclins have to be degraded by proteases [12–14].

CDKs are regulated by phosphorylation and dephosphorylation of critical residues. Important targets of this regulation are some threonine and tyrosine residues present in the ATP-binding domain. The cyclin kinase inhibitors (CKI), also called CDKIs, negatively regulate CDK activity. There are two CKI classes. The first one includes p21Cip1, p27Kip1, and p57Kip2. These bind to G1/S CDKs; second class is the INK4 family (p15/INK4B, p16/INK4A, p18/INK4C, and p19/INK4D). These act on cyclin D complexed to CDK4 or CDK6 [15–18].

2 The G1 Phase

In a cell, following extrinsic or intrinsic cell growth signaling, the cyclin/CDK complexes of G1 phase are activated to drive the cell in S phase. This is accomplished by promoting the expression of transcription factors that in turn induce the expression
of S cyclins and of enzymes required for DNA replication. The G1 cyclin/CDK complex-
exes also promote the degradation of molecules that function as S phase inhibitors
by targeting them for ubiquitination, that is, a proteolytic process that degrades pro-
teins. The G1/S transition is the key step for cell cycle progression and is controlled
by D-type cyclins/CDK4, D-type cyclins/CDK6, which act in mid-G1, and by cyclin
E/CDK2, which operates in late G1 [1, 19–23].

Unlike many other cyclins, the level of D-type cyclins (cyclin D1, D2, and
D3) does not change during the cell cycle, but rather their levels are controlled
mainly by mitogens. Several evidences have proved that D-type cyclins are func-
tionally redundant. Nevertheless, each of them has unique tissue-specific functions
[24, 25].

One key substrate of cyclin D/CDK complexes is the nuclear tumor suppressor
pRb (and its related proteins pRb2/p130 and p107), which is phosphorylated on
serine and threonine residues during G1 phase. pRb phosphorylation results in the
liberation of E2F transcription factors, whose activity is required for entry into S
phase [1, 19–23].

There are at least eight members of E2F transcription factor family [26]. In addi-
tion, several E2F isoforms are generated by alternative splicing. E2F1–E2F5 are the
most studied members of this family and have a well-recognized role in cell cycle
regulation through interaction with RB family proteins. These E2F proteins can
recognize specific DNA cis-elements forming heterodimers with partially related
proteins called DP. In this way they activate transcription from genes responsible
for cell cycle control, initiation of replication, and DNA synthesis (DHFR, thymi-
dine kinase, HsOrc1, and DNA polymerase alpha, PCNA, cyclin E, cyclin A, cdc2),
as well as several proto-oncogenes such as c-myb, B-myb, and c-myc [26, 27].

Cell cycle progression is strictly associated with Rb activity. The cell responds to
mitogenic stimuli and progresses through the various phases of the cell cycle only
during a limited phase of its cycle. In fact, the cell needs stimulation only during
the first two-thirds of its G1 phase where it may decide to continue its advance
and complete its cell cycle. This point is termed “restriction point” (R point) or
“checkpoint”; it is a central event in normal cellular proliferation control. It has
been demonstrated that pRb is the molecular device that serves as the R point switch
[28–30].

pRb is hypophosphorylated in resting G0 cells, is increasingly phosphorylated
during progression through G1, and is maintained in a hyperphosphorylated state
until late mitosis [31, 32]. pRb phosphorylation seems to be related to mitogenic
signals, which converge on the cell cycle machinery, represented by the cyclin
D1/CDK4 (CDK6) complex in the early and mid-G1, and composed of cyclin
E/CDK2 in late G1 [1, 20, 22].

The phosphorylation Rb protein releases E2F transcription factors and allows the
expression of proteins required for S phase progression. Among these the expres-
sion of cyclin E determines a positive feedback of Rb phosphorylation, since cyclin
E in complex with CDK2 will continue to phosphorylate Rb, contributing to an
irreversible transition into the S phase [1, 20, 22].

The G1/S progression is regulated also by members of CKI family. In fact, forma-
tion of active complexes among D-type cyclins and CDK4 and CDK6 is influenced
by the INK4 proteins, namely p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. These proteins bind to the catalytic subunits and inhibit the association of D-type cyclins with CDKs [5, 15, 16].

Proteins of the second class of CKI (p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1}, and p57\textsuperscript{KIP2}) are broad-spectrum inhibitors of different cyclin–CDK complexes [10, 15, 16, 33].

Delineating the mode of action for this protein family is more complicated, since these proteins can have a role both in promoting cyclin/CDK complex formation and in inhibiting their activity. It has been observed that p21\textsuperscript{CIP1} levels increase immediately following mitogenic stimulation of serum-starved human fibroblasts, before declining at the G\textsubscript{1}/S boundary [34]. Moreover, the assembly of cyclin D/CDK4 correlates concomitantly with the binding of CIP–KIP proteins and is impaired in mouse embryonic fibroblasts lacking either p21\textsuperscript{CIP1} or p27\textsuperscript{KIP1}. In order to explain this double role of CIP–KIP family member, a titration model has been proposed, where cyclin D/CDK4/6 complexes act as activators of cyclin E/CDK2 complexes by titrating CIP/KIP proteins away from, and thus releasing the inhibition of, cyclin E/CDK2 complexes [2, 15, 16, 33, 35].

Cyclin D/CDK4/6 and cyclin E/CDK2 complexes are regulated by several proteins, including Wee1/Myt1/Mik1 kinases, CDK-activating kinase (CAK), and Cdc25 phosphatase. CAK is a complex that is composed of a catalytic subunit, p40\textsuperscript{MO15}, also called CDK7, a regulatory subunit, cyclin H, and an assembly factor MAT1. CAK adds an activating phosphate to the CDK proteins, while Wee1 adds an inhibitory phosphate. The presence of both activating and inhibitory phosphates inactivates cyclins/CDKs complexes. To activate the complexes, component of the Cdc25 phosphatase family activates CDK/cyclin complexes deleting the Wee1 phosphorylation [16, 36–39].

In summary, biochemical events are protein synthesis and degradation, primarily phosphorylation, dephosphorylation, promoting the complex formation and/or inhibiting its activity, with the overall mission to either prevent or induce a new cell cycle through the Rb pathway.

3 The S and G\textsubscript{2} Phases

In S phase of cell cycle DNA replication occurs. Main regulator of this and related events is the cyclin A. In fact, cyclin A is thought to contribute to the G\textsubscript{1}/S transition, S phase progression, and G\textsubscript{2}/M transition. This protein is expressed at low levels in G\textsubscript{1}, then its levels increase from S phase through G\textsubscript{2}, finally during M phase it declines again [40, 41].

Support for a key role of cyclin A in S phase comes from the observations that microinjection of neutralizing antibodies against cyclin A resulted in a failure to replicate DNA in fibroblasts. Moreover, cyclin A knockout drosophila embryos cannot enter mitosis. Cyclin A associates with two CDKs: CDK2 and Cdc2 (now called CDK1). Cyclin A/CDK2 complex is present in both S and G\textsubscript{2} phase, cyclin A/Cdc2 activity is present only in G\textsubscript{2} [42–44].
Several studies suggest that cyclin A/CDK2 complex is required for S phase progression, and cyclin A/Cdc2 activity is required for G2/M progression. In fact, in cell-free extracts CDK2 is essential for DNA synthesis, while mouse cells expressing temperature-sensitive mutated Cdc2 arrest specifically in G2 phase. A few targets for CDK2 and Cdc2 kinases have been identified; among these are histone proteins. This is in agreement with the observation that cyclin A/CDKs phosphorylate proteins that make up the pre-replication complexes assembled during G1 phase on DNA replication origins. These phosphorylation events activate already assembled pre-replication complex and, at the same time, prevent formation of new complexes to ensure that every region of DNA will be replicated once and only once [2, 42–44].

The RB protein is also a target of cyclin A/CDK complexes. It has been demonstrated that RB, with a mutation in its phosphorylation sites, was capable of blocking progression through S phase, suggesting that the continued hyperphosphorylation of RB could be a key event for cell cycle progression. It is interesting to note that RB represses both cyclin A and Cdc2 expression; this could create a fine “feedback regulation loop” [2, 20, 22].

4 The M Phase

During S and G2 phases are synthesized mitotic cyclin/CDK complexes that are kept inactive till entry into M phase when they promote the initiation of mitosis by stimulating downstream proteins involved in chromosome condensation and mitotic spindle assembly [45, 46].

Cyclin B/Cdc2 complex is the main component of the mitotic promoting factor (MPF), whose activity triggers mitotic entry. The expression of cyclin B changes through the cell cycle: cyclin B is initially synthesized during S phase, increases in G2, and declines by proteasome degradation during the anaphase of mitotic division. Cyclin B1, B2, and B3 are the three cyclin B isoforms so far described [47–50].

The activity of MPF complex is determined through phosphorylation of Cdc2 as well as subcellular localization of the cyclin B/Cdc2 complex. Cyclins B1 and B2 are cytoplasmic till mitotic division, whereas cyclin B3 appears to be nuclear. At the beginning of mitosis, cyclin B1 and B2 translocate to the nucleus prior to nuclear-envelope breakdown, this nuclear localization is necessary for normal cyclin B activity [48, 51, 52].

During G2 phase, the Wee1 and Myt1 kinases phosphorylate and inactivate Cdc2. At mitotic onset, these inhibitory phosphorylations are removed by Cdc25 phosphatases, such as Cdc25A, Cdc25B, and Cdc25C. Also Polo-like kinase-1 protein has a role in mitotic entry through activation of Cdc25C and nuclear translocation of Cdc25C and cyclin B. Moreover, as for other CDKs, CAK phosphorylation is required for complete activation of Cdc2 [40, 53–57].

As described, progression from G2 to M phase is driven by activation of the cyclin B/Cdc2. However, this complex must be active from the prophase to the
metaphase of mitotic division. Subsequent entry into the anaphase critically relies on the sudden destruction of the cyclin B/Cdc2 activity. At the end of the metaphase, the anaphase-promoting complex (APC) destroys cyclin B to allow mitosis to proceed. The APC, a polyprotein complex with ubiquitin ligase activity, recruits cyclin B, causes its ubiquitination, and thus targets it for degradation by the 26S proteasome. To complete mitosis, APC promotes also degradation of structural proteins associated with the chromosomal kinetochore [58–60].

5 Cell Cycle Checkpoints

In normal cells, there are several “checkpoints” that allow the cell to determine if conditions are favorable for the cell cycle to continue. If a cell fails to meet the requirements of a phase it will not be allowed to proceed to the next phase until the requirements have been met. Alternatively, cells may undergo apoptosis with or without growth arrest or enter an irreversible G0 state, called senescence [61, 62].

Several checkpoints are designed to ensure that damaged or incomplete DNA is not inherited by daughter cells. During a life cycle, continuous DNA damages occur into cells. These are caused either by endogenous phenomena, such as the activity of reactive oxygen species (ROS), DNA replication errors, stalling of DNA replication forks, or by exogenous factors, such as ionizing radiation, UV lights, chemicals. Check on DNA status occurs at the end of the G1 phase, G2 phase, and after replication (S phase). Moreover, at the end of the M phase a checkpoint is present to stop cytokinesis in case the chromosomes are not properly aligned on the mitotic spindle (spindle checkpoint). A delay in activation or inactivation of a particular set of cyclin/CDK complex is the way through which checkpoints arrest cell cycle progression [61, 62].

6 The G1 Checkpoint

The strict regulation of CDK phosphorylation on tyrosine and residues is a key event of G1 checkpoint. For example, UV lights induce a phosphorylation on Tyr 17 of CDK4, and this blocks the activity of cyclin/CDK complex and a G0/G1 arrest will occur. During cell cycle, CDK2 is inhibited by phosphorylation on Tyr 15, and Cdc25 phosphatases relieve this block. DNA damaging agents interfere with CDC25 enzymes and maintain CDK2 in its phosphorylated form to arrest cell cycle progression [63–67].

G1 arrest associated with DNA damages is heavily dependent on p53-related pathways. This is a transcription factor that is mutated in a high percentage of human tumors. Several carcinogens that cause DNA damages (such as ionizing radiations) trigger a strong p53 activation and G1 arrest. Cells lacking a functional p53 enter S phase regardless of radiations [68–73].
Fig. 1.1 Schematic diagram of cell cycle. Cyclins/CDKs and some of the proteins involved in their regulation are depicted in the picture. Cyclin/CDK complexes promote cell cycle progression through phosphorylation of several targets. Cyclin/CDK complexes are inactivated by cyclin kinase inhibitors, such as those belonging to INK4 family and to Cip/Kip family. Cyclin kinase activators (CAK) act on cyclin/CDKs and promote cell cycle progression. APC: anaphase-promoting complex.

The promoter of p21\(^{CIP1}\) genes has binding sites for p53 transcription factor. For this reason, p53 activation can induce p21\(^{CIP1}\) gene expression, besides several other genes. p21\(^{CIP1}\) can inhibit the activity of cyclin/CDK complexes acting in G\(_1\) phase and arrest cell cycle. In agreement, the overexpression of p21\(^{CIP1}\) in cycling cells determines G\(_1\) arrest, while cells lacking this protein exhibit a reduced arrest in G\(_1\) phase, following treatment with ionizing radiations [33, 74–76].

Also the proteins of INK4 family play a role in regulation of cell cycle progression. However, it is not clear if they contribute to cell cycle checkpoint.
7 The S Checkpoint

Few minutes following exposure to DNA damaging agents, eukaryotic cells in cultures show a significant reduction in DNA synthesis. In detail, there is a quick suppression of formation of new DNA replicons and, subsequently, the arrest of initiated replicons. The block of DNA synthesis is accomplished through the inhibition of cyclin A/CDK2 complex. ATM gene has a key role in S checkpoint. This gene is mutated in patients suffering from ataxia-telangiectasia. Mutation of ATM causes defective cell cycle checkpoint along with reduced capacity for repair of DNA double-strand breaks and abnormal apoptosis, all of which contribute to the major features of ataxia-telangiectasia including genome instability, increased cancer risk, and neurodegeneration [29, 77–80].

8 The G2 Checkpoint

As for the others, also in G2 checkpoint, regulation of CDK phosphorylation on tyrosine residues is a key event. For example, treatment of cell cultures with DNA damaging agents induces phosphorylation on Thr 14 and Tyr 15 of cdc2 protein, resulting in the inhibition of cyclin B/cdc2 activity. In cell lines harboring mutated cdc2 that cannot be phosphorylated, cell cycle arrest in G2 phase is partially eliminated. The phosphorylation/dephosphorylation of cdc2 relies on a complex pathway including the Cdc25 phosphatases. These proteins have to be phosphorylated to be active on cdc2. In cells treated with DNA damaging agents the Cdc25 enzymes are not phosphorylated, which in turn maintain cdc2 in inactive state and block cell cycle progression [81–85].

Inactivation of cdc2 can be reached also by regulation of cyclin B/cdc2 cellular distribution. During S/G2 phases, this complex is present in cytoplasm, then it is transferred to nucleus as cell traverses G2/M phases. Ionizing radiation treatment of cell cultures can induce an arrest of cell cycle progression and accumulation of cyclin B/cdc2 into cytoplasms [86, 87].

9 The Spindle Checkpoint

This checkpoint ensures proper chromosome segregation, avoiding aneuploidia. The spindle checkpoint delays anaphase onset until all chromosomes are correctly attached in a bipolar fashion to the mitotic spindle. The core spindle checkpoint proteins are Mad and Bub proteins that were identified in budding yeast by genetic screens for mutants that failed to arrest in mitosis when the spindle was destroyed [88–91].

Complex molecular interactions regulate both chromosome attachment and formation of microtubules. Checkpoint proteins can monitor these interactions. In fact,
lack of microtubule attachment elicits the checkpoint response. This is due to a careful screening of tension forces. Tension is established across the sister kinetochores by the pulling forces of the spindle when a chromosome is attached to microtubules from opposite poles. Experiment aiming to the laser ablation of the last unattached kinetochore induced the elimination of the checkpoint-dependent arrest and the cell reached anaphase without a proper completion of mitosis [92–94].

Spindle checkpoint has a major role also in controlling duplication of centrosomes. These organelles duplicate before cells enter M phase. Failure in duplication event can determine polyploidy because most cells return to interphase without division. On the other side, if centrosomes duplicate more than once in a cell cycle, chromosomes are unequally distributed to daughter cells because of multipolar spindle assembly [92–94].

References

Chapter 2
The Basic Helix-Loop-Helix Transcription Factors in Neural Differentiation

Toshiyuki Ohtsuka and Ryoichiro Kageyama

Abstract During the development of the central nervous system, neural stem cells initially expand their own population by symmetric cell divisions, in which both progeny re-enter the cell cycle. By mid-gestation, the cells initiate neurogenesis by adopting a mode of asymmetric cell division, in which one daughter cell differentiates into a neuron while the other continues to cycle in the ventricular zone. Neural stem cells gradually alter their characteristics during development and thus give rise to different types of neurons over time, and finally switch to gliogenesis. The basic helix-loop-helix (bHLH) genes coordinately govern these processes and play a key role in the fate choice and the cell diversity. The repressor-type bHLH gene \( Hes \) is essential for maintenance of neural stem cells. \( Hes \) genes antagonize the activator-type bHLH genes such as \( Mash1, Math, \) and \( Neurogenin (Ngn) \), which induce neuronal differentiation by activating the neuronal-specific genes. The activator-type bHLH genes not only promote the neuronal fate determination but also regulate the neuronal subtype specification. They also induce expression of Notch ligands such as Delta, which activate Notch signaling and upregulate \( Hes1 \) and \( Hes5 \) expression in neighboring cells, thereby maintaining these cells undifferentiated. Thus, the activator-type and repressor-type bHLH genes regulate each other, allowing only subsets of cells to undergo differentiation while keeping others to stay neural stem cells. This regulation is essential for generation of complex brain structures of appropriate size, shape, and cell arrangement.

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1 Introduction

During the development of the central nervous system (CNS), multipotent neural progenitor cells in the ventricular zone (VZ) initially expand their own population by symmetric cell divisions, in which both progeny re-enter the cell cycle. By mid-gestation, the cells initiate neurogenesis by adopting a mode of asymmetric cell division, in which one daughter cell differentiates into a neuron while the other continues to cycle [1, 2]. Many of the progenitor cells in the VZ exhibit characteristics of neural stem cells, which include the capacity to generate many cell types, including neurons, astrocytes, and oligodendrocytes (multipotentiality), and the ability to divide symmetrically to duplicate their own population or divide asymmetrically to continually replenish the progenitor pool (self-renewal) [3, 4].

Neuroepithelial cells are first generated from the ectoderm, forming the neural plate. These cells are neural stem cells and undergo a symmetric cell division to produce more neural stem cells [5, 6]. After neural tube is formed from the neural plate, neuroepithelial cells become radial glia by acquiring some glial features. Radial glia has a cell body in the ventricular zone and long radial fibers extending from the internal surface to the pial (outer) surface. This cell type was long thought as specialized glia that guides neuronal migration along the radial fibers, but recent studies revealed that it is an embryonic neural stem cell [5–9]. Radial glia undergoes many rounds of asymmetric cell divisions, forming one radial glial cell and one neuron (or a neuronal precursor) from each cell division. After production of neurons, radial glial cells finally give rise to glial cells such as oligodendrocytes and astrocytes. Thus, neural stem cells change their characteristics of both morphology and competency over time during development. It takes a certain period of time for neural stem cells to change their characteristics, and maintenance of these cells until late stages is essential to generate all cell types. Premature differentiation allows differentiation of early-born cell types only and disorganizes the shape and cytoarchitecture of the brain. It has been shown that multiple bHLH genes play a critical role in regulation of neural stem cell differentiation [10, 11].

There are two types of bHLH genes, the repressor type and the activator type. The repressor-type bHLH genes include Hes genes, mammalian homologs of Drosophila hairy and Enhancer of split [E(spl)], while the activator-type bHLH genes include Mash1, Math, Neurogenin (Ngn), and NeuroD, homologs of Drosophila proneural genes achaete-scute complex and atonal. Hes genes negatively regulate neuronal differentiation and maintain neural stem cells while Mash1, Math, Ngn, and NeuroD promote neurogenesis. This chapter describes an overview of the roles of bHLH genes in neural differentiation and their significance in brain morphogenesis.
2 Maintenance of Neural Stem Cells by the Repressor-Type bHLH Genes: *Hes*

2.1 Structure and Transcriptional Activities of *Hes* Factors

There are seven members in the *Hes* family. Among them, *Hes1*, *Hes3*, and *Hes5* are highly expressed by neural stem cells in the developing nervous system [12–14]. Hes factors have three characteristic conserved domains: the bHLH domain, the Orange domain (the helix 3-helix 4 domain), and the WRPW (Trp-Arg-Pro-Trp) domain, which are essential for transcriptional activities (Fig. 2.1A).

The bHLH domain in the amino-terminal region is important for dimer formation and DNA binding [12]. bHLH factors form homodimers or heterodimers through the HLH domain and bind to DNA targets via the basic regions. Hes factors have a conserved proline residue in the middle of the basic region (Fig. 2.1A, asterisk), suggesting that this may be involved in the specificity of the target DNA sequences, although the exact significance of this proline residue remains to be determined. Hes1 exhibits a higher binding affinity to the N box (CACNAG) and the class C site (CACGCG) than to the E box (CANNTG) sequence and represses the expression of neurogenic bHLH factors such as Mash1 (active repression, Fig. 2.1B), while other activator-type bHLH factors bind to the E box with a higher affinity (Fig. 2.1D).

Hes factors also have a less conserved region, called the Orange domain [15], located just downstream of the bHLH domain (Fig. 2.1A). This domain is suggested to consist of two amphipathic helices and is known to confer specificity for protein–protein interaction between the bHLH factors [15, 16]. For example, the Hes-related bHLH factor Hairy interacts with the bHLH factor Scute efficiently, while another Hes-related bHLH factor E(spl)m8 does not, and this difference in the interaction specificity is attributed to the Orange domain [15]. This domain is also shown to mediate transcriptional repression [17], although a co-repressor interacting with this domain is not known yet.

Another structural feature is a repression domain called the WRPW domain (Trp-Arg-Pro-Trp sequence) located at or near the carboxyl terminus (Fig. 2.1A). The co-repressor TLE/Grg, a homolog of *Drosophila* Groucho, interacts with the WRPW domain and modifies the chromatin structure by recruiting the histone deacetylase Rpd3, thereby inactivating the chromatin and transcription [18, 19]. It is suggested that Groucho mediates long-range transcriptional repression that can affect over distances of several kilobases in *Drosophila* embryos [20]. *Hes* genes thereby actively repress transcription and thus are classified into the repressor-type bHLH genes.

The target genes for Hes factors include the activator-type bHLH genes such as *Mash1*. Hes1 represses *Mash1* expression by directly binding to the promoter (Fig. 2.1B) [21]. Hes1 can bind to these sites not only as a homodimer (Fig. 2.1Ba) but also as a heterodimer with Hes-related bHLH factors such as Hesr [22] and exerts strong repressor activity (Fig. 2.1Bb). The activator-type bHLH factors form a heterodimer with another bHLH activator E47 (e.g., Mash1–E47 heterodimer)
Fig. 2.1  Structure and transcriptional activities of Hes factors. (A) The conserved domains of Hes factors. The basic, HLH, Orange, and WRPW domains and their functions are indicated. Asterisk indicates a conserved proline residue in the basic region. (B) Active repression: Hes factors bind to the N box or class C site by forming homodimers (a) or heterodimers with Hesr (b) and actively repress transcription by interacting with co-repressors, such as Groucho homologs. (C) Passive repression: Hes factors form non-DNA-binding heterodimers with bHLH activators such as E47 and inhibit transcriptional activation. (D) Activation: activator-type bHLH factors such as Mash1 and E47 form heterodimers that bind to the E box and activate transcription by recruiting co-activators such as CBP/p300.