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A SURVEY OF CELL BIOLOGY

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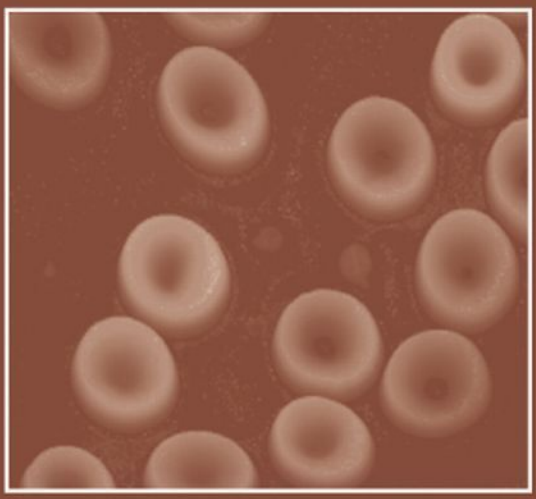
Kwang W. Jeon



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CHAPTER ONE

Cross-Talk Among Integrin, Cadherin, and Growth Factor Receptor: Roles of Nectin and Nectin-Like Molecule

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Abstract

Integrin, cadherin, and growth factor receptor are key molecules for fundamental cellular functions including cell movement, proliferation, differentiation, adhesion, and survival. These cell surface molecules cross-talk with each other in the regulation of such cellular functions. Nectin and nectin-like molecule (Necl) have been identified as cell adhesion molecules that belong to the immunoglobulin superfamily. Nectin and Necl play important roles in the integration of integrin, cadherin, and growth factor receptor at the cell–cell adhesion sites of contacting cells and at the leading edges of moving cells, and thus are also involved in the fundamental cellular functions together with integrin, cadherin, and growth factor receptor. This chapter describes how newly identified cell adhesion molecules, nectin and Necl, modulate the cross-talk among integrin, cadherin, and growth factor receptor and how these integrated molecules act in the regulation of fundamental cellular functions.

Key Words: Cadherin, Cell functions, Growth factor receptor, Integrin, Necl, Nectin.

1. Introduction

Cell movement, proliferation, adhesion, differentiation, and survival are fundamental cellular functions in multicellular organisms (Gumbiner, 1996; Guo and Hay, 1999; Lauffenburger and Horwitz, 1996; Lin and Bissell, 1993; Perez-Moreno *et al.*, 2003; Vaux and Korsmeyer, 1999). Cell adhesion molecules (CAMs) including integrin and cadherin and growth factor receptors such as platelet-derived growth factor (PDGF) receptor play pivotal roles in these cellular functions. In addition to these CAMs and growth factor receptors localized at the surface of cells, a number of intracellular signaling molecules, which are positively and negatively controlled by extracellular stimuli through these CAMs and receptors, are also involved in the regulation of the cellular functions. The roles of CAMs and receptors and their related intracellular signalings are mutually well organized to maintain the cellular functions and are thus essential for living of not only the cells but also multicellular organisms. The disruption of the organized functions of CAMs and receptors causes the cellular dysfunction and may lead to pathological, even more life-threatening, disorders of individuals.

On the other hand, cell differentiation and survival are directly related to the cell fate. These phenomena are known to be mainly regulated by the growth factor-induced signals (Birling and Price, 1995; Pettmann and Henderson, 1998). However, it has been demonstrated that integrin is also involved in the differentiation of certain tissues such as the epidermis (Watt, 2002). Moreover, cell adhesion to the substratum is indispensable for the survival of cells, and in turn, loss of cell–matrix or cell–cell adhesion causes cell death by apoptosis or anoikis (Bergin *et al.*, 2000; Fouquet *et al.*, 2004; Hofmann *et al.*, 2007; Ruoslahti and Reed, 1994). Thus, cell differentiation and survival are also correlated with the relationship between growth factors and CAMs.

Cultured normal cells move and proliferate until they become confluent and form cell–cell junctions. After the establishment of cell–cell junctions, they arrest cell movement and proliferation. This phenomenon was identified over a half century ago as contact inhibition of cell movement and proliferation (Abercrombie and Heaysman, 1953; Farquhar and Palade, 1963). In contrast, transformed cells abnormally continue to move and proliferate even after they contact each other, and thus contact inhibition of transformed cells is disrupted, resulting in the enhancement of their invasiveness into neighboring tissues and metastasis to other organs. Although CAMs and receptors essentially act in cell movement and proliferation and their contributions to cell movement and proliferation have been extensively studied for a long time (Benito and Lorenzo, 1993; Lauffenburger and Horwitz, 1996), the molecular mechanism for contact inhibition of cell movement and proliferation has been poorly elucidated to date.

Cell adhesion includes cell–cell and cell–matrix junctions. At cell–matrix junctions, integrin is a key CAM and comprises heterodimers with α and β subunits (Geiger *et al.*, 2001). To date, 18 α subunits and 8 β subunits have been identified and 25 of their combinations were reported (Kinbara *et al.*, 2003). It is involved in the formation of focal adhesions and focal complexes, which are specialized subcellular apparatuses including several signaling molecules that transduce integrin-initiated outside-in signals and also modulate the affinity of integrin to the extracellular matrix (inside-out signals). Integrin is also reported to be important for cell movement, proliferation, and differentiation as well as cell adhesion (Geiger *et al.*, 2001; Kinbara *et al.*, 2003; Watt, 2002). On the other hand, the molecular mechanism of cell–cell junctions has been well characterized in epithelial cells. Epithelial cells contain at least four types of junctional apparatuses: tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions, all of which collectively form junctional complexes at the intercellular adhesion sites. TJs are localized at the most apical side of cell–cell junctions. Two major functions of TJs are to act as a barrier, preventing the passage of soluble molecules through the gaps between cells, and as a fence, keeping the cell surface proteins and lipids in the basolateral region separate from those in the

apical region (Tsukita and Furuse, 2002; Tsukita *et al.*, 1999). However, the fence function of TJs is controversial, because the apical marker proteins and the basolateral ones are normally distributed on the plasma membrane even in the epithelial cells in which TJs are completely disrupted (Umeda *et al.*, 2006). AJs serve as a mechanically adhesive apparatus between neighboring cells. The functions of AJs and TJs are mainly regulated by several CAMs and their related peripheral membrane proteins. Claudin, occludin, and junctional adhesion molecule (JAM) are major Ca^{2+}-independent CAMs located at TJs (Tsukita *et al.*, 1999), while at AJs, E-cadherin and nectin are major Ca^{2+}-dependent and -independent CAMs, respectively (Takai and Nakanishi, 2003; Takeichi, 1991). The claudin and cadherin families consist of a large number of members: more than 20 and 80 members, respectively (Mitic *et al.*, 2000; Yagi and Takeichi, 2000). The JAM family is also composed of four members and JAM-like molecule (Ebnet *et al.*, 2004), but occludin-related genes have not been identified yet (Tsukita *et al.*, 2001). There is a cross-talk between cell–cell and cell–matrix junctions and the formations of both types of junctions are mutually regulated (Pignatelli, 1998; Siu and Cheng, 2004). We mainly describe the features of AJs and TJs in this chapter. For the concerns on desmosomes and gap junctions, other excellent reviews would be helpful (Garrod *et al.*, 2002; Kumar and Gilula, 1996).

Although the molecular mechanisms of the cellular functions including cell movement, proliferation, adhesion, differentiation, and survival have been individually studied, the integrated investigation of these cellular functions is quite important to understand the whole cell architecture. Moreover, the integrated and harmonized regulation of these cellular functions is indispensable for living of multicellular organisms. Thus, in this chapter, we focus on the newly identified CAMs nectin and nectin-like molecule (Necl), of which the molecular structure is similar to that of nectin, and describe the molecular mechanism of the cross-talk between CAMs and growth factor receptors.

2. Molecular and Structural Features of Nectin and Nectin-Like Molecule

Nectin is a Ca^{2+}-independent cell–cell adhesion molecule with three immunoglobulin (Ig)-like loops at its extracellular region, single transmembrane segment, and one intracellular region (Takai *et al.*, 2003; Takai and Nakanishi, 2003) (Fig. 1.1A). To date the nectin family consists of four members: nectin-1, nectin-2, nectin-3, and nectin-4. The genetic distance of the nectin and Necl (see later) family members is estimated by construction of a phylogenetic tree (Fig. 1.1B). Each nectin member has two or

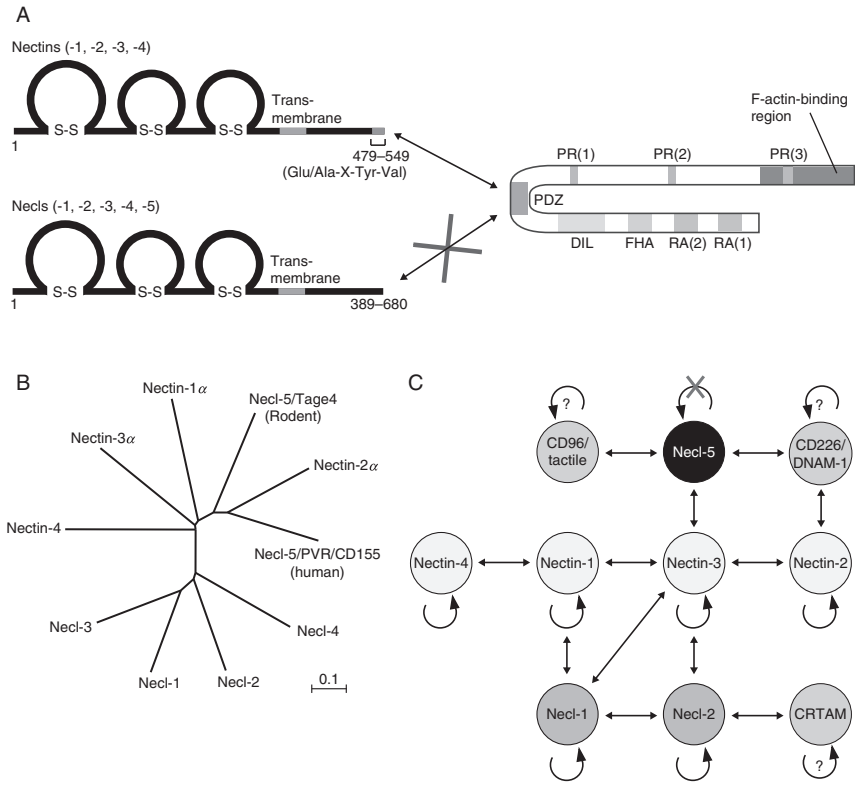


Figure 1.1 Molecular structures of nectins, Necls, and afadin and their interactions. (A) Schematic representatives of molecular structures of nectins, Necls, and afadin. Nectins and Necls both contains three immunoglobulin-like loops in their extracellular region, one membrane-spanning region, and one cytoplasmic region. Nectin family members, except nectin-4, possess a consensus motif of four amino acids (E/A-X-Y-V) for interaction with afadin. Nectin-4 has another C-terminal motif, but also binds afadin. Afadin has two Ras-association (RA) domains, a forkhead-associated (FHA) domain, a DIL domain, a PDZ domain, three proline-rich (PR) domains, and an F-actin-binding domain. Direct binding between nectins and afadin is conducted through the C-terminal motif of nectins and the PDZ domain of afadin, which links nectin to the actin cytoskeleton. (B) Phylogenetic analysis of nectins and Necls. The nectin and Necl amino acid sequences were aligned using the CLASTALW program and a phylogenetic tree was constructed using tree-drawing software (TreeViewPPC). Branch lengths are drawn to scale and the longer branches represent more genetic changes. (C) Homophilic and heterophilic transinteractions among nectins, Necls, and other immunoglobulin-like molecules. Only known homophilic (looped arrows) and heterophilic (double arrows) interactions are indicated in this figure.

three alternative splicing isoforms. For nectin-1, there are three isoforms: nectin-1α, nectin-1β, and nectin-1γ; nectin-2 is composed of two isoforms: nectin-2α and nectin-2δ; nectin-3 has three isoforms: nectin-3α, nectin-3β, and nectin-3γ; and nectin-4 also contains two splicing isoforms, whose

name has not yet been determined (Reymond *et al.*, 2001; Satoh-Horikawa *et al.*, 2000; Takahashi *et al.*, 1999). Among these isoforms, only nectin-1γ lacks the transmembrane and cytoplasmic regions and is secreted from its producing cells.

Nectin-1α and nectin-2α were originally isolated as the poliovirus receptor-related proteins, PRR-1 and PRR-2, respectively (Aoki *et al.*, 1997; Eberle *et al.*, 1995; Lopez *et al.*, 1995; Morrison and Racaniello, 1992). Actually, neither of them functions as a receptor for poliovirus entry, and later, they have been identified as receptors for α-herpes virus (HSV-1, HSV-2, and pseudorabies virus) entry, helping its infection into animal cells and tissues (Geraghty *et al.*, 1998; Warner *et al.*, 1998). Thus, they were renamed herpes virus entry mediator C (HveC) and B (HveB), respectively. Nectin-1, nectin-2, and nectin-3 are widely expressed in adult tissues and various kinds of cells including fibroblasts, epithelial cells, and neurons, together with another cell–cell adhesion molecule cadherin. Nectin-2 and nectin-3 are also expressed in cells that lack cadherin, such as hematopoietic cells and cells in the testis (Lopez *et al.*, 1998; Ozaki-Kuroda *et al.*, 2002). On the other hand, nectin-4 is expressed mainly in placenta in humans, while it is broadly expressed in mouse tissues and is also detected in mouse embryo (Reymond *et al.*, 2001). Moreover, the production of nectin-4 is highly upregulated in breast cancer (Fabre-Lafay *et al.*, 2005). The functions of nectins are represented in Table 1.1.

Each nectin first forms homo-*cis*-dimers similar to cadherin, and then makes the formation of homophilic or heterophilic *trans*-dimers (*trans*-interaction) in contrast to cadherin, which *trans*-interacts only homophilically **(**Fig. 1.1C). Nectin-1 heterophilically *trans*-interacts with nectin-3 and nectin-4, and nectin-2 also *trans*-interacts with nectin-3. These heterophilic *trans*-interactions exhibit a significantly higher affinity than the homophilic *trans*-interactions. For example, the K_d values of nectin-3 for nectin-1 and nectin-2 are 2.3 nM and 360 nM, respectively, as estimated by surface plasmon resonance analysis (Ikeda *et al.*, 2003). Nectin-2 also *trans*-interacts with CD226/DNAM-1 in addition to the nectin family members. CD226/DNAM-1 is a single membrane-spanning molecule possessing two Ig-like loops at its extracellular region and it supports the differentiation and proliferation of T cells in which this molecule is mainly expressed (Chen *et al.*, 2003; Shibuya *et al.*, 2003). The first Ig-like loop at the extracellular region of nectin is necessary for the formation of the *trans*-dimers, but not for *cis*-dimers, whereas the second Ig-like loop contributes to the formation of *cis*-dimers (Momose *et al.*, 2002; Yasumi *et al.*, 2003). The function of the third Ig-like loop is currently unknown. Intermolecular force microscopy (IFM) measurement has revealed that these multiple tandem aligned Ig-like loops of nectin act uncooperatively, as a zipper-like multiply bonded system, whereas five-tandemly-repeated ectodomains of cadherin act cooperatively, as a parallel-like multiply bonded system (Tsukasaki *et al.*, 2007).

Table 1.1 Roles of nectin and Necl family members

	Old nomenclature	Functions
Nectin-1	PRR-1/HveC	Cell–cell adhesion molecule Receptor for α-herpes virus (HSV-1, HSV-2, and pseudorabies virus) entry into cells Defects in humans: cleft lip/palate-ectodermal dysplasia syndrome, also known as Zlotogora–Ogur syndrome Knockout mice: microphthalmia, skin abnormalities, and abnormal mossy fiber trajectories in the hippocampus
Nectin-2	PRR-2/HveB	Cell–cell adhesion molecule Receptor for α-herpes virus entry into cells Knockout mice: male-specific infertility
Nectin-3	PRR-3	Cell–cell adhesion molecule Knock-out mice: male-specific infertility, microphthalmia, and abnormal mossy fiber trajectories in the hippocampus
Nectin-4		Cell–cell adhesion molecule Overexpressed in breast carcinoma
Necl-1	TSLL1/ SynCAM3	Cell–cell adhesion molecule Neural tissue-specific expression Localized at contact sites between axons and glial cells or Schwann cells, not at synaptic junctions
Necl-2	IGSF4/RA175/ SgIGSF/ TSLC1/ SynCAM1	Cell–cell adhesion molecule Localized on the basolateral membranes in epithelia Involved in spermatogenesis and synapse formation Tumor suppressor in lung carcinoma
Necl-3	Similar to Necl3/ SynCAM2	Putative cell–cell adhesion molecule
Necl-4	TSLL2/ SynCAM4	Cell–cell adhesion molecule Possible involvement in tumor suppression
Necl-5	Tage4/PVR/ CD155	Enhancement of cell movement and proliferation cooperatively with integrin $\alpha_v\beta_3$ and PDGF receptor Overexpressed in various cancer cells Receptor for poliovirus

The IFM system is one of the cutting-edge technologies in the field of biophysics and has force resolution to the subpicoNewton level with a response time at the submillisecond level, an approximately several tenfold higher force sensitivity than conventional atomic force microscopy. The results obtained from the IFM system are consistent with the previous analysis on nectin.

All the nectin family members directly bind afadin, which links nectin to the actin cytoskeleton as catenins connect cadherin to the actin cytoskeleton (Takahashi *et al.*, 1999). This binding is mediated by a C-terminal four amino acid conserved motif of nectin (E/A-X-Y-V; X is any amino acid) and the PDZ domain of afadin. Although nectin-4 has a C-terminal motif (G-H-L-V) different from the other nectin family members, it also directly interacts with the PDZ domain of afadin through its C-terminus (Reymond *et al.*, 2001). Afadin has two splicing variants, l-afadin and s-afadin/AF-6 (Mandai *et al.*, 1997) (Fig. 1.1A). Both afadin variants share the two Ras-associated (RA) domains, with which activated Rap1 small G protein interacts, a forkhead-associated (FHA) domain, a dilute (DIL) domain, and two proline-rich (PR) domains. Only l-afadin, a larger variant, contains an actin-filament (F-actin)-binding domain and the third PR domain, whereas s-afadin/AF-6, a smaller variant, lacks these domains and is thus unable to interact with F-actin. l-Afadin is broadly expressed in tissues, but the expression of s-afadin is relatively specific in the brain. The human *s-afadin/AF-6* gene was originally identified as an *ALL-1* fusion partner involved in acute myeloid leukemias (Prasad *et al.*, 1993). It has also been reported that s-afadin/AF-6 directly binds to a subset of Eph receptor tyrosine kinases (Buchert *et al.*, 1999; Hock *et al.*, 1998) and interacts with a deubiquitinating enzyme, Fam (Taya *et al.*, 1998). Hereafter, unless otherwise specified, afadin refers to l-afadin in this chapter.

Necl is genetically and structurally similar to nectin; it has an extracellular region with three Ig-like loops, a membrane-spanning region, and a short cytoplasmic tail **(**Fig. 1.1A and B). Necl has a Ca^{2+}-independent cell adhesion activity like nectin but does not bind afadin at its C-terminal cytoplasmic tail (Takai *et al.*, 2003). Each Necl family member also exhibits several other cellular functions in addition to cell adhesion activity as summarized in Table 1.1. The Necl family is composed of five members: Necl-1 (TSLL1/SynCAM3), Necl-2 (IGSF4/RA175/SgIGSF/TSLC1/SynCAM1), Necl-3 (similar to Necl3/SynCAM2), Necl-4 (TSLL2/SynCAM4), and Necl-5 (Tage4/PVR/CD155). Necl-1 homophilically *trans*-interacts with itself and heterophilically *trans*-interacts with nectin-1, nectin-3, and Necl-2, but not nectin-2 or Necl-5 (Kakunaga *et al.*, 2005) (Fig. 1.1C). Necl-2 also homophilically *trans*-interacts and heterophilically *trans*-interacts with nectin-3 and Necl-1 and another Ig-like molecule CRTAM, which is reported to enhance the cytotoxicity of natural killer (NK) cells (Boles *et al.*, 2005; Kennedy *et al.*, 2000; Shingai *et al.*, 2003).

On the other hand, Necl-5 does not homophilically *trans*-interact but heterophilically *trans*-interacts with nectin-3 and other Ig-like molecules CD96/Tactile and CD226/DNAM-1 (Bottino *et al.*, 2003; Fuchs *et al.*, 2004; Ikeda *et al.*, 2003; Mueller and Wimmer, 2003). CD96/Tactile, a member of the Ig superfamily, is expressed in T cells and promotes adhesion of T cells to target cells expressing Necl-5, triggering T cell activation (Fuchs *et al.*, 2004; Wang *et al.*, 1992). The binding partners of Necl-3 and Necl-4 have not been identified yet.

Although the general properties of Necl-3 and Necl-4 remain to be elucidated, the roles of Necl-1, Necl-2, and Necl-5 have partly been discovered. Necl-1 is specifically expressed in the neural tissue and localizes at the contact sites between two axon terminals, between an axon terminal and an axon shaft, and between an axon terminal and glia cell processes in the cerebellum (Kakunaga *et al.*, 2005). In the peripheral myelinated nerve fibers, Necl-1 localizes at the contact sites between the cellular processes of Schwann cells at the nodes of Ranvier. Necl-2 is expressed in various tissues (Shingai *et al.*, 2003) and acts as a tumor suppressor in human non-small cell lung cancer (Kuramochi *et al.*, 2001). In normal epithelial cells, Necl-2 localizes at the basolateral portion of the cell–cell adhesion sites, but not at the cell–cell junctional apparatus, such as TJs, AJs, or desmosomes. Human PVR/CD155 was originally identified as the human poliovirus receptor (Koike *et al.*, 1990; Mendelsohn *et al.*, 1989), while rodent Tage4 was originally identified as the product of a gene overexpressed in rodent colon carcinoma (Chadeneau *et al.*, 1994). PVR/CD155 was subsequently shown to be overexpressed in many human cancer cells (Masson *et al.*, 2001; Sloan *et al.*, 2004). For a long time, the physiological role of Necl-5 has been largely unclear; however, its various cellular functions have been clarified. A detailed description is given later in this chapter.

3. Formation of Adherens Junctions Induced by the Nectin–Afadin System

3.1. Interaction between the nectin–afadin and cadherin–catenin systems

The nectin–afadin system physically associates with the cadherin–catenin system and both cell–cell adhesion systems cooperatively promote the formation of AJs (Nakanishi and Takai, 2004; Takai and Nakanishi, 2003). Cadherins are key Ca^{2+}-dependent CAMs and are classified in several groups, such as classical cadherins, desmosomal cadherins, and protocadherins (Takeichi, 1991; Yagi and Takeichi, 2000). Classical cadherins of the cadherin superfamily, such as E-cadherin and N-cadherin, play an

essential role in cell–cell adhesion at AJs. The extracellular region of E-cadherin, which is the best characterized cadherin and is exclusively expressed in epithelial cells, contains five-tandemly-repeated ectodomains, EC1–EC5. E-cadherin homophilically *trans*-interact with each other in a Ca^{2+}-dependent manner. At the cytoplasmic region of E-cadherin, it directly binds β-catenin or γ-catenin (plakoglobin) through its C-terminal tail and $p120^{ctn}$ through its juxtamembrane portion (Anastasiadis and Reynolds, 2000; Takeichi, 1995). β-Catenin also directly interacts with α-catenin, which then binds to F-actin and F-actin-binding proteins, such as α-actinin and vinculin. Thus, cadherin is anchored to the actin cytoskeleton through several peripheral membrane proteins including catenins.

The mode of physical association between the nectin–afadin and cadherin–catenin systems has largely been revealed by more recent studies (Asada *et al.*, 2003; Mandai *et al.*, 1999; Ooshio *et al.*, 2004) (Fig. 1.2). Evidence has accumulated that nectin recruits cadherin to the nectin-based cell–cell adhesion sites through their cytoplasm-associated proteins, afadin and catenins, in the formation of AJs. Both afadin and α-catenin are involved in this physical association by the direct interaction of afadin with α-catenin, although the affinity of these two molecules is not high (Pokutta *et al.*, 2002; Tachibana *et al.*, 2000). The direct binding of these proteins may occur *in vivo*, but it is more likely that a posttranslational modification(s) of either or both proteins and/or an unidentified molecule(s) are required for the

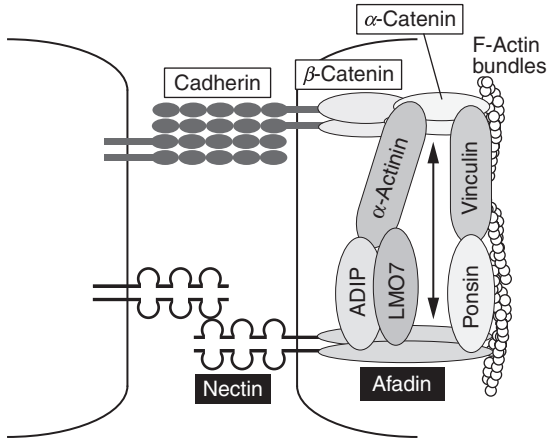


Figure 1.2 **Association of the nectin–afadin system with the cadherin–catenin system. After nectins first transinteract at the initial cell–cell contacts, three connector units beside the actin cytoskeleton are involved in the recruitment of the cadherin–catenin system to the nectin-based cell–cell adhesion sites. The first connector unit is the ponsin–vinculin unit, the second is the ADIP–α-actinin unit, and the third is the LMO7–α-actinin unit. Moreover, the direct interaction of afadin with α-catenin is assumed, but this interaction does not seem to be strong.**

efficient binding between afadin and α-catenin (Pokutta *et al.*, 2002; Tachibana *et al.*, 2000). To date, three putative units that link the nectin–afadin complex to the cadherin–catenin complex have been identified. The first one is a ponsin–vinculin unit (Mandai *et al.*, 1999). Ponsin, an afadin- and vinculin-binding protein, and vinculin, an F-actin-binding protein localized at AJs and focal adhesions, colocalize with nectin and afadin at AJs. Vinculin directly binds to α-catenin (Aberle *et al.*, 1996). Thus, vinculin and ponsin are involved in the connection of nectin and cadherin. Although ponsin forms a binary complex with either afadin or vinculin, it does not form a ternary complex with afadin and vinculin, probably due to the competitive interaction of ponsin with afadin or vinculin. The reason why ponsin, afadin, and vinculin do not form a ternary complex remains to be clarified, but ponsin may regulate the linkage between afadin and vinculin to promote the connection between nectin and cadherin. The second one is an afadin DIL-domain-interacting protein (ADIP)–α-actinin unit (Asada *et al.*, 2003). ADIP has been identified as an afadin- and α-actinin-binding protein by yeast two-hybrid screening and the direct binding of ADIP with afadin and α-actinin is further confirmed by the immunoprecipitation assay and the experiments using these recombinant proteins. In addition, the localization of ADIP is limited at AJs in epithelial cells of small intestine. Because α-actinin is known to associate with E-cadherin through α-catenin, ADIP is likely to connect the nectin–afadin system to the cadherin–catenin system through α-actinin. However, it is unclear whether ADIP forms a ternary complex with afadin and α-actinin. The third one is an LIM domain only 7 (LMO7)–α-actinin unit (Ooshio *et al.*, 2004). An immunoprecipitation assay has revealed that LMO7 associates with afadin and α-actinin. LMO7 also colocalizes with afadin at AJs in epithelial cells. LMO7 is assembled at AJs after the nectin-induced formation of cadherin-based AJs is established at the cell–cell adhesion sites. Thus, LMO7 does not appear to function as a molecule that recruits the cadherin–catenin system to the nectin–afadin system; it may, rather, stabilize both systems at the cell–cell adhesion sites by connecting them. The relationship among these connector units in the interaction between the nectin–afadin and cadherin–catenin systems has not been fully elucidated.

3.2. Cross-talk between nectin and integrin

We have described in the previous section that the nectin–afadin system is linked to the cadherin–catenin system through several molecules including ponsin, vinculin, ADIP, LMO7, and α-actinin. This linkage is important for the prompt recruitment of cadherin to the nectin-based cell–cell adhesion sites and the efficient formation of AJs. Nectin also interacts with a cell–matrix adhesion molecule integrin in the formation of cell–cell adhesion (Sakamoto *et al.*, 2006). Several studies indicate that there is cross-talk between

cell–cell and cell–matrix junctions (Pignatelli, 1998; Siu and Cheng, 2004). Cell–matrix junctions are formed by interactions of integrins with extracellular matrix (ECM) proteins, such as collagen, fibronectin, laminin, and vitronectin (Geiger *et al.*, 2001; Jin and Varner, 2004). The integrin-mediated cell–matrix junctions positively or negatively regulate the formation and stability of cell–cell junctions through protein tyrosine kinases associated with integrins, such as FAK and c-Src (Geiger *et al.*, 2001; Parsons, 2003). As an example, this regulation may be important for the epithelial–mesenchymal transition of epithelial cells in both physiological and pathological states (Monier-Gavelle and Duband, 1997; Schreider *et al.*, 2002). During embryonic development, integrins β_1 and β_3 promote epithelial cell remodeling, which appears to be related to reduced interactions of CAMs at AJs with the cytoskeleton (Monier-Gavelle and Duband, 1997). In a colon cancer cell line, the attachment of integrin β_1 to ECM proteins induces functional polarization of the cells and reinforces the E-cadherin-based AJs (Schreider *et al.*, 2002). Moreover, the integrin-induced signaling molecules FAK and paxillin have been shown to regulate the N-cadherin-based cell–cell adhesion in HeLa cells (Yano *et al.*, 2004). These data support the importance of cross-talk between cell–cell and cell–matrix junctions with physiological and pathological relevance.

It has been revealed that the cross-talk between the cell–cell adhesion molecule nectin and cell–matrix adhesion molecule integrin is essential for the formation of AJs (Sakamoto *et al.*, 2006). Integrin $\alpha_v\beta_3$ and nectin physically interact with each other at the nectin-based cell–cell adhesion sites through their extracellular regions. Integrin $\alpha_v\beta_3$ has at least two forms: the low- and high-affinity forms (Takagi *et al.*, 2002). The low-affinity form shows weak adhesion activity for extracellular matrix proteins and is inactive, whereas the high-affinity form exhibits increased adhesion activity for its extracellular ligands and is active (Calderwood, 2004). Binding of talin, an actin-binding protein, to the cytoplasmic tail of the β_3 subunit of integrin is one of the mechanisms with which to activate integrin through inside-out signaling (Tadokoro *et al.*, 2003). At the initial step of the formation of AJs, nectin associates with the high-affinity form of integrin $\alpha_v\beta_3$, which is then gradually converted into the low-affinity form by the establishment of AJs (Ozaki *et al.*, 2007; Sakamoto *et al.*, 2006). Because nectin is capable of associating with both the low- and high-affinity forms of integrin $\alpha_v\beta_3$ as estimated by the immunoprecipitation assay, nectin always colocalizes and interacts with integrin from the initial to the final stage of the formation of AJs. Given that the high-affinity form of integrin $\alpha_v\beta_3$ upregulates cell movement and proliferation, which tend to disrupt cell–cell adhesion, it may be difficult to keep AJs for a long time if integrin $\alpha_v\beta_3$ continues to be activated even after the establishment of AJs. Thus, inactivation of integrin $\alpha_v\beta_3$ after the formation of AJs seems to be physiologically reasonable to maintain the nectin- and cadherin-based AJs. The mature transinteraction of

nectin is involved in this integrin inactivation. Nectin associates with and induces the activation of the phosphatase PTPμ at cell–cell adhesion sites (Sakamoto *et al.*, 2007). Activated PTPμ inhibits phosphatidylinositol phosphate kinase type Iγ90 (PIPKIγ90) after the formation of cell–cell junctions. PIPKIγ90 is shown to be involved in the activation of integrin by increasing the generation of phosphatidylinositol 4,5-bisphosphate and promoting the binding of talin to integrin (Martel *et al.*, 2001; Di Paolo *et al.*, 2002; Ling *et al.*, 2002). Thus, apart from the functions during the formation of cell–cell adhesion, nectin negatively regulates PIPKIγ90 through the PTPμ and inhibits integrin $\alpha_v\beta_3$ after the formation of cell–cell junctions.

3.3. Cooperative roles of nectin and integrin $\alpha_v\beta_3$ in intracellular signaling

Nectin induces the activation of intracellular signaling and formation of AJs in cooperation with activated integrin $\alpha_v\beta_3$ (Sakamoto *et al.*, 2006). Detailed investigations have largely uncovered this signaling (Ogita and Takai, 2006; Shimizu and Takai, 2003) (Fig. 1.3). At the beginning of the formation of AJs, nectin *trans*-interacts with each other to form the primordial cell–cell adhesion. This *trans*-interaction of nectin first induces the activation of c-Src, which is also regulated by activated integrin $\alpha_v\beta_3$ and its downstream signaling molecules protein kinase C (PKC) and FAK (Ozaki *et al.*, 2007; Sakamoto *et al.*, 2006). Thus, the activation of c-Src is doubly controlled by nectin and integrin $\alpha_v\beta_3$. c-Src is activated in this way, then tyrosine phosphorylates FRG, a GDP/GTP exchange factor (GEF) for Cdc42, and Vav2, a GEF for Rac, and induces the activation of Rap1 small G protein through an adaptor protein Crk and a Rap1-GEF C3G (Fukuhara *et al.*, 2004, Fukuyama *et al.*, 2005; Kawakatsu *et al.*, 2005). Activated Rap1 fully activates phosphorylated FRG, resulting in the activation of Cdc42 and the formation of filopodia. Similarly, activated Cdc42 also enhances the activation of phosphorylated Vav2 and eventually induces the activation of Rac and the formation of lamellipodia. The cell protrusions such as filopodia and lamellipodia formed by this signaling contribute to facilitate the formation of cell–cell junctions, because filopodia increase the contact sites between neighboring cells and lamellipodia efficiently zip up the gaps between these contact sites. On the other hand, activated Cdc42 and Rac reorganize the actin cytoskeleton and are involved in the recruitment of the cadherin–catenin complex to the nectin-based cell–cell adhesion sites through F-actin-binding proteins such as IQGAP1 (Fukuhara *et al.*, 2003; Sato *et al.*, 2006). An increased number of cadherin clusters at the cell–cell adhesion sites and finally the formation of AJs is established. These F-actin-binding proteins primarily play a pivotal role in cell movement. In addition, when afadin does not bind to nectin in moving cells, it accumulates at the leading edge and is involved in the local activation of Rap1

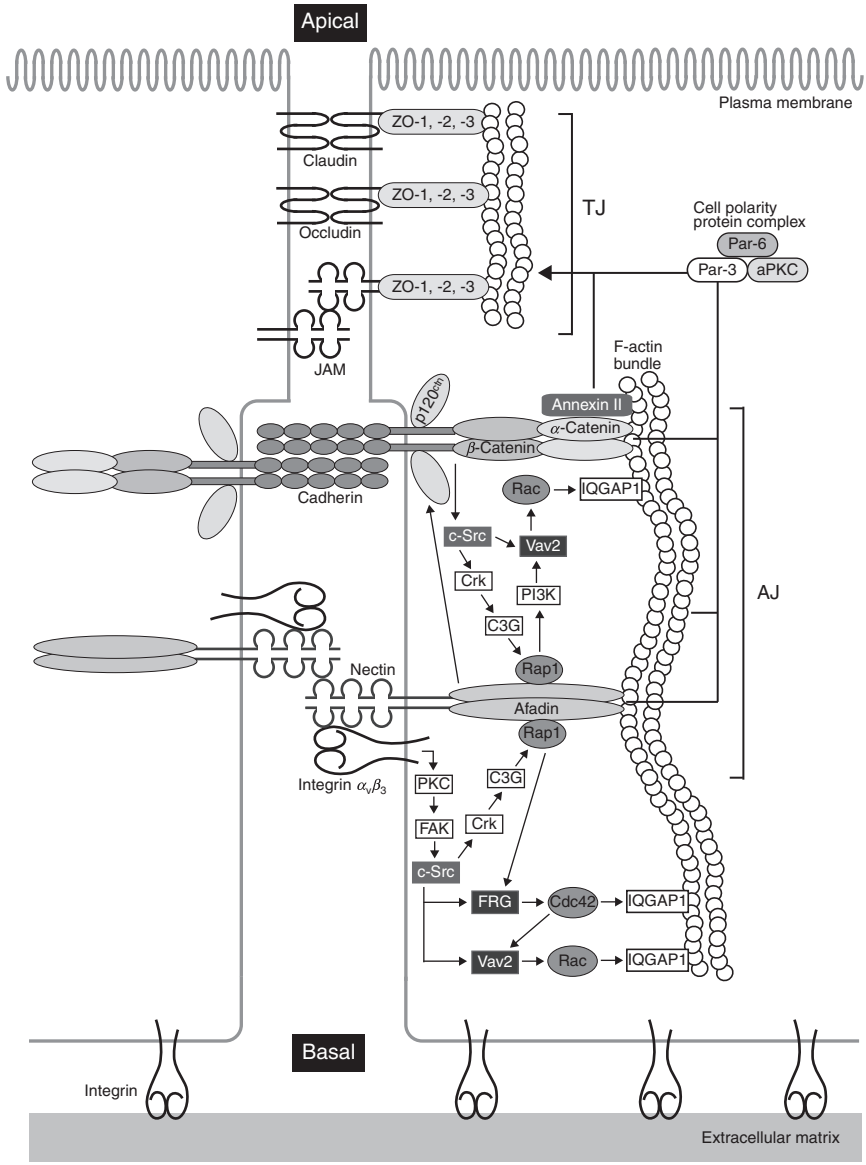


Figure 1.3 Nectin- and cadherin-induced formation of adherens junctions (AJs) and tight junctions (TJs). *Trans*-interacting nectin at the initial cell–cell adhesion induces the activation of Rap1, Cdc42, and Rac mediated by c-Src, Crk, C3G, FRG, and Vav2. This nectin-induced signaling is dependent on integrin $\alpha_v\beta_3$, which physically associates with nectin, and its downstream signaling molecules PKC and FAK. Activated Cdc42 and Rac reorganize the actin cytoskeleton and recruit the cadherin–catenin system to the nectin-based cell–cell adhesion sites. At this phase, cadherin has only weak adhesion activity. However, afadin interacting with activated Rap1 also associates with $p120^{ctn}$, leading to increased adhesion activity of cadherin and the establishment of

and Rac there (unpublished data), increasing the formation of the leading edge structure and the velocity of cell movement. Taken together, the molecules related to cell movement at the leading edge also participate in the formation of cell–cell junctions, indicating the relationship between cell movement and cell–cell adhesion.

A more recent study has explored the phenomenon that E-cadherin and its associating proteins, such as α-catenin, β-catenin, and $p120^{ctn}$, are not recruited to the nectin-based cell–cell adhesion sites in Madin–Darby canine kidney (MDCK) cells during the Ca^{2+} assay in the presence of a protein synthesis inhibitor, cycloheximide, or a proteasome inhibitor, *N*-acetyl-Leu-Leu-norleucinal (ALLN) (Yamada *et al.*, 2005). This suggests that degradation of one or more protein factor(s) and *de novo* synthesis of the same or other proteins factor(s) are necessary for the formation of E-cadherin-based AJs. The biochemical experiment has identified the annexin II-S100A10 complex as at least one of the protein factors by analysis of two-dimensional gel electrophoresis. Annexin II, also called calpactin I heavy chain, is a member of the annexin family of Ca^{2+}- and phospholipid-binding protein and usually forms a heterotetrameric complex with S100A10, also called calpactin I light chain (Gerke and Moss, 2002). Annexin II binds to F-actin in a Ca^{2+}- and phospholipid-dependent manner. Actually, the assembly of E-cadherin and its associating proteins is not observed at the nectin-based cell–cell adhesion sites in annexin II-knockdown MDCK cells and the formation of AJs is impaired during the Ca^{2+} switch experiment in these cells. During the formation of AJs, annexin II cooperates with other actin-binding proteins such as IQGAP1 and α-catenin and essentially functions in the formation of AJs (Yamada *et al.*, 2006). Taken together, the first step of the reorganization of the actin cytoskeleton occurs by the *trans*-interaction of nectin at the initial cell–cell contact sites (Fig. 1.4A). Next, the nectin-induced activation of Cdc42 and Rac and their effecter IQGAP1 contribute to the second step of the reorganization of the actin cytoskeleton and recruit the cadherin–catenin complex to the nectin-based cell–cell adhesion sites (Fig. 1.4B). Then, accumulating cadherin at the cell–cell adhesion sites *trans*-interacts with each other and this *trans*-interaction of cadherin finally builds up the third step of the reorganization of the actin cytoskeleton and the firm F-actin bundles in cooperation with several actin-binding proteins, which in turn supports the *trans*-interaction of cadherin, eventually resulting in the establishment of AJs (Fig. 1.4C).

cadherin-based AJs. On the other hand, the *trans*-interaction of cadherin induces the activation of Rap1 and Rac through c-Src, Crk, C3G, PI3K, and Vav2 to maintain cadherin-based AJs by reorganizing the actin cytoskeleton and inhibiting the endocytosis of cadherin. After the formation of AJs, both the nectin–afadin and cadherin–catenin systems cooperatively play an essential role in the formation of TJs as well as AJs. The Par cell polarity protein complex, annexin II, and the IQGAP1-dependent actin cytoskeleton organized by the nectin-induced activation of Cdc42 and Rac are also involved in the formation of TJs.

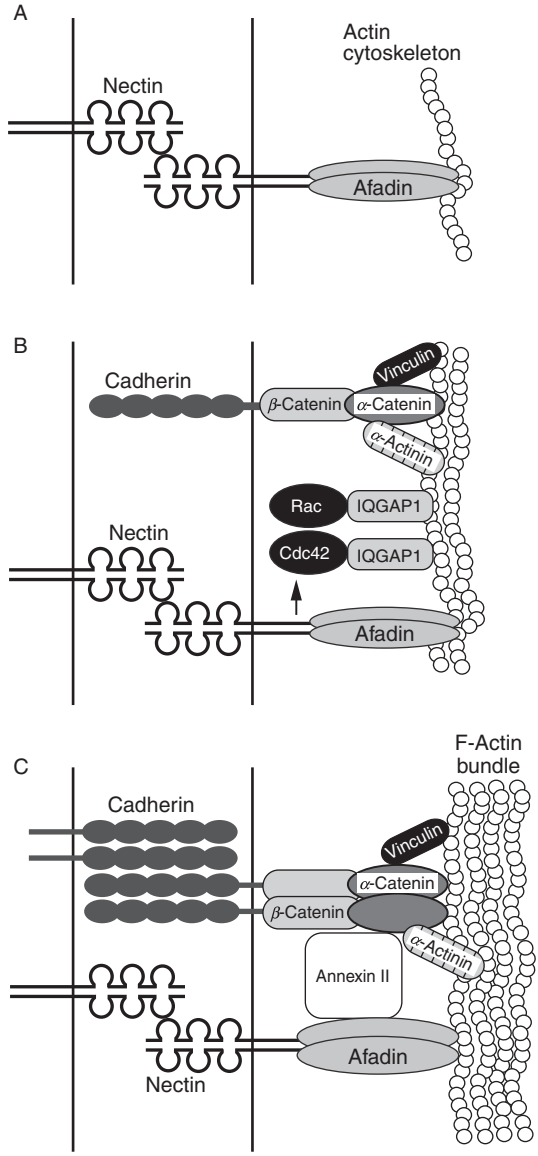


Figure 1.4 Reorganization of the actin cytoskeleton in the formation of adherens junctions. (A) The first step of the reorganization of the actin cytoskeleton. The *trans*-interaction of nectin initially causes the reorganization of the actin cytoskeleton through afadin. (B) The second step of the reorganization of the actin cytoskeleton. The nectin-induced activation of Cdc42 and Rac and one of their effecters IQGAP1 is involved in this step of the reorganization of the actin cytoskeleton, which assembles the cadherin–catenin complex at the nectin-based cell–cell adhesion sites. (C) The third step of the reorganization of the actin cytoskeleton. The *trans*-interaction of

3.4. Regulation of adhesion activity of cadherin by nectin

When *trans*-interacting nectin and its related intracellular signaling recruit the cadherin–catenin complex to the nectin-based cell–cell adhesion sites, cadherin shows only weak adhesion activity and thus does not *trans*-interact at this time (Sato *et al.*, 2006). Non-*trans*-interacting cadherin located at the cell surface tends to be internalized by endocytosis. However, when afadin interacts with Rap1 activated by *trans*-interacting nectin, it is capable of inhibiting the endocytosis of non-*trans*-interacting cadherin through the association between afadin and $p120^{ctn}$, which directly binds to the juxtamembrane domain of cadherin (Hoshino *et al.*, 2005). The Rap1-dependent association of afadin with $p120^{ctn}$ also enhances the binding of $p120^{ctn}$ to the juxtamembrane region of cadherin and increases the adhesion activity of cadherin through $p120^{ctn}$, resulting in the induction of the *trans*-interaction of non-*trans*-interacting cadherin that clusters near the nectin-based cell–cell adhesion sites (Hoshino *et al.*, 2005; Sato *et al.*, 2006). As a consequence, the number of *trans*-interacting cadherin increases at the nectin-based cell–cell adhesion sites. This induces the activation of Rac and consequent formation of lamellipodia (Fukuyama *et al.*, 2006; Kovacs *et al.*, 2002; Nakagawa *et al.*, 2001). The formation of lamellipodia is important not only for cell movement but also for cell–cell junction formation (Yonemura *et al.*, 1995; Ehrlich *et al.*, 2002). During the formation of AJs, filopodia-mediated microdomains are first formed between initially contacted cells and then lamellipodia close the gaps between the microdomains as a ''zipper,'' resulting in the establishment of AJs.

3.5. Roles of nectin and growth factor receptor in cell survival

After cells become confluent and establish the formation of cell–cell junctions, they arrest cell movement and proliferation but continue to survive. There are a number of reports that demonstrate the physical and functional association between CAMs and growth factor receptors (Comoglio *et al.*, 2003; Perez-Moreno *et al.*, 2003; Yap and Kovacs, 2003). It has been proposed that three types of signal transduction pathways are mediated by CAMs, such as integrin and cadherin, and growth factor receptors, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and vascular endothelial growth factor receptor-2 (Comoglio *et al.*, 2003);

cadherin finally induces the formation of firm F-actin bundles together with several actin-binding proteins and establishes adherens junctions. These F-actin bundles preferentially back up cadherin-based adherens junctions. It is unclear when α-actinin and vinculin bind to α-catenin in this process.

the first one is the collaborative signaling in which the CAM- and growth factor receptor-mediated signalings are individually or cooperatively transduced to the intracellular signaling molecules, the second is the CAM-dependent activation of growth factor receptor signaling that first requires the formation of cell–cell or cell–matrix junctions by CAMs before the growth factor receptor mediates its signaling, and the last one is the growth factor receptor-dependent activation of CAM signaling in which the growth factor receptor is first activated by its cognate growth factor and sequentially the function of CAM-related molecules is regulated.

PDGF-induced cell survival signaling is modified by nectin and its binding protein afadin (unpublished data) (Fig. 1.5). Nectin-3 associates with the PDGF receptor at the cell–cell adhesion sites and both nectin-3 and PDGF receptor function in a cooperative manner in the PDGF-induced activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. The PDGF-induced phosphorylation of Akt in nectin-3- or afadin-knockdown NIH3T3 cells is attenuated as compared with that in wild-type

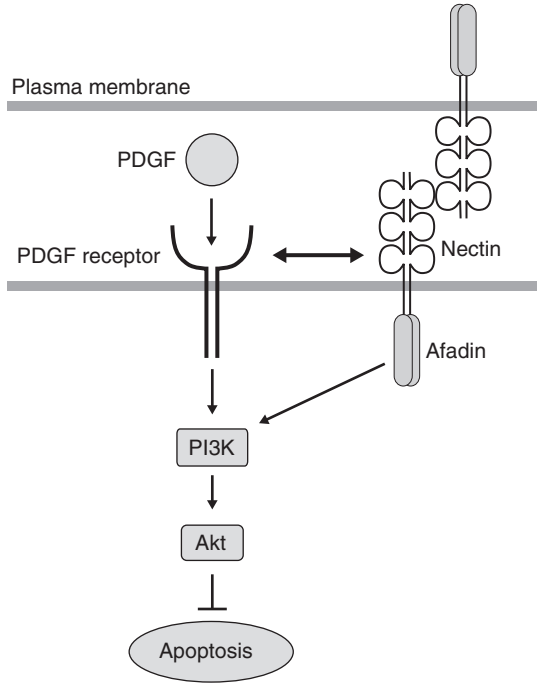


Figure 1.5 Cross-talk between the platelet-derived growth factor (PDGF) receptor and nectin in PDGF-induced cell survival signaling. Nectin interacts with PDGF receptor through their extracellular regions and regulates the PDGF-induced activation of PI3K and Akt and inhibition of apoptosis in cooperation with afadin at PI3K activation.

NIH3T3 cells. PI3K activity enhanced by PDGF is also suppressed in nectin-3- or afadin-knockdown NIH3T3 cells, indicating that the regulation of PI3K/Akt signaling by nectin and afadin is conducted at the step of the activation of PI3K. Moreover, the linkage of nectin with afadin is necessary for the activation of PI3K/Akt signaling, because the transfection of the nectin-3 mutant that is unable to bind to afadin in NIH3T3 cells fails to mediate the PDGF-induced phosphorylation of Akt. Similarly, embryoid bodies derived from afadin$^{-/-}$ embryonic stem (ES) cells have an enormous number of apoptotic cells in their cavity compared with those from wild-type ES cells, indicating the inhibitory effect of afadin on apoptosis during early embryogenesis.

3.6. Disassembly of adherens junctions and epithelial-to-mesenchymal transition

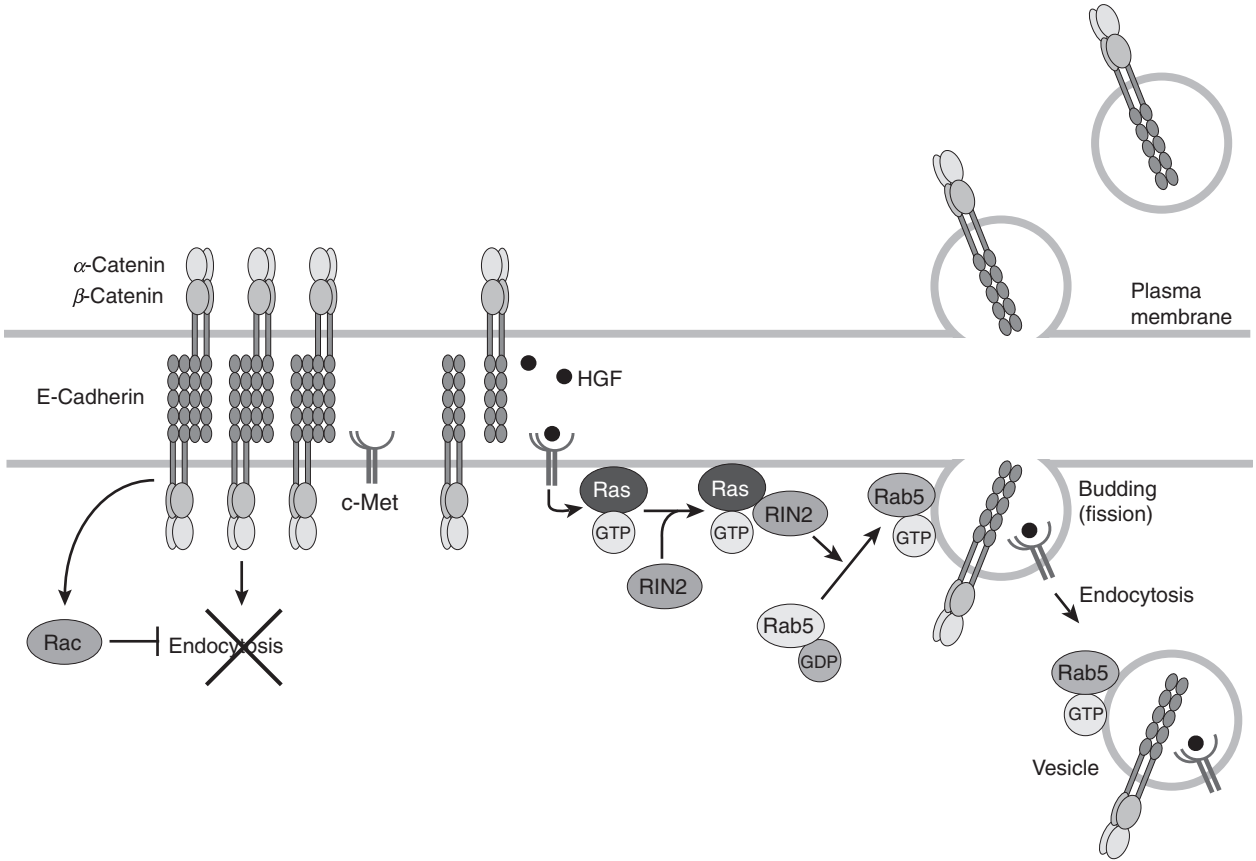
The formation of TJs and desmosomes is dependent on the formation of AJs, while the breakdown of AJs causes the disruption of TJs and desmosomes, indicating the essential role of AJs in the formation and maintenance of cell–cell junctions. Disassembly of AJs and subsequent disruption of cell–cell junctions are related to the epithelial-to-mesenchymal transition (EMT) and are necessary for the normal development of embryos and physiological process of ordered tissue turnover (Gumbiner, 2005). In terms of pathological implications, disruption of E-cadherin-based AJs in cancer cells enhances tumor development, invasion, and metastasis. The disassembly of AJs and TJs increases the paracellular permeability of epithelium and endothelium and the extravasation of monocytes and macrophages, which are involved in the induction of inflammation. Thus, to elucidate the molecular mechanism of disassembly of AJs is important for proper understanding of the cellular functions in both physiological and pathological statuses and the phenomenon of EMT.

In epithelial cells, endocytosis of E-cadherin is one of the major reasons to break AJs. Various growth factors such as hepatocyte growth factor (HGF)/scatter factor (SF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) enhance endocytosis of E-cadherin and induce disruption of cell–cell junctions of epithelial cells through their cognate receptors, resulting in cell migration and EMT (Bryant *et al.*, 2005; Kamei *et al.*, 1999; Lu *et al.*, 2003). The molecular mechanism of the HGF/SF-induced endocytosis of E-cadherin has been clarified in several studies. The c-Cbl-like protein, Hakai, which directly interacts with E-cadherin and is identified as an E3 ubiquitin-ligase, enhances endocytosis of E-cadherin by ubiquitination of the E-cadherin–catenin complex and causes cell scattering (Fujita *et al.*, 2002). The interaction of Hakai with E-cadherin is dependent on the c-Src-mediated phosphorylation of the cytoplasmic region of E-cadherin, and the activation of c-Src is regulated by receptor tyrosine kinases including c-Met, a receptor of HGF/SF, and the EGF receptor. On the other

hand, Rab5 small G protein plays a role in HGF/SF-induced E-cadherin endocytosis and cell scattering (Imamura *et al.*, 1998; Kamei *et al.*, 1999). More recently, the Ras-activated Rab5-GEF, RIN2, is reported to be involved in the HGF/SF-induced endocytosis of E-cadherin through the activation of Rab5 (Kimura *et al.*, 2006) (Fig. 1.6). RIN2, a member of the RIN family, is expressed ubiquitously and contains Src homology 2, two proline-rich, Vps9p-like (Rab5-GEF), and Ras association domains in this order from the N-terminus (Saito *et al.*, 2002). The GTP-bound form of Ras and Rab5 is capable of binding to RIN2. Thus, RIN2 functions as a connector between Ras and Rab5 in the HGF/SF-induced signaling of E-cadherin endocytosis.

HGF/SF also induces proteolytic cleavage of nectin-1α in the ectodomain, resulting in the generation of the 80-kDa extracellular fragment and the 33-kDa fragment composed of the transmembrane and cytoplasmic regions (Kim *et al.*, 2002; Tanaka *et al.*, 2002). This HGF/SF-induced shedding of nectin-1 may cause the disruption of the nectin-based cell–cell adhesion. However, as this possibility has not been studied in detail yet, it is not clear whether the proteolytic cleavage of nectin-1 is indeed sufficient to disrupt the nectin-based cell–cell adhesion. On the other hand, the 80-kDa extracellular fragment of nectin-1, which is released from the cell surface and into the culture medium, can *trans*-interact with nectin-1 or nectin-3 located at the free cell surface different from the cell–cell adhesion sites. This *trans*-interaction of nectins may induce the activation of Rac and Cdc42 and contribute to cell spreading and scattering through these activated small G proteins, although the supportive data are not yet available. Finally, the residual 33-kDa transmembrane and cytoplasmic regions may also transduce an intracellular signal as suggested for PECAM-1 (Ilan *et al.*, 2001).

It is well known that the *trans*-interaction of E-cadherin induces the activation of small G proteins, preferentially Rac (Fukuyama *et al.*, 2006; Kovacs *et al.*, 2002; Nakagawa *et al.*, 2001) as described above, although there is also a report that it induces the activation of Cdc42 in addition to Rac (Kim *et al.*, 2000). However, the physiological relevance of Rac activated by *trans*-interacting E-cadherin has not been fully elucidated. Rac activated by *trans*-interacting E-cadherin inhibits the endocytosis of E-cadherin through the IQGAP-dependent reorganization of the actin cytoskeleton and stabilizes E-cadherin on the plasma membrane (Izumi *et al.*, 2004) (Fig. 1.6). On the other hand, Rac is famous for participating in the formation of cell protrusions such as lamellipodia at the cell periphery, which facilitates cell spreading and migration. Taken together, it is likely that the robust activation of Rac at the cell periphery negatively regulates cell–cell adhesion and induces cell scattering, but that spatiotemporally controlled activation of Rac by the *trans*-interaction of E-cadherin at AJs upregulates the stability of AJs by inhibiting the endocytosis of E-cadherin.


α-Catenin
β-Catenin
E-Cadherin
c-Met
HGF
Plasma membrane
Ras
GTP
Ras
GTP
RIN2
RIN2
Rab5
GTP
Rab5
GDP
Budding (fission)
Endocytosis
Rac
Endocytosis
Rab5
GTP
Vesicle

4. Formation of Tight Junctions Regulated by the Nectin–Afadin System

4.1. Components of tight junctions

Three distinct types of CAMs are mainly found in TJs: occludin (Furuse *et al.*, 1993), claudins (Furuse *et al.*, 1998a), and JAMs (Martin-Padura *et al.*, 1998). Some Ig-like CAMs such as Coxsackie and adenovirus receptor molecule (CAR) and endothelial cell-selective adhesion molecule (ESAM), which was originally identified in endothelial cells (Hirata *et al.*, 2001), are also reported to be constituents of TJs (Cohen *et al.*, 2001; Nasdala *et al.*, 2002). Among these CAMs at TJs, occludin was first identified as a transmembrane protein that localizes within TJ strands (Fujimoto, 1995), and has four transmembrane domains, two extracellular loops, and the N- and C-termini facing the cytoplasm. *In vitro* transfection studies with deletion mutants of occludin have revealed that last ~150 amino acids from the C-terminus are required for its localization at TJs (Furuse *et al.*, 1994). Consistent with this, MDCK cells stably expressing a C- terminal-truncated mutant of occludin exhibit a discontinuous junctional staining pattern of mutant occludin and also disrupt the continuous junctional ring formed by endogenous occludin, resulting in an increase in the paracellular flux of tracers (Balda *et al.*, 1996). In contrast, the expression of full-length occludin in MDCK cells induces an increase in transcellular electrical resistance compared with wild-type MDCK cells, indicating the upregulation of electrically sealed tight junctions by overexpression of occludin (Balda *et al.*, 1996). An *in vivo* study has demonstrated that when the RNAs of truncated occludin constructs are injected into *Xenopus* embryos, the truncated occludin is correctly targeted to TJs, but the barrier function is disrupted (Chen *et al.*, 1997). Thereafter, the loss-of-function study clearly reveals that occludin is not essentially required for the formation of TJs, because embryonic stem cells lacking both alleles of the *occludin* gene still develop a normal network of TJ strands between adjacent epithelial cells of differentiated embryoid bodies (Saitou *et al.*, 1998). Thus, the role of occludin in the formation of TJs appears to be inconclusive.

Figure 1.6 **Mechanism of the hepatocyte growth factor (HGF)-induced disassembly of E-cadherin-based adherens junctions. *Trans*-interacting E-cadherin is resistant to endocytosis by activating Rac. However, stimulation of growth factors such as HGF induces the disruption of the *trans*-interaction of E-cadherin and the disassembly of adherens junctions by the upregulation of E-cadherin endocytosis. This HGF-induced endocytosis of E-cadherin is mediated by Ras-RIN2-Rab5 signaling.**

To identify the key molecule that is critically required for the formation of TJs and controls the barrier and fence functions of TJs, extensive studies were done, followed by studies on occludin. These great efforts have contributed to the identification of claudins as structural and functional components of TJs essentially involved in paracellular transport (Furuse *et al.*, 1998a). Interestingly, expression of claudin-1 and claudin-2 into mouse L fibroblasts lacking TJs induces the formation of the TJ strands (Furuse *et al.*, 1998b). Claudins localize at the site of close membrane apposition within TJs and are detected in both epithelial and endothelial cells in all tissues that bear TJs. Claudins are small proteins of 20 to 24 kDa, with four transmembrane domains, two extracellular loops, one intracellular turn, and the N- and C-termini, structurally similar to occludin. Some claudins have restricted and different distribution patterns among cell types and tissues (Kiuchi-Saishin *et al.*, 2002; Morita *et al.*, 1999a,b; Rahner *et al.*, 2001), suggesting their tissue-specific physiological properties. Studies on mice lacking claudin family members clearly show the physiological significance of claudin. Claudin-11 knockout mice have demonstrated the absence of the TJ strands in myelin sheets of oligodendrocytes and Sertoli cells in the testis (Gow *et al.*, 1999). These mice show delayed axonal conduction rates in the central nervous system and male-specific infertility. Mice lacking claudin-14 are deaf due to rapid degeneration of cochlear outer hair cells (Ben-Yosef *et al.*, 2003). In addition to the knockout mice studies, human mutations of claudin-16 (also called paracellulin-1), a specific claudin expressed in kidney epithelial cells, exhibit an abnormal paracellular passage of Mg^{2+} ions, resulting in excessive loss of Mg^{2+} in the urine (Simon *et al.*, 1999).

JAMs are structurally different from occludin and claudins; they are single-span transmembrane proteins possessing two Ig-like loops in the extracellular region and Ca^{2+}-independent cell adhesion activity (Martin-Padura *et al.*, 1998). So far, four members of JAMs and one JAM-like molecule have been identified: JAM-A (also referred to JAM-1) (Malergue *et al.*, 1998; Martin-Padura *et al.*, 1998), JAM-B (also known as VE-JAM/mouse JAM-3/human JAM2) (Aurrand-Lions *et al.*, 2000; Cunningham *et al.*, 2000; Liang *et al.*, 2002; Palmeri *et al.*, 2000), JAM-C (also known as mouse JAM-2/human JAM3) (Aurrand-Lions *et al.*, 2000, 2001), and JAM-4 (Hirabayashi *et al.*, 2003), and JAM-Like (JAML) (Moog-Lutz *et al.*, 2003). Among these JAM family members, JAM-A homophilically *trans*-interacts, whereas JAM-B and JAM-C are heterophilic binding partners (Arrate *et al.*, 2001), although neither of them homophilically *trans*-interacts. JAMs localize at TJs not only in epithelial and endothelial cells but also in hematopoietic cells of all lineages (Liu *et al.*, 2000). Although L cells exogenously expressing JAM-A do not form TJ strand-like structures in contrast to claudins, they contribute to the junctional organization of TJs and the regulation of TJ permeability (Itoh *et al.*, 2001). JAM-A binds to Par-3, of which the homologue in *Caenorhabditis elegans* is involved in

asymmetric cell division and polarized cell growth, and thus recruits the cell polarity protein complex Par-3/Par-6/aPKC to TJs (Ebnet *et al.*, 2001; Itoh *et al.*, 2001), indicating that JAMs appear to regulate epithelial cell polarity during the formation of cell–cell junctions.

These CAMs at TJs including occludin, claudin, and JAMs are all connected to the actin cytoskeleton mediated by ZO proteins. The ZO protein family consists of three members: ZO-1, ZO-2, and ZO-3. Both claudins and JAMs possess the PDZ-binding motif at their C-termini and bind ZO proteins at their PDZ domains. In contrast, occludins bind ZO proteins through their guanylate kinase domains (Tsukita *et al.*, 2001). TJs also contain cell polarity proteins including the Par complex and many signaling molecules and serve as a regulatory center for coordinating multiple cell processes (Aijaz *et al.*, 2006; Ohno, 2001; Tsukita *et al.*, 2001).

As described previously, claudin and occludin are concentrated at so-called bicellular TJs where two apposing epithelial cells adhere. Tricellulin has been identified as a novel molecule localized at tricellular TJs where three epithelial cells form tricellular contacts (Ikenouchi *et al.*, 2006). The predicted molecular structure of tricellulin is related to that of claudin or occludin: it has four membrane-spanning domains and its N- and C-termini are located in the cytoplasm. The existence of tricellulin is required for the maintenance of not only tricellular TJs but also bicellular TJs, because knockdown of tricellulin disrupts both types of TJs and impairs the epithelial barrier function. As mentioned previously, some Ig-like CAMs, CAR (Cohen *et al.*, 2001) and ESAM (Nasdala *et al.*, 2002), which resemble JAMs in their structure, have been identified at TJs. It is assumed that the functions of these two Ig-like molecules are different from those of JAMs, but their molecular functions have not been fully elucidated.

4.2. Integrity of tight junctions mediated by nectin

During the formation of cell–cell junctions between adjacent epithelial cells, AJs are first formed and TJs are then formed at the apical side of AJs accompanied by the formation of cell polarity, resulting in the establishment of cell–cell junctions and the development of the epithelial cell monolayer. In contrast, in the breakdown of AJs, TJs are consequently disrupted. Therefore, the formation of AJs is important and indispensable for the formation and maintenance of cell–cell junctions (Yap *et al.*, 1997). As described previously, the *trans*-interaction of nectin is essential for the formation of cadherin-based AJs. Accumulating evidence has shown the importance of nectin in the formation of TJs as well as AJs (Takai and Nakanishi, 2003). Actually, the inhibition of the intercellular interaction of nectin by the recombinant protein of Nef, which is the extracellular fragment of nectin fused to the IgG Fc portion, significantly blocks the formation of TJs (Fukuhara *et al.*, 2002a,b). After or during the formation of

AJs, nectin recruits JAMs first and then claudins and occludin to the apical side of AJs in cooperation with cadherin, leading to the formation of TJs (Takai and Nakanishi, 2003). Although the entire molecular mechanism of the recruitment of the TJ constituents such as claudins, occludin, and JAMs at the apical side of the cell–cell adhesion sites remains unclear, this recruitment is at least mediated by the nectin–afadin and cadherin–catenin systems, the Par cell polarity protein complex, annexin II, and the IQGAP1-dependent actin cytoskeleton organized by the nectin-induced activation of Cdc42 and Rac (Ooshio *et al.*, 2007; Yamada *et al.*, 2006). In addition, ZO proteins are reported to be critically involved in the formation of TJs (Umeda *et al.*, 2004, 2006). Therefore, the molecular mechanism in the formation of TJs seems to be quite complicated and has not been completely elucidated.

It has been proposed on the basis of circumstantial evidence that cadherin plays a key regulatory role in the formation and maintenance of TJs; (1) the formation and maintenance of TJs are dependent on extracellular Ca^{2+}, which is necessary for the cell–cell adhesion activity of cadherin (Gonzalez-Mariscal *et al.*, 1985); (2) E-cadherin-blocking antibodies inhibit the formation of TJs as evaluated by electron microscopy and barrier assay (Gumbiner *et al.*, 1988); and (3) AJs and TJs are not formed in PC9 carcinoma cells, which do not express α-catenin (Watabe *et al.*, 1994). Only one report has demonstrated the AJ-independent formation of TJs, in which the treatment of MDCK cells cultured in a low Ca^{2+} medium with tumor-promoting phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), causes the formation of a TJ-like structure at the cell–cell adhesion sites in the absence of E-cadherin-based cell–cell adhesion (Balda *et al.*, 1993). However, one study has discovered in such conditions that non-*trans*-interacting E-cadherin, which has only weak cell–cell adhesion activity but associates with α-catenin, β-catenin, and $p120^{ctn}$, is actually recruited to the nectin-based cell–cell adhesion sites (Okamoto *et al.*, 2005), indicating the necessity of the nectin–afadin system for the formation of TJs. The reason why the existence of non-*trans*-interacting E-cadherin is not recognized is likely to be due to the fact that several antibodies against E-cadherin can detect only the signal for *trans*-interacting E-cadherin, but not that for non-*trans*-interacting E-cadherin.

In addition to these previous reports, annexin II, a protein factor necessary for the formation of AJs, is also involved in the formation of TJs even in the absence of E-cadherin-based AJs (Yamada *et al.*, 2006). In annexin II-knockdown MDCK cells, the formation of E-cadherin-based AJs is inhibited but not that of TJs, suggesting the inhibitory effects of annexin II on the formation of TJs. Experimental results prove the structurally and functionally normal formation of TJs in these annexin II-knockdown cells: (1) the concentration of immunofluorescence signals for all the major TJ components including claudin-1, occludin, JAM-A, and ZO-1 at the cell–cell adhesion sites; (2) the existence of junctional strands assessed by

electron microscopy; and (3) the normal barrier function evaluated by the paracellular diffusion of a nonionic solute, FITC-conjugated dextran. Thus, cadherin-based AJs are not absolutely required for the formation of TJs under these experimental conditions. This is also supported by other studies using MDCK cells or *Drosophila* models (Harris and Peifer, 2004; Capaldo and Macara, 2007). In contrast to cadherin, the nectin–afadin system plays a critical role in the formation of TJs in annexin II-knockdown MDCK cells, because the formation of TJs is disrupted in afadin- and annexin II-double knockdown MDCK cells.

4.3. The nectin and Par complex in the formation of cell polarity and tight junctions

The Par complex including Par-3, Par-6, and aPKC is also reported to be involved in the formation of TJs as well as apicobasal polarization in epithelial cells (Ohno, 2001; Roh and Margolis, 2003). At the beginning of the cell polarization, Par-6 and aPKC form a ternary complex with Lgl, but not Par-3 (Yamanaka *et al.*, 2003). However, binding of activated Cdc42 to Par-6 induces the activation of aPKC, which then phosphorylates Lgl (Plant *et al.*, 2003). It has not been established how Cdc42 is activated, but it is likely that this Cdc42 is activated by the *trans*-interaction of nectin formed at the initial cell–cell contact sites (Takai and Nakanishi, 2003). Phosphorylated Lgl is released from the Par-6/aPKC complex and in turn Par-3 binds to this complex, resulting in the formation of the new ternary complex of Par-3/Par-6/aPKC, which interacts with JAM through the PDZ domain of Par-3 and the C-terminal four amino acids of the cytoplasmic tail of JAM. The dynamic formation of the Par-3/aPKC/Par-6 complex regulated by Lgl and Cdc42 and the interaction between this complex and JAM are important for the formation of TJs (Macara, 2004; Plant *et al.*, 2003; Yamanaka *et al.*, 2003), but it remains unknown how these cell polarity proteins regulate the formation of TJs, following the formation of AJs. A more recent study has demonstrated crosstalk between the Par complex and another cell polarity protein complex including Crumbs, PATJ, and Pals1 and the involvement of these two complexes in the formation of apicobasal cell polarity and consequent formation of TJs (Hurd *et al.*, 2003). Thus, accumulating evidence on cell polarity proteins provides new insight into the molecular mechanisms of cell polarization during the establishment of cell–cell junctions.

Par-3 directly binds to nectin-1 and nectin-3, but not nectin-2, between the first PDZ domain of Par-3 and the C-terminal four amino acids of the cytoplasmic tails of these nectins (Takekuni *et al.*, 2003). From the results of experiments using Par-3-knockdown MDCK cells, Par-3 is necessary for the formation of both AJs and TJs, although Par-3 is dispensable for the formation of nectin-based cell–cell adhesion (Ooshio *et al.*, 2007). At the

initial phase of cell–cell contact, Par-3 contributes to the interaction of afadin with nectin, facilitating the formation of AJs. However, this role of Par-3 is not sufficient for the formation of AJs and TJs, because overexpression of afadin in Par-3 knockdown MDCK cells exhibits the assembly of afadin at the nectin-based cell–cell adhesion sites but does not induce the formation of either AJs or TJs. This indicates the cooperative role of Par-3 and afadin in the formation of AJs and TJs. Taken together with the facts described in this section, the nectin–afadin system is involved not only in the formation of AJs but also in the regulation of cell polarization and the subsequent formation of TJs, indicating the essential contribution of nectin in the whole process of the formation of cell–cell junctions.

5. Roles of Nectin and Necl-5

5.1. Roles of nectin in nectin-mediated cell–cell adhesions

Nectins are expressed in almost all the tissues of both embryos and adult individuals and in a variety of cell types including epithelial cells, neurons, and fibroblasts (Irie *et al.*, 2004; Ogita and Takai, 2006; Takai *et al.*, 2003; Takai and Nakanishi, 2003). As described previously, the molecular characteristics of nectins and Necls have been most extensively studied in single-layered epithelia (Fig. 1.7A). The role and behavior of nectins and Necls in other tissues are demonstrated later.

5.1.1. In embryonic development

At the early stages of mouse development, nectin-1, nectin-2, and nectin-3 are equally concentrated at AJs of homotypic columnar epithelia such as neuroepithelia and epithelial somites. These nectins and afadin are highly expressed during somitogenesis (Okabe *et al.*, 2004a). The nectin-1 mRNA is expressed in the presomitic mesoderm and dorsal part of mature somitomeres, but is downregulated in the anterior presomitic mesoderm as it is condensing into somites. In the mature somites, nectins are concentrated at AJs of the dorsal region of each somite (dermomyotome). When elongated cells of myotome are differentiated, the concentration of nectins and afadin at AJs of the epithelial dermatome, which will finally provide the dermis, is maintained.

5.1.2. In brain

At the CA3 region of the hippocampus, nectin-1 and nectin-3 asymmetrically localize at the presynaptic and postsynaptic sides of puncta adherentia junctions (PAJs), respectively (Mizoguchi *et al.*, 2002) (Fig. 1.7B). PAJs as well as synaptic junctions (SJs) are intercellular junctions in the synapse, where asymmetric junctions form between two different neurons

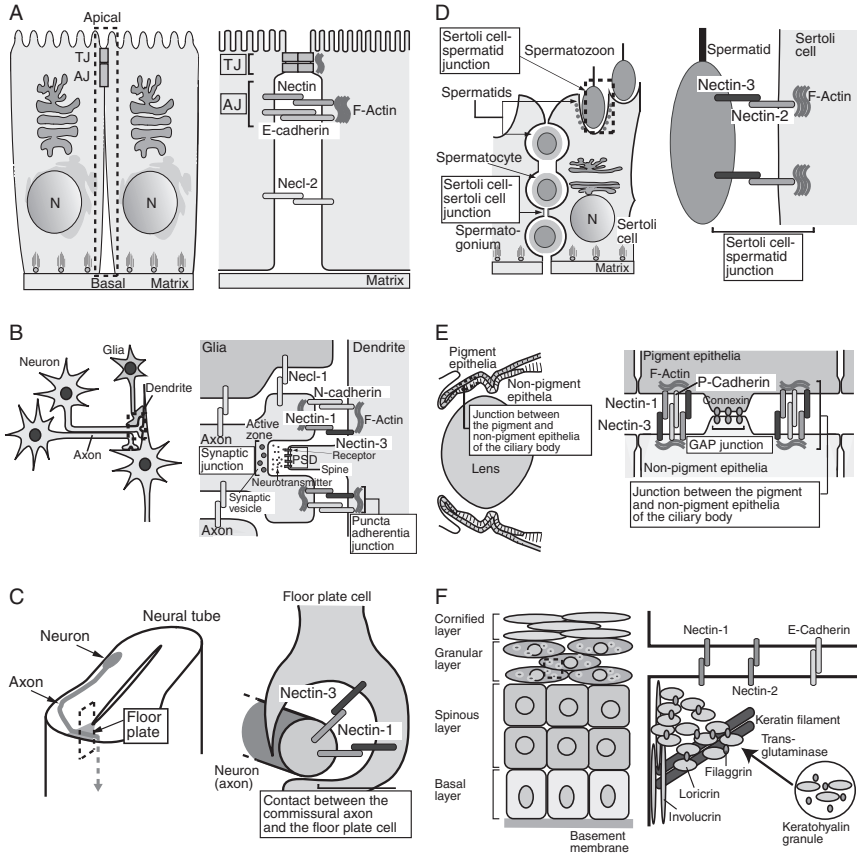


Figure 1.7 **Involvement of nectins, Necls, and cadherins in the formation of various kinds of cell–cell adhesion. (A) Cell–cell junctions in epithelial cells in tubular organs. (B) Junctions in neurons and glias in the brain. (C) Adhesion between the commissural axons and the floor plate cells. (D) Sertoli cell–spermatid junctions in the testis. (E) Junctions between the apical membranes of the pigment and nonpigment epithelia in the ciliary body. (F) Junctions in the epidermis. The dashed boxes in the left panels correspond to the right panels.**

(Spacek and Lieberman, 1974). SJs function as sites for neurotransmission, while PAJs are regarded as mechanical adhesion sites between axon terminals and their targets. Consistent with this observation, the number of PAJs at the synapses between the mossy fiber terminals and the dendrites of the CA3 pyramidal cells in hippocampus was reduced in nectin-1$^{-/-}$ and nectin-3$^{-/-}$ mice, resulting in the abnormal mossy fiber trajectory (Honda *et al.*, 2006). In the case of interactions between hippocampal pyramidal neurons, axons attach to dendrites for their synaptogenesis, although dendrites do not form functional contacts with each other. In these neurons,

nectin-1 preferentially localizes in axons, and its heterophilic partner, nectin-3, is present in both axons and dendrites. Because the homophilic binding between nectin-3 is less strong than the heterophilic *trans*-interaction of nectin-1 with nectin-3 (Honda *et al.*, 2003), normal axodendritic interactions would be formed between nectin-1 and nectin-3. When nectin-1 is ectopically overexpressed, nectin-1 abnormally localized in dendrites beside axons and atypical dendrodendritic as well as excessive axodendritic interactions occur (Togashi *et al.*, 2006), indicating that the controlled *trans*-interaction of nectin-1 in axons and nectin-3 in dendrites is important for sustaining the normal interactions between axons and dendrites.

Nectin-1 and nectin-3 also asymmetrically localize at the commissural axon side and the floor plate cell side, respectively, and they *trans*-interact between commissural axons and floor plate cells (Okabe *et al.*, 2004b) (Fig. 1.7C). After commissural axons that grow toward the ventral midline cross the floor plate, they abruptly change their trajectory from the circumferential to the longitudinal axis. Because the cadherin–catenin system is not concentrated at the contact sites between commissural axons and floor plate cells, the *trans*-interaction between nectin-1 and nectin-3 appears to be critically involved in the alteration in the trajectory of commissural axons. This hypothesis is supported by the data that *in vitro* inhibition of the endogenous *trans*-interaction of nectins by the inhibitor actually impairs the contacts between commissural axons and floor plate cells and the longitudinal turn of the commissural axons at the contralateral sites of the rat hindbrain. Because the commissural axons and the floor plate cells communicate or transfer signals through their contact sites (Stoeckli and Landmesser, 1998), the abnormal turn and loss of proper direction of commissural axons by inhibition of the *trans*-interaction of nectins may be due to the failure of the transduction of signals in addition to mechanically weak contact between the commissural axons and the floor plate cells.

5.1.3. In testis

In the testis, nectin-2 and nectin-3 reside specifically in Sertoli cells and spermatids, respectively (Ozaki-Kuroda et al., 2002) (Fig. 1.7D). Because the existence of the cadherin–catenin system has been questionable at Sertoli cell–spermatid junctions (Cheng and Mruk, 2002), these junctions are likely to mainly depend on the *trans*-interaction between nectin-2 and nectin-3, whereas Sertoli cell–Sertoli cell junctions are formed through the cooperation of multiple intercellular adhesion molecules including nectin-2, N-cadherin, claudin-11, and occludin. Consistent with the importance of nectin-2 and nectin-3 for the formation of Sertoli cell–spermatid junctions, nectin-$2^{-/-}$ and nectin-$3^{-/-}$ mice show differentiation abnormalities in spermatogenesis, resulting in male-specific infertility (Inagaki *et al.*, 2006; Mueller and Wimmer, 2003; Ozaki-Kuroda *et al.*, 2002). These knockout

mice also exhibit distorted nuclei and an abnormal distribution of mitochondria in sperm morphogenesis. It should be noted that the signal for nectin-2 at Sertoli cell–spermatid junctions completely disappears in the nectin-$3^{-/-}$ testis, while the signal for nectin-3 is disorganized but still remains in the nectin-$2^{-/-}$ testis, suggesting that nectin-3 in spermatids also *trans*-interacts with CAM(s) other than nectin-2 in Sertoli cells. However, such interactions might not be as important as those between nectin-2 and nectin-3, because loss of nectin-2 in Sertoli cells dramatically affects the organization of nectin-3 at Sertoli cell–spermatid junctions. In the process of the spermatogenesis and release of spermatozoa from the seminiferous epithelium, germ cells have to move across the blood–testis barrier, which is composed of inter-Sertoli cell junctions, in the seminiferous epithelium. Based on the fact that nectins are expressed in both Sertoli cells and spermatids, nectins may additionally contribute to the passing of spermatids through the blood–testis barrier.

Necl-2 is also expressed in the testis, but the distribution of Necl-2 is limited in spermatids, but not in Sertoli cells (Wakayama *et al.*, 2003). Although nectin-3 has the ability to heterophilically *trans*-interact with Necl-2, Necl-2 is not a binding partner of nectin-3, because these two molecules localize only in spermatids. Necl-$2^{+/-}$ mice that show over a 90% reduction in Necl-2 expression exhibit reduced male-specific fertility due to the reduced motility and maturation of spermatozoa (Surace *et al.*, 2006). Thus, Necl-2 as well as nectin-2 and nectin-3 plays an important role in the proper development of germ cells.

5.1.4. In ciliary body of the eyes

Nectins in the ciliary body also contribute to its morphogenesis. The ciliary body produces both aqueous humor and some components of the vitreous body and is the source of the zonules that support the lens (Raviola, 1977). The ciliary body consists of epithelia, vascular layers, and muscles. The ciliary epithelia is divided into two layers, the pigment and non-pigment epithelia. The lateral membrane of each layer is held together primarily by AJs and TJs. The apices of the pigment and non-pigment epithelia are apposed and adhere to each other by fascia adherens and gap junctions. Fascia adherens junctions resemble typical AJs in their structure, but are macular in shape instead of encompassing the cell perimeter in a belt-like fashion. One of the TJ components occludin and one of the classical cadherins P-cadherin localize at the apex–apex junctions between the pigment and non-pigment epithelia in the ciliary body (Wu *et al.*, 2000), although it is still unclear whether these CAMs are essential for the formation of junctions between the pigment and non-pigment epithelia. Nectin-1, nectin-2, and nectin-3 localize at the lateral junctions of each of the pigment and non-pigment epithelia, while nectin-1 and nectin-3, but not nectin-2, localize at the junctions between the apposed pigment and

non-pigment epithelia (Inagaki *et al.*, 2005) (Fig. 1.7E). As expected, both types of knockout mice, nectin-$1^{-/-}$ and nectin-$3^{-/-}$, show a separation of the apex–apex adhesion between the pigment and non-pigment epithelia and have a phenotype of microphthalmia (Inagaki *et al.*, 2005). In contrast, these mice do not exhibit any change in the lateral junctions between each of the pigment and non-pigment epithelia, probably because residual nectins contribute to the maintenance of the lateral junctions in nectin-$1^{-/-}$ and nectin-$3^{-/-}$ mice. These results indicate the significance of the heterophilic *trans*-interaction between nectin-1 and nectin-3 for the apex–apex adhesion between the pigment and non-pigment epithelia in the ciliary body.

5.1.5. In epidermis

Human as well as mouse epidermis expresses nectin-1 at the cell–cell junctions where nectin-1 colocalizes with E-cadherin (Matsushima *et al.*, 2003; Wakamatsu *et al.*, 2007) (Fig. 1.7F). Newborn nectin-$1^{-/-}$ pups showed a shiny and slightly reddish skin due to the reduced amount of loricrin, which is one of the differentiation markers and also a major component of cornified cell envelopes, in the epidermis (Wakamatsu *et al.*, 2007). Consistent with loricrin$^{-/-}$ mice (Koch *et al.*, 2000), cornified cells from nectin-$1^{-/-}$ mice were sensitive to mechanical stress. The Ca^{2+}-induced differentiation assay using primary keratinocytes from nectin-$1^{-/-}$ mice has shown impaired phosphorylation of ERK mediated by Rap1 activation as compared with that from wild-type mice, resulting in the reduced expression of loricrin. It has been reported that the transcription of the *loricrin* gene is regulated by binding of protein factors to an AP-1 consensus site in the loricrin proximal promoter sequence (DiSepio *et al.*, 1995). Because ERK is known to be one of the activators for AP-1 transcription factors, a decrease in ERK phosphorylation seems to cause the downregulation of loricrin expression.

5.1.6. Compensatory mechanisms of nectin and critical roles of afadin *in vivo*

The *in vivo* studies shown previously clearly demonstrate the importance of nectins for the maintenance of various kinds of cell–cell adhesion and functions of many organs in individuals. However, each of the nectin-null mice is viable and shows the relatively moderate phenotypes, but not the life-threatening disorder. This may depend on the functional redundancy in each of the nectin-null mice, as many tissues express multiple nectins and Necls and these nectins and Necls homophilically or heterophilically *trans*-interact with each other in a variety of combinations (Ogita and Takai, 2006; Takai *et al.*, 2003; Takai and Nakanishi, 2003). In contrast to each of the nectin-null mice, afadin$^{-/-}$ mice are embryonic lethal because of no redundancy in the function of afadin (Ikeda *et al.*, 1999). During embryonic development, afadin is highly expressed in the embryonic ectoderm and the

mesoderm at E6.5–E8.5, but is hardly detected in the extraembryonic regions such as the visceral endoderm at that time. In agreement with the expression pattern of afadin during gastrulation, afadin$^{-/-}$ embryos display major defects in the embryonic ectoderm and mesoderm relative to endoderm. The disruption of afadin in mice causes the disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and other structures derived from the ectoderm and the mesoderm at stages during and after gastrulation of embryonic development (Ikeda *et al.*, 1999). Cell–cell junctions including AJs and TJs were improperly achieved in the ectoderm of afadin$^{-/-}$ embryos and embryonic bodies. Thus, at least during early embryogenesis, afadin plays a key role in actively rearranging epithelia of the embryonic ectoderm. As the mesoderm can be generated in afadin$^{-/-}$ embryos, gastrulation itself appears to occur.

5.2. Physiological roles of Necl-5

5.2.1. Enhancement of cell movement and proliferation by Necl-5

As described previously, Necl-5 is identified as a poliovirus receptor (Koike *et al.*, 1990; Mendelsohn *et al.*, 1989) and is overexpressed in several kinds of carcinomas (Chadeneau *et al.*, 1994; Masson *et al.*, 2001; Sloan *et al.*, 2004). However, the physiological role of Necl-5 remained unknown for a long time. In moving cells, Necl-5 localizes at the leading edge of the cell, where integrin $\alpha_v\beta_3$ also exists (Ikeda *et al.*, 2004). Necl-5 associates functionally with integrin $\alpha_v\beta_3$ to facilitate the PDGF-induced cell movement. More recently, Necl-5 has been found to directly interact in *cis* with integrin $\alpha_v\beta_3$ through their extracellular regions, resulting in clustering of integrin $\alpha_v\beta_3$ at the leading edge and enhanced cell movement (Minami *et al.*, 2007a). Not only the extracellular but also the cytoplasmic regions of Necl-5 are necessary for cell movement (Ikeda *et al.*, 2004). The intracellular signaling initiated by binding of growth factors such as PDGF and FGF to their receptors is regulated by Necl-5 at the step downstream of the receptors and upstream of Ras small G protein (Kakunaga *et al.*, 2004). Necl-5 enhances the activation of the Ras-Raf-MEK-ERK signaling and causes up- and downregulation of cell cycle regulators, including cyclins D2 and E and cyclin-dependent kinase inhibitor $p27^{Kip1}$, in NIH3T3 cells. These effects contribute to shortening of the G_1 phase of the cell cycle and enhancement of growth factor-induced cell proliferation.

Necl-5 forms complexes with integrin $\alpha_v\beta_3$ and PDGF receptor at the leading edge of the moving cell upon PDGF stimulation, such that Necl-5 promotes both cell movement and proliferation by enhancing integrin $\alpha_v\beta_3$- and PDGF receptor-induced signalings simultaneously (Amano *et al.*, 2007) (Fig. 1.8A). Detailed observation of the leading edge has revealed that it is composed of three different peripheral structures:

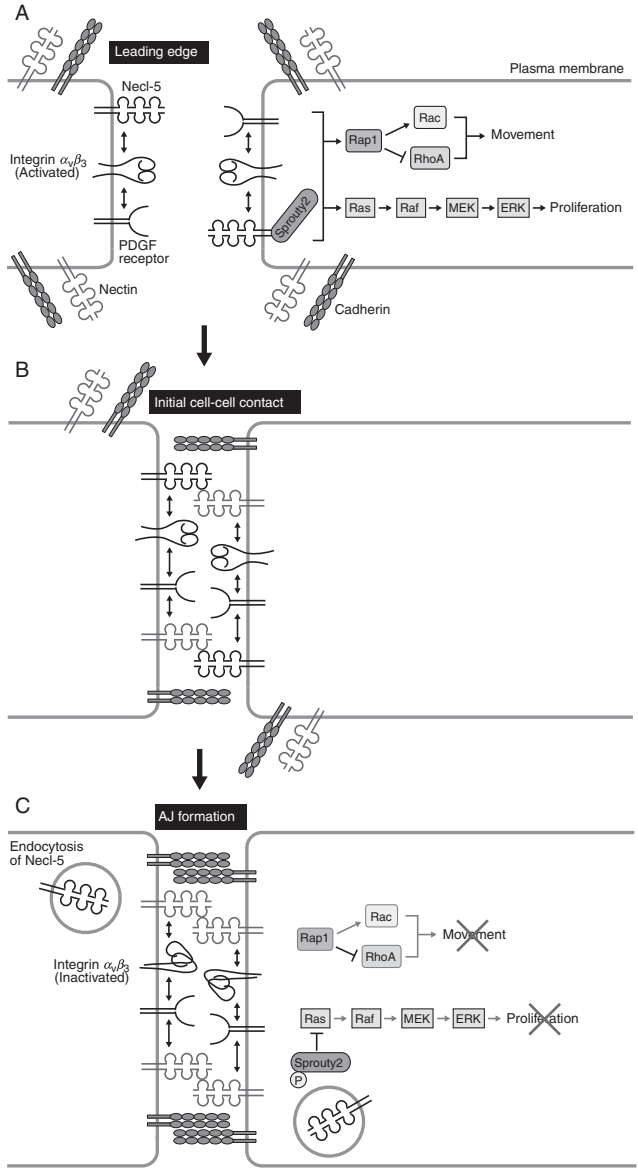


Figure 1.8 Cross-talk of the signalings among cell movement, proliferation, and adhesion and contact inhibition of cell movement and proliferation. (A) At the leading edge of moving cells, Necl-5, integrin $\alpha_v\beta_3$, and the platelet-derived growth factor (PDGF) receptor mutually form a ternary complex. This complex activates Ras-Raf-MEK-ERK signaling for cell proliferation by suppressing the inhibitory effect of Sprouty2, which associates with Necl-5, on cell proliferation. This ternary complex also enhances cell movement by activation of the Rap1–Rac pathway and inhibition of Rho. (B) When moving cells collide with each other, the initial cell–cell contact occurs by

peripheral membrane ruffles, lamellipodia, and focal complexes (Geiger and Bershadsky, 2001; Suetsugu *et al.*, 2003) (Fig. 1.9). Peripheral ruffles and focal complexes are formed over and under lamellipodia, respectively. All of three molecules including Necl-5, the PDGF receptor, and integrin $\alpha_v\beta_3$ are concentrated at peripheral ruffles; however, only two molecules, Necl-5 and integrin $\alpha_v\beta_3$, but not the PDGF receptor, are concentrated at the focal complex. The PDGF-induced formation of peripheral ruffles, lamellipodia, and focal complexes is enhanced by the interaction of Necl-5 with integrin $\alpha_v\beta_3$ and is necessary for effective cell movement.

The local activation of Rap1 at peripheral ruffles is necessary for the PDGF-induced activation of Rac and the formation of the leading edge of moving cells (unpublished data). Inactivation of Rap1 by induction of Rap1GAP into NIH3T3 cells inhibits the accumulation of not only Rac but also Rac-GEFs and an Rac effector PAK at peripheral ruffles and the formation of the leading edge toward the higher concentration of PDGF. None of the signals for Necl-5, integrin β_3, or the PDGF receptor is concentrated at any regions in these cells, resulting in a reduction in cell movement. Activated Rap1 directly interacts with afadin and recruits afadin to the leading edge when afadin does not bind to nectin. This interaction is necessary for the activation of Rac and inactivation of RhoA at the leading edge, which enhances cell movement (unpublished data). The inhibition of RhoA is mediated by a Rho-GAP ARAP1, of which activation is induced by binding of Rap1. On the other hand, at the rear side of the leading edge, Rap1 is immediately inactivated by one of the Rap-GAPs SPA-1. Thus, at this site, Rap1-mediated negative regulation of RhoA is blocked and RhoA is activated, followed by the activation of Rho-kinase that upregulates the phosphorylation of the myosin light chain by inhibiting myosin phosphatase and thus increases the formation of stress fibers. This RhoA-Rho-kinase signaling enhances the formation of focal adhesions at the rear side of focal complexes by increasing the transformation of focal complexes to focal adhesions (unpublished data) (Fig. 1.9). Afadin has been found to play an essential role in this dynamic and cyclical activation of Rap1, Rac, and RhoA small G proteins at the leading edge.

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the *trans*-interaction of Necl-5 with nectin-3. At this phase, integrin $\alpha_v\beta_3$ remains active. (C) The *trans*-interaction of Necl-5 with nectin-3 is tentative, and nectins and cadherins *trans*-interact with each other to form adherens junctions after Necl-5 is endocytosed in a clathrin-dependent manner and sequestered from the cell surface. At this time, integrin $\alpha_v\beta_3$ becomes inactive. Sprouty2, which is released from Necl-5, is tyrosine phosphorylated and blocks Ras-mediated cell proliferation signaling. These cause the suppression of intracellular signaling induced by integrin $\alpha_v\beta_3$ and PDGF receptor, resulting in the inhibition of cell movement and proliferation. Thus, downregulation of Necl-5 is at least partly involved in the contact inhibition of cell movement and proliferation.

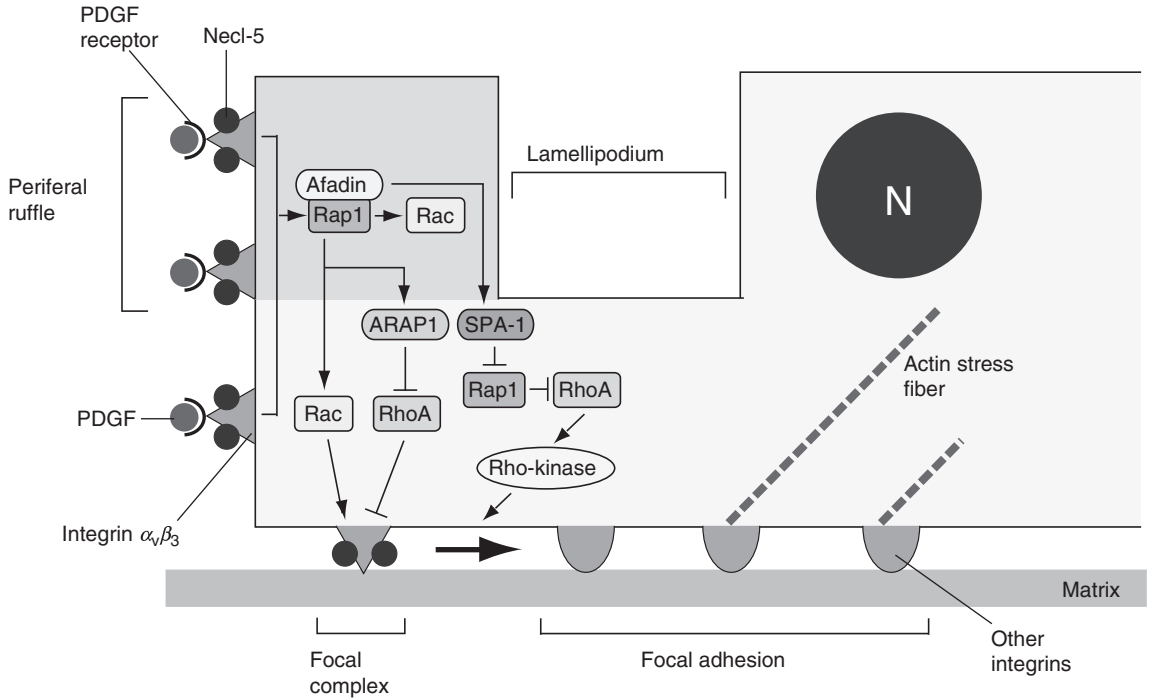

Figure 1.9 Peripheral membrane structures and local signal activation at the leading edges of moving cells. At the leading edge of moving cells, peripheral membrane ruffles are formed over lamellipodia, and focal complexes are formed under membrane ruffles. Focal adhesions are formed at the rear side of focal complexes. All of these three molecules, Necl-5, platelet-derived growth factor (PDGF) receptor, and integrin $\alpha_v\beta_3$, are observed at membrane ruffles; however, only two molecules, Necl-5 and integrin $\alpha_v\beta_3$, but not PDGF receptor, are concentrated at focal complexes. Focal adhesions include integrins other than integrin $\alpha_v\beta_3$. Rap1 locally activated at the leading edge interacts with afadin

Necl-5 binds to the dynein light chain Tctex-1 in the cytoplasmic region of Necl-5 (Mueller *et al.*, 2002). Dynein is a member of microtubule (MT) plus-end-binding proteins (+TIPs) and participates in searching and capturing MTs as well as the intracellular retrograde transport of molecules (Mimori-Kiyosue and Tsukita, 2003). In the directional cell movement, the reorientation of the MT network is necessary and makes it possible to search for the membrane cue toward the cell movement. The +TIPs are involved in this searching and the determination of the direction of cell movement. The direct interaction of Necl-5 with Tctex-1 targets the dynein/dynactin complex to the leading edges and recruits plus ends of MTs there (unpublished data). Localization of MT-stabilizing proteins, such as LL5β, at the rear area of leading edges is also regulated by Necl-5. Taken together, Necl-5 preferentially corresponds to the search for and reorientation of MT networks and the directional cell movement through the MT-related proteins.

5.2.2. Involvement of Necl-5 in contact inhibition of cell movement

Necl-5 does not homophilically *trans*-interact, but heterophilically *trans*-interacts with nectin-3 among the nectin and Necl family members (Takai *et al.*, 2003). Thus, the initial cell–cell contact occurs by the heterophilic *trans*-interaction of Necl-5 at the leading edges with nectin-3 on the adjacent cell surface when individually moving cells collide with each other (Ikeda *et al.*, 2003) (Fig. 1.8B). This *trans*-interaction induces the activation of Cdc42 and Rac (Sato *et al.*, 2005), both of which reorganize the actin cytoskeleton and increase the number of cell–cell adhesion sites. However, the *trans*-interaction of Necl-5 with nectin-3 is transient, and downregulation of Necl-5 from the cell surface occurs by endocytosis in a clathrin-dependent manner (Fujito *et al.*, 2005) (Fig. 1.8C). This downregulation of Necl-5 leads to the reduction in cell movement and proliferation by inhibiting the signalings initiated by integrin $\alpha_v\beta_3$ and growth factor receptors. On the other hand, nectin-3 dissociated from Necl-5 is retained on the cell surface and subsequently *trans*-interacts with nectin-1, which most feasibly *trans*-interacts with nectin-3 among the nectin family members (Ikeda *et al.*, 2003). This *trans*-interaction of nectins induces cadherin recruitment to the nectin-based adhesion sites, eventually establishing AJs as described previously.

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and consequently activates Rac but inhibits Rho through Rap1-dependent RhoGAP ARAP, enhancing the formation of focal complexes. At the rear side of focal complexes, Rap1 is immediately inactivated by Rap1GAP SPA-1. This Rap1 inactivation reverses the Rap1-mediated inhibition of RhoA and thus the RhoA-Rho-kinase signaling is activated. This increases the formation of focal adhesions by upregulation of the transformation of focal complexes to focal adhesions at the rear side of the focal complexes.

When Necl-5 is knocked down in NIH3T3 cells, these cells do not form the leading edges toward the higher concentration of PDGF (Minami *et al.*, 2007a). The accumulation of the PDGF receptor and integrin $\alpha_v\beta_3$ at the peripheral membrane is not observed in these cells, resulting in the inability of the formation of focal complexes. Moreover, the local activation of Rap1 and Rac at the peripheral membrane is not detected in the Necl-5-knock-down cells. These cause the reduction of cell movement and indicate the importance of Necl-5 for cell movement.

5.2.3. Negative regulation of cell proliferation by sprouty induced by the downregulation of Necl-5

It has been found that the PDGF-induced activation of Ras-mediated cell proliferation signaling is regulated by Necl-5 and Sprouty2 (Kajita *et al.*, 2007). Sprouty was originally identified as an antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways (Hacohen *et al.*, 1998). It was then reported to be a negative regulator of growth factor-induced signaling (Christofori, 2003; Kim and Bar-Sagi, 2004). c-Src-catalyzed tyrosine-phosphorylated Sprouty inhibits the growth factor-induced activation of Ras and subsequent activation of Raf-MEK-ERK signaling at the site upstream of Ras and downstream of growth factor receptors (Gross *et al.*, 2001; Hanafusa *et al.*, 2002; Li *et al.*, 2004; Mason *et al.*, 2004). This site of action of Sprouty in growth factor-induced signaling is similar to that of Necl-5, although these two molecules show opposite roles in growth factor-induced signaling. A more recent study has certified that Necl-5 interacts with Sprouty2 and that this interaction reduces the inhibitory effect of Sprouty2 on PDGF-induced Ras signaling (Kajita *et al.*, 2007) (Fig. 1.8). However, when Necl-5 is downregulated from the cell surface by *trans*-interacting with nectin-3 at the cell–cell adhesion sites, Sprouty2 is released from Necl-5 and thus exhibits an inhibitory effect on Ras signaling. Taken together, both the downregulation of Necl-5 by the cell–cell contact-induced *trans*-interaction of Necl-5 with nectin-3 and the release of Sprouty from Necl-5 cooperatively suppress cell proliferation. This is at least one of the mechanisms underlying contact inhibition of cell proliferation, the molecular mechanism of which has not been fully elucidated. This phenomenon has been observed in normal cultured cells for a long time; when moving and proliferating cultured cells become confluent and form cell–cell junctions, they cease both movement and proliferation (Abercrombie and Heaysman, 1953; Fisher and Yeh, 1967). Therefore, the discovery of Necl-5 provides new insight into the elucidation of the mechanism on contact inhibition of cell movement and proliferation.

5.3. Pathological implications of nectin and Necl-5

The expression of nectin-1 at cell–cell junctions was reduced in human epithelial cancer cells located at the advancing border of the tumor, losing the cell–cell junctions and facilitating the invasion of cancer cells into the neighboring tissue (Matsushima *et al.*, 2003). On the other hand, mutations in human nectin-1 are responsible for cleft lip/palate-ectodermal dysplasia, which includes Zlotogora–Ogur syndrome and Margarita Island ectodermal dysplasia (Sozen *et al.*, 2001; Suzuki *et al.*, 1998, 2000) and is an autosomal recessive disorder, clinically characterized by unusual faces, dental anomalies, hypotrichosis, palmoplantar hyperkeratosis and onychodysplasia, syndactyly, cleft lip/palate, and in some cases, mental retardation. These phenotypes are mainly correlated with the places where nectin-1 is specifically expressed (Okabe *et al.*, 2004a). As indicated previously, human nectin-1 allows entry of all α-herpes viruses including HSV-1, HSV-2, pseudorabies virus, and bovine herpesvirus type 1 (Geraghty *et al.*, 1998), while human nectin-2 can mediate entry of a restricted number of α-herpes viruses (Warner *et al.*, 1998). The interaction of nectin-1 or nectin-2 with one of the HSV envelope glycoproteins recruits other viral glycoproteins to initiate fusion between the viral envelope and a cell membrane, thereby mediating entry of the viral nucleocapsid into the cell (Spear and Longnecker, 2003). The usual manifestations of HSV disease are mucocutaneous lesions. HSV establishes latent infection of neurons in sensory ganglia and causes recurrent lesions at the sites of primary infection. Thus, the intercellular spreading of HSV significantly contributes to the pathogenesis of HSV disease. The nectin–afadin system increases the efficiency of intercellular spreading, but not the entry of HSV-1, whereas the cadherin–catenin system increases the efficiency of both entry and intercellular spreading of HSV-1 (Sakisaka *et al.*, 2001).

The expression of Necl-5 is known to be upregulated in transformed cells (Chadeneau *et al.*, 1994; Ikeda *et al.*, 2003; Masson *et al.*, 2001; Mendelsohn *et al.*, 1989). Necl-5 is also upregulated in NIH3T3 cells overexpressing oncogenic Ki-Ras (V12Ras). The Necl-5 promoter has an AP-1-binding site and the upregulation of Necl-5 is mediated by the transcriptional activation of the *Necl-5* gene through the V12Ras-Raf-MEK-ERK-AP-1 pathway (Hirota *et al.*, 2005). This uncontrolled excess expression of Necl-5 overwhelms the rate of Necl-5 internalization upon cell–cell adhesion, resulting in the loss of contact inhibition in V12Ras-NIH3T3 cells (Minami *et al.*, 2007b). Consistent with this, an *in vivo* study showed that V12Ras-NIH3T3 cells gain metastatic ability by the upregulation of Necl-5 (Ikeda *et al.*, 2003). Moreover, the *trans*-interaction of Necl-5 in cancer cells with another Ig-like adhesion molecule CD226 in platelets enhances the metastasis of cancer cells to the lung (Morimoto *et al.*, 2007). On the other hand, poliovirus infects susceptible cells through Necl-5. It is thought that binding of Necl-5 to poliovirus, the outer coat of which is an icosahedral protein shell, initiates conformational changes that enable

the altered virion to bind to membranes and to invade cells even in the absence of the receptor (Hogle, 2002). It is not clear whether the target membrane for entry is the plasma membrane or an endosomal membrane. Poliovirus is the causative agent of poliomyelitis, manifestations of which are the spread and replication of virus in the central nervous system, particularly in the motor neurons. The cytoplasmic domain of Necl-5 on the surface of endosomes that might enclose an intact poliovirion could interact with a dynein subunit Tctex-1, and the endosomes could be transported in a retrograde direction along microtubules through the axon to the neural cell body where replication of poliovirus occurs.

Necl-2/TSLC1 has been shown to be a tumor suppressor in human non-small-cell lung cancer (NSCLC) (Kuramochi *et al.*, 2001). The expression of Necl-2/TSLC1 is reduced or absent in the A549 NSCLC line as well as several other NSCLC, hepatocellular carcinoma, and pancreatic cancer cell lines. Necl-2/TSLC1 expression or suppression is correlated with a promoter methylation state in these cell lines. Restoration of Necl-2/TSLC1 expression to normal or higher levels suppresses tumor formation by A549 cells in nude mice.

6. Common Utilization of Signaling Molecules in Forming Leading Edges and Cell–Cell Adhesion

The formation of the leading edge in moving cells depends on the cooperative roles of the PDGF receptor, integrin $\alpha_v\beta_3$, and Necl-5 upon stimulation of chemoattractants and is critical for effective and directional cell movement, as described previously. The dynamic restructure of cell protrusions occurs at the leading edge. The cell protrusions include specialized cellular structures such as filopodia, lamellipodia, focal complexes, and focal adhesions. The formation of cell protrusions requires spatiotemporal activation and inactivation of various signaling molecules, especially small G proteins such as Rap1, RhoA, Cdc42, and Rac, and their related actin-binding proteins such as IQGAP1, WAVE, and NWASP. In addition, afadin is recruited to the leading edge of moving cells and plays roles in the cyclical activation of Rap1 and Rac there (unpublished data). In moving cells, there is a pool of afadin that does not bind to nectin, although afadin primarily interacts with nectin at cell–cell adhesion sites. These molecules contribute to the reorganization of the actin cytoskeleton that mainly regulates the formation of the leading edge through the coordination of the cellular structures.

Interestingly, these signaling molecules and the cell adhesion molecules Necl-5 and integrin $\alpha_v\beta_3$, all of which localize at the leading edge, also participate in the formation of cell–cell adhesion. As shown in the previous

section, at the initial phase of cell–cell adhesion, Necl-5 *trans*-interacts with nectin-3 at primordial cell–cell contact sites and this *trans*-interaction replaces nectin-3 with nectin-1 due to the downregulation of Necl-5 from the cell surface. *Trans*-interacting nectin induces the activation of Rap1, Cdc42, and Rac cooperatively with integrin $\alpha_v\beta_3$, followed by the reorganization of the actin cytoskeleton through the actin-binding proteins. Then, cadherin is recruited to the nectin-based cell–cell adhesion sites and AJs are eventually formed. After the establishment of cell–cell junctions, both the PDGF receptor and nectin are involved in cell survival. It appears to be spatially reasonable that same molecules are utilized in the formation of both leading edge and cell–cell adhesion, because cell–cell adhesion occurs at the place where the leading edges of two moving cells meet.

7. Concluding Remarks

In this chapter, we have described our recent findings on molecular mechanisms of cell adhesion focusing on nectins and Necls and their cross-talk with other cell adhesion molecules including integrin and cadherin and growth factor receptors such as PDGF receptor. As described previously, recently identified cell–cell adhesion molecules such as nectins have greatly contributed to providing a new paradigm for understanding the regulatory mechanism of cell–cell adhesion. A number of studies on the mode of action of nectins and Necls have revealed at least partly the underlying mechanisms on contact inhibition of cell movement and proliferation, which have been known for a long time. This controlled cell movement and proliferation are critical for the physiological organization of tissues. If this process is disrupted, cells grow in an unlimited fashion and invade neighboring tissues, leading to pathogenesis such as cancer and atherosclerosis. Furthermore, nectins and Necl-5 play an essential role in the cross-talk between cell–matrix and cell–cell adhesion cooperatively with integrin $\alpha_v\beta_3$. However, there are still unresolved issues concerning the formation and maintenance of cell–cell adhesion. It is not fully understood how the elongation of axons ceases after synapse formation, although information on the attachment between axons and dendrites is sent to the cell body in a retrograde manner. It also remains to be elucidated how TJs are formed at the apical side of AJs in the process of apicobasal cell polarization at cell–cell adhesion sites in epithelial cells. Further studies on nectins and Necls may address these unresolved issues, and provide further insight on the mode of action of nectins and Necls regarding not only cell adhesion but also various cellular functions including cell proliferation, movement, differentiation, and survival.

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CHAPTER TWO

Neural Stem Cells in the Mammalian Brain

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Contents

Abstract

New fundamental results on stem cell biology have been obtained in the past 15 years. These results allow us to reinterpret the functioning of the cerebral tissue in health and disease. Proliferating stem cells have been found in the adult brain, which can be involved in postinjury repair and can replace dead cells under specific conditions. Numerous genomic mechanisms controlling stem cell proliferation and differentiation have been identified. The involvement of stem cells in the genesis of malignant tumors has been demonstrated. Neural stem cell tropism toward tumors has been shown. These findings suggest new

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lines of research on brain functioning and development. Stem cells can be used to develop radically new treatments of neurodegenerative and cancer diseases of the brain.

Key Words: Stem cells, Stem cell self-renewal, Neuron, Brain, Transplantation, Nerve cell differentiation.

1. Introduction

The stem cell is one of the most popular topics in current biology and medicine. The increasing number of publications indicates that stem cells are of great interest to a wide range of biological and medical scientists. The topic of stem cells is central in developmental biology. Studying molecular mechanisms of stem cell self-renewal and differentiation control promises to shed light on key problems in cell biology. A great number of publications form a base of new data; however, these data often do not solve the problem posed but rather raise new and more complex questions. One of these unclear issues is the definition of the stem cell or, to be more specific, what distinguishes a stem cell from other cells. The idea of stem cells was proposed by the Russian histologist Alexander Maximov (1907). It was accepted that the adult body lacks stem cells and their existence is limited to the earliest period of embryonic development. Another Russian histologist, Friedenstein (1976), found these cells in the mesenchyme (stroma) of the adult bone marrow. Based on their localization, these cells were later assigned to stromal or mesenchymal stem cell groups.

Stem cells are divided into embryonic stem cells (ESCs) isolated from blastocyst stage embryos and regional stem cells isolated from later embryonic or adult tissues. In ontogeny, all organs and tissues result from the proliferation and differentiation of blastocyst cells, which are ESCs in the strict sense (Brustle *et al.,* 1999; Gage, 2000). ESCs are pluripotent (i.e., they give rise to derivatives of all germ layers including nervous system cells). The multistage development of ESCs results in pools of regional stem cells varying by their potential for differentiation in the developing and adult body.

Most adult stem cells have a limited differentiation potential and can largely give rise to derivatives of a single germ layer, ectoderm in the case of neural stem cells (NSCs). They also represent a substantial repair reserve and can correct various defects in different organs including the nervous system (Loseva, 2001).

The most common definition of stem cells involves their conformity to three main conditions: (1) multipotency (i.e., the capacity to give rise to different cell types); (2) high proliferative potential; and (3) self-renewal

(i.e., the capacity to reproduce identical descendants by symmetrical divisions) (Hall and Watt, 1989; Potten and Loeffler, 1990). However, the diversity of cells assigned to stem cells can go beyond this definition. For instance, germline cells, often considered as stem cells, are unipotent. Other cells can self-renew only within a limited time period or under specific conditions. For instance, cells of the hippocampal dentate gyrus (DG) (described later) divide only asymmetrically in adult mammals to yield committed progenitors (Encinas *et al.,* 2006); however, in culture these cells self-renew and generate neurospheres containing the whole range of neural progenitors including NSCs (Mignone *et al.,* 2004). On the other hand, descendants of stem cells not recognized as stem cells in many cases meet all three conditions. For instance, type C cells in the subventricular zone (SVZ) of the lateral ventricles (see later), called amplifying progenitors rather than stem cells in culture medium supplemented with epidermal growth factor (EGF), satisfy all three conditions of stem cells (Doetsch *et al.,* 2002). Hence, the above definition is vague and needs to be refined in the future. For instance, Mikkers and Frisen (2005) proposed defining stem cells as cells halted somewhere along the line of specialization and dividing to give rise to cells of their own type and to cells progressing along the line. In terms of formal logic, this definition seems more consistent; however, it also does not cover the whole range of stem cell properties. This chapter concerns some problems related to NSCs that we consider of primary importance for neurobiology and developmental biology.

2. Neural Stem Cells and Their Niches in the Adult Mammalian Brain

2.1. General description of NCSs

NSCs are classified as regional stem cells. The finding of stem cells in the nervous system has shaken a number of established concepts, particularly concerning recovery processes in the central nervous system. NSC has the same properties as the stem cell in general. The molecular markers that allow the identification of NSCs as well as the subsequent stages of their differentiation are known (Gage *et al.,* 1995). Note, however, that these markers are relative and their significance depends, in particular, on the cell environment and state. For instance, in the adult brain, the standard NSC marker nestin can be found in stem cells as well as in endothelial and reactive glial cells (e.g., in injury). Moreover, a single cell can express two or more of the above-mentioned markers under particular conditions. A virtually unlimited proliferative capacity allows stem cells to self-renew after symmetric divisions or to give rise to precursor cells after asymmetric divisions (Gage, 2000; van der Kooy and Weiss, 2000; Watt and Hogan, 2000).

Stem cells have been found in the central nervous system of adult animals and humans. First, they have been found in the brain parts known for active neurogenesis throughout the life span: the SVZ of the lateral ventricles and the dentate gyrus (DG) of the hippocampal formation. Proliferative activity of cells in these parts was reported long ago (Altman and Das, 1965; Altman, 1969). The ability of cells in these parts to give rise to both astrocytes and neurons was later demonstrated (Reynolds and Weiss, 1992; Luskin, 1993; Palmer *et al.,* 1995).

Both proliferative zones of the adult mammalian brain, the subgranular layer of the DG and the subependymal layer of the SVZ, demonstrate that cells glial by morphology and protein markers, but essentially stem cells can divide to generate both glial cells and neurons (Seri *et al.,* 2001). Notably, these DG and SVZ stem cells first give rise to glial fibrillary acidic protein (GFAP)-negative actively dividing progenitor cells (type C in the subependymal zone and type D in the subgranular zone of the DG) and only then to neuroblasts (Seri *et al.,* 2001; Alvarez-Buylla and Garcia-Verdugo, 2002).

In the adult brain, stem cells are localized to particular niches, structural elements of the microenvironment allowing stem cells to maintain their identity and modulate their proliferative activity and fate (Watt and Hogan, 2000). Extensive cell–cell interactions, close association with blood vessels, abundant extracellular matrix (ECM), and specialized basal lamina are the key components of this microenvironment (Palmer *et al.,* 2000; Mercier *et al.,* 2002; Doetsch, 2003). In the SVZ, the proximity of the cerebrospinal fluid of the lateral ventricle, which is the target for factors secreted by the choroid plexus, is significant (Doetsch, 2003).

The vascular niche plays an important role in the fate of NSCs. For instance, neurogenesis in the DG takes place in the foci associated with blood vessels (Palmer *et al.,* 2000). The vasculature is an integral component of the stem cell niche. Endothelial cells, perivascular macrophages, and fibroblasts release mitogens, trophic factors, and neural differentiation signals: basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), interleukin-8, and brain-derived neurotrophic factor (Leventhal *et al.,* 1999; Grothe *et al.,* 2001) that exert an effect on NSCs (Vescovi *et al.,* 1993; Kuhn *et al.,* 1997; Gritti *et al.,* 1999). Angiogenesis and neurogenesis can be coregulated by reciprocal signals. Both processes share common regulatory factors: bFGF, VEGF, IGF-1, and transforming growth factor-α (TGF-α) (Leventhal *et al.,* 1999; Louissaint *et al.,* 2002). The functional relationship between NSCs and blood vessel cells has been experimentally confirmed by Shen *et al.* (2004), who demonstrated that endothelial cells (rather than vascular smooth muscle cells) release soluble factors inducing self-renewal, inhibiting differentiation, and promoting neuronal production of NSCs.

Epithelial cells, pericytes, perivascular macrophages, and fibroblasts line the vascular lumen and are separated from the brain by the basal lamina (Mercier *et al.,* 2002). The basal lamina contains considerable quantities of heparan sulfate glycosaminoglycans, which have high affinity to growth factors such as bFGF (Yayon *et al.,* 1991). Hence, the basal lamina can tether and accumulate factors, anchor cells, and provide spatial signals in the stem cell niche. Carbohydrates associated with the ECM increase the ligand activity and bind them for storage. The molecules associated with the ECM and cell surface can be cleaved to release active ligands and soluble inhibitors. For instance, the effect of factors of endothelial and other cells surrounding the stem cell niche can be regulated by their binding to the ECM and basal lamina (Heissig *et al.,* 2002; Leventhal *et al.,* 1999; Mercier *et al.,* 2002).

The above-mentioned proliferative zones in the adult brain have both common and specific structural and functional properties. Nerve cells derived from these zones have different migration routes and different functional patterns; however, the structural properties of the germinal zones in the adult brain discussed later do not exclude the common origin of the principal components of these structures.

2.2. Neural stem cells in the subventricular zone

The largest germinal zone in the adult brain is located along the lateral wall of the lateral ventricles (Doetsch *et al.,* 1996, 1999). *In vivo* clonal analysis has demonstrated that NSCs amount to less than 0.2 to 0.4% of cells in the SVZ germinal zone (Morshead *et al.,* 1998). Retroviral-mediated transfer of bacterial β-galactosidase and alkaline phosphatase genes has demonstrated the postnatal production of cells later differentiated into neurons and astrocytes as well as into oligodendrocytes (Gage *et al.,* 1995; Levison and Goldman, 1993).

The views concerning the identification of stem cells in the germinal zone of the lateral ventricles have changed. Previously, ependymal cells giving rise to actively proliferating cells migrating to the subependymal layer of the SVZ and further in the rostral migratory stream (RMS) to the olfactory bulb to differentiate into neurons and glia were considered stem cells (Johansson *et al.*, 1999). The significance of the mitotic spindle orientation for the production of postmitotic daughter cells in the ventricular zone has been demonstrated. If the plane of mitotic division is parallel to the ependymal layer, an asymmetric division occurs to give rise to a stem cell staying in the ependyma and a daughter cell migrating to the subependymal zone, where it divides several times to generate many neural precursor cells (progenitors). If the plane of mitotic division is perpendicular to the ependymal surface, a symmetric division takes place (self-renewal) (Chenn and McConnel, 1995; Johansson *et al.*, 1999). Indeed, such processes are observed during the embryonic development of the forebrain (Gotz and Huttner, 2005).

Later publications demonstrated that cultured ependymal cells indeed form spherical clones but can differentiate only into astrocytes, while subependymal cells form true neurospheres including cells of both neuronal and glial lineages (Laywell *et al.*, 2000; Chiasson *et al.*, 1999). In addition, ependymal cells do not express the important marker of NSCs Lewis X (LeX), a carbohydrate found in embryonic pluripotent stem cells (Capela and Temple, 2002). These facts are inconsistent with the views that ependymal cells are SVZ stem cells.

It is currently accepted that true stem cells are localized to the subependymal layer of the SVZ of the lateral ventricles. The population of neural cells in the subependymal layer includes three cell types (Doetsch *et al.*, 1997, 1999). Type B cells in the subependymal layer of rat brain lateral ventricle express glial marker, GFAP, and surround the streams of rostral migration of type A cells (immature neuroblasts). Type B cells divide relatively rarely to give rise to actively proliferating type C cells expressing neither glial nor neuronal markers. Consequently, type C cells actively replicate and are referred to as amplifying. Later they give rise to type A cells expressing markers of migrating neuroblasts, polysialylated neural cell adhesion molecule (PSA-NCAM), doublecortin, and TuJ1 (Rousselot *et al.*, 1995; Doetsch *et al.*, 1999; Gleeson *et al.*, 1999). In the intact adult brain, type A cells migrate to the olfactory bulb along the RMS. The rodent RMS consists of longitudinal chains of neuroblasts possessing a leading process with a growth cone (Doetsch *et al.*, 1996; Wichterle *et al.*, 1997). The neuroblast chains are ensheathed by type B cells. In the olfactory bulb, neuroblasts incorporate into the granular cell layer and periglomerular region, where some of them become differentiated neurons (Luskin, 1993). The germinal zone also exists in the subventricular zone in the human brain; however, nascent cells do not migrate to the olfactory bulb in chains but leave the periventricular zone one by one. Their final migratory goal remains unclear (Sanai *et al.*, 2004).

GFAP-immunopositive type B cells are considered regional NSCs. Type B cells closely interact with ependymal (type E) cells and at least some of them contact the lumen of the lateral ventricle. Processes of type B cells contacting the lateral ventricle have a single short cilium projecting into the lumen (Doetsch *et al.*, 1999). Although GFAP is considered as an astroglial marker, $GFAP^+$ type B SVZ cells essentially differ from $GFAP^+$ astroglial cells from other brain parts by phenotype and morphology. Both primary and adhesive cultures of $GFAP^+$ cells from the SVZ coexpress nestin and LeX/CD15 (NSC markers) and can form true neurospheres including all three cell types of the neural lineage. The primary and adhesive cultures of astrocytes from the cortex and white matter do not express these markers and have no neurogenic potential (Imura *et al.*, 2006).

The stemness of GFAP$^+$ SVZ cells has been confirmed by the study of the brain in transgenic mice carrying an inducible Cre recombinase (Cre-ER[T2]) controlled by the human GFAP promoter (hGFAP). 4-Hydroxytamoxifen injections induced Cre recombination in astroglial cells at postnatal day 5 and allowed these cells and their descendants to be permanently tagged. Reporter-tagged cells first appeared among quiescent astroglial cells expressing the stem cell marker LeX in the SVZ and DG. After 2 to 4 weeks, proliferating progenitors expressing the neuronal marker Doublecortin (Dcx) appeared among reporter-tagged cells. After 4 weeks, the reporter tag could be found in mature neurons in the olfactory bulb, DG, and even neocortex (Ganat *et al.*, 2006).

Cultured SVZ cells exposed to EGF form self-renewing multipotent neurospheres. Doetsch *et al.* (2002b) have demonstrated that EGF-responsive cells descend from actively dividing type C cells expressing distal-less homeobox transcription factor Dlx2 rather than from relatively rarely dividing stem (type B) cells. Type C cells exposed to EGF decrease the expression of Dlx2, stop neurogenesis, and start active proliferation by symmetric divisions. Thus, type C cells considered as committed amplifying cells can reproduce stem cell properties after exposure to growth factors (Doetsch *et al.*, 2002b).

A study of 2′,3′-cyclic nucleotide 3′-phosphodiesterase-enhanced green fluorescent protein (CNP-EGFP) transgenic mouse with NG2 chondroitin sulfate proteoglycan-expressing cells tagged by green fluorescent protein (GFP) has demonstrated that NG2$^+$ cells in the SVZ can self-renew *in vitro* and have phenotypic properties of transit-amplifier type C-like multipotent cells. They actively proliferate and express EGF receptors as well as transcription factors Dlx, Mash1, Olig2, and LeX antigen but not GFAP. After transplantation into the lateral ventricles of mice at postnatal day 2, NG2$^+$ cells migrate to the hippocampus and give rise to GABAergic neurons (Aguirre *et al.*, 2004). NG2$^+$/CNP$^-$EGFP$^+$ cells can migrate throughout the RMS and contribute to both neurogenesis and gliogenesis by generating interneurons and oligodendrocytes in the olfactory bulb (Aguirre and Gallo, 2004). The process of type C cell proliferation is controlled by p27Kip1, a regulator of G_1 phase transition. A study of p27Kip1 null mice has demonstrated an increased total number of type C cells in the SVZ as well as the number of proliferating type C cells identified by [^{3}H]thymidine labeling. At the same time, the number of type A cells decreased (Doetsch *et al.*, 2002a). In contrast, cortical, olfactory bulb, or cerebellar NG2$^+$ cells have a very limited migratory potential and give rise to glia in the subcortical white matter and striatum (Aguirre and Gallo, 2004).

In vitro clonal analysis has demonstrated that resident NSCs are localized to both the subependymal layer of the SVZ and its rostral extension, RMS, along which neuroblasts migrate from the subependymal layer to the olfactory bulb. Accurate isolation of the proximal RMS within the olfactory

bulb demonstrated the presence of multipotent self-renewing cells distinct from those that migrated from the subependymal layer of the lateral ventricles. This confirms that stem type B cells are components of the walls of migratory streams for type A neuroblasts (Gritti *et al.*, 2002).

Despite cardinal postnatal changes in the germinal zone arrangement, there is a continuity between the embryonic and adult germinal centers in the brain. Overall, the observed morphological and marker changes in the SVZ cell phenotype argue that subependymal glial stem cells (type B) in adult animals are modified descendants of the radial glia (Tramontin *et al.*, 2003). Postnatally, these cells gradually lose the radial glial markers while maintaining the above-mentioned contact with the lumen of the lateral ventricle. This contact is mediated by a process with a single cilium similar to that in fetal radial glial cells. Ependymal cells also descend from a fraction of radial glial cells. They gradually replace radial glial cells in postnatal development to eventually line the entire wall of the lateral ventricle. In contrast to type B cells and radial glial cells, they have many long cilia. Morphological analysis has identified a series of stages of radial glial cell transformation into ependymal cells, which has been confirmed using a Cre-lox recombination strategy (Spassky *et al.*, 2005). The assignment of SVZ stem (type B) cells and radial glia to the same lineage was demonstrated by retroviral-mediated transfer of activated Notch1 with alkaline phosphatase as a reporter gene to the mouse embryonic forebrain before neurogenesis started. During embryogenesis, Notch1-transfected cells transformed into radial glia. In postnatal mice, many Notch1-transfected cells transformed into paraventricular astrocytes (i.e., stem cells of the subependymal layer of the SVZ) (Gaiano *et al.*, 2000).

Apparently, the contact between SVZ stem cells and the lumen of the lateral ventricle, where choroid plexus factors are released, is important for the regulation of proliferative activity and differentiation (Doetsch, 2003). The contact between these cells and the basal lamina is an essential neuroepithelial component of the embryonic niche for radial glia. This contact is likely in the niche for SVZ stem cells in the adult brain. The proliferation and differentiation in the subependyma of the lateral ventricles are closely associated with the vasculature and its basal lamina (Mercier *et al.*, 2002). The vascular basal lamina in the subependymal zone forms processes extending from the tips of perivascular macrophages toward the ependyma (Fig. 2.1). The extravascular processes of the basal lamina consist of stems 0.1 to 0.5 μm in thickness and 5 to 50 μm in length and bulbs 1 to 4 μm in diameter (Fig. 2.2). Under the electron microscope, the bulbs look like labyrinths with the basal lamina contacting numerous processes of ependymal and subependymal parenchyma cells including type A, B, and C cells. Hence, the extravascular basal lamina is associated, on the one hand, with the fibroblast/macrophage network and, on the other hand, with the whole set of parenchymal components of the niche for subependymal

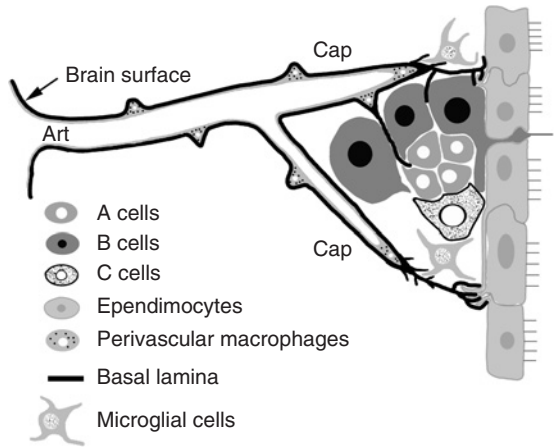

Figure 2.1 Schematic representation of the neurovascular niche in the subependymal layer of the rat subventricular zone. The extravascular processes of the basal lamina extend from the tips of perivascular macrophages. Their stems go along the subependymal layer and end in bulbs directly under the ependyma. Perivascular macrophages belong to the fibroblast/macrophage network starting on the brain meninges. The bulbs are compacted multifolded terminations of the basal lamina projection engulfing processes of ependymal cells and cells of the subependymal layer. Art, artery; Cap, capillary. (Modified with permission from Mercier *et al.*, 2002.)

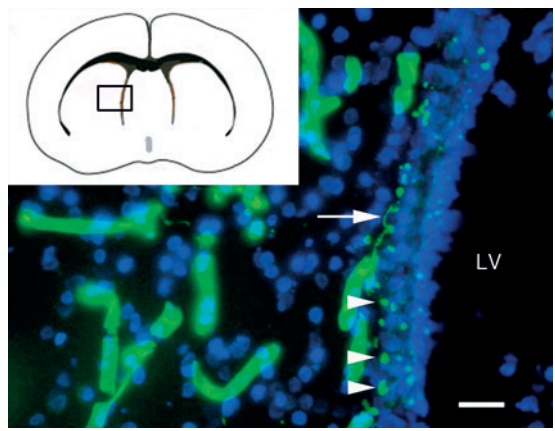

Figure 2.2 Immunocytochemical detection of the basal lamina protein laminin in the subventricular zone of the rat brain. Laminin was stained with Cy2-conjugated antibodies. Extravascular processes of the basal lamina consist of thin stems (arrow) and bulbs (arrowheads) positioned directly under the ependyma. Cell nuclei were counterstained with Hoechst 33342. The position of the frame on the section of rat forebrain is shown schematically in the upper left inset. LV, lateral ventricle. Scale bar = 20 μm.

stem cells. These associations can underlie the joint regulation of angiogenesis and neurogenesis and gliogenesis in the SVZ of the lateral ventricles (Mercier *et al.*, 2002).

The notion of a neurovascular niche in the mammalian brain under pathological conditions was further studied in the laboratory of S. Thomas Carmichael. These studies demonstrated that stroke induces neurogenesis in the rat SVZ and migration of new neurons descending from $GFAP^+$ cells to the periinfarct region. This migration is regulated by the vascularly produced chemokines stromal-derived factor 1 (SDF1) and angiopoietin 1 (Ang1). As a result, neurogenesis and vasculogenesis become causally connected and a neurovascular niche is formed in the periinfarct region, where new neurons eventually appear (Ohab *et al.*, 2006).

2.3. Neural stem cells in the dentate gyrus

The second main area in which NSCs are located is the DG of the hippocampal formation. NSCs have been found in the adult hippocampus of rats (Palmer *et al.*, 1997), mice (Kempermann *et al.*, 1997), primates (Gould *et al.*, 1999), and humans (Eriksson *et al.*, 1998; Kukekov *et al.*, 1999; Roy *et al.*, 2000). According to Kempermann *et al.* (1997), the DG of 9-week-old mice daily generates one neuron per 2000 DG neurons. The rate of neurogenesis decreases with age, although it is still observed in the mature and aged brain of rodents and primates including humans (Rao *et al.,* 2006). Certain pathologies such as epileptogenesis can increase neurogenesis (Parent and Lovenstein, 2002).

Stem cells in the subgranular layer of the DG give rise to precursor cells that differentiate into mature granule cells and glial elements. Precursor cells descend from stem cells located in the basal region of the granular cell layer (Seaberg and van der Kooy, 2002; Encinas *et al.,* 2006). Axons of new neurons in the DG can be traced up to the CA3 area, which suggests the involvement of these cells in hippocampal functions (Hastings and Gould, 1999). Double labeling ([^{3}H]thymidine and neuron-specific enolase or glial fibrillary acidic protein) allowed Cameron *et al.* (1993) to demonstrate that most newly born cells (about 85%) differentiate into neurons. The significance of neurogenesis in the adult hippocampus remains unclear. Aimone *et al.* (2006) proposed that new neurons are involved in the formation of the temporal clusters of long-term memory.

Substantial data on neurogenesis in the hippocampus of adult mammals have been obtained in studies on transgenic mice, in which NSCs were marked by the expression of GFP under the nestin promoter (Mignone *et al.*, 2004; Encinas *et al.*, 2006). These studies demonstrate that nestin-expressing cells are localized only on the subgranular layer in the DG (Fig. 2.3). The cells have a unipolar process crossing the granular layer and extensive branching in the molecular layer. In addition to nestin,

these cells express GFAP, vimentin, transcription factor Sox2 crucial for the maintenance of the pluripotent state of ESCs, and brain fatty acid-binding protein (B-FABP) (it is first expressed in neuroepithelial precursor cells and later becomes restricted to radial glial cells and immature astrocytes (Feng *et al.*, 1994). They divide at a relatively low rate and thus were called quiescent neural progenitors (QNPs). The observed QNP divisions were exclusively asymmetric with the division plane parallel or inclined to the subgranular layer. These asymmetric divisions give rise to cells in the subgranular layer with low levels of the reporter protein. They stain very weakly for nestin and do not express other QNP markers mentioned above. A bromodeoxyuridine (BrdU) assay demonstrated the high rate of their division; accordingly, these cells were called amplifying. Their descendants proceed from proliferation to differentiation and migrate to the granular layer to become largely granule cells with neuronal markers. *In vivo,* QNPs are capable of asymmetric divisions and were thus assigned to stem-like

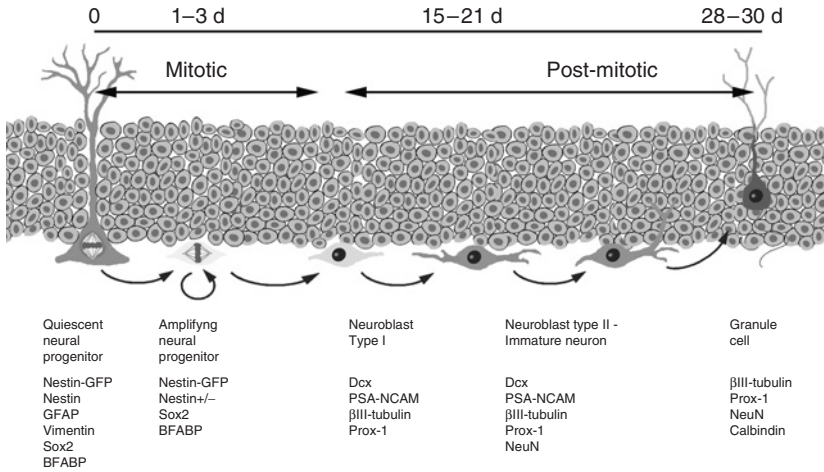

Figure 2.3 **The process starts with an asymmetric division of quiescent neural progenitors (QNPs) expressing several markers of neural stem cells and early neural progenitors: nestin, glial fibrillary acidic protein, vimentin, Sox2, and brain fatty acid binding protein (BFABP). Asymmetric division of quiescent neural progenitors gives rise to amplifying neural progenitors, expressing nestin, Sox2, and BFABP, but not glial fibrillary acidic protein and vimentin. After several symmetric divisions, they withdraw from the cell cycle to become type I neuroblasts after 1 to 3 days expressing markers of early neuroblasts: doublecortin (Dcx), polysialic acid neural cell adhesion molecule (PSA-NCAM), *β*III-tubulin, as well as a marker of differentiated neurons homeobox prospero-like protein (Prox-1). After 15 to 20 days, type I neuroblasts reach the stage of immature neurons expressing DNA-binding neuron-specific protein NeuN in addition to markers expressed by type I neuroblasts. Finally, 10 to 15 days later, immature neurons become mature granule cells of the dentate gyrus expressing *β*III-tubulin, Prox-1, NeuN, and calbindin. (Modified with permission from Encinas *et al.*, 2006.)**

rather than stem cells. However, in culture these cells can form neurospheres with the whole range of neural progenitors including cells expressing nestin. As a result, QNPs can be considered stem cells (Mignone *et al.,* 2004).

Drugs and physiological factors affecting the rate of proliferation in the DG proliferative zone have been identified. For instance, antidepressants of the selective serotonin reuptake inhibitor group such as fluoxetine increase neurogenesis in the DG, while behavioral stress decreases it (Malberg *et al.*, 2000; Malberg and Duman, 2003; Warner-Schmidt and Duman, 2006). Moreover, enhanced neurogenesis was required for antidepressant effects (Santarelli *et al.*, 2003). A similar effect was observed for electroconvulsive seizure (ECS) treatment, which also has an antidepressant effect (Madsen *et al.*, 2000; Newton *et al.*, 2006). It is of interest that ECS also enhances angiogenesis, which agrees with the previously observed relationship between angiogenesis and neurogenesis in the hippocampus (Palmer *et al.*, 2000). Encinas *et al.* (2006) demonstrated that antidepressant fluoxetine has no effect on asymmetric division of stem QNP cells in the DG but increases symmetric divisions of amplifying cells and increases their number in the adult brain. Clearly, these cells are the target for this antidepressant in drug therapy for depression.

Similar to the subependymal layer of the lateral ventricles, an important vascular component of the stem cell niche has been identified in the DG (Palmer *et al.*, 2000). It was shown that 37% of proliferating cells in the DG are endothelial precursors. Neural precursors and angioblasts proliferate in common nests associated with microvessels, and hence with the basal lamina.

2.4. Neural stem cells in other brain parts

Seri *et al.* (2006) have described a new important population of NSCs localized in the subcallosal zone (SCZ) of the mouse brain. This laminar structure is a caudal extension of the SVZ and resides between the dorsal hippocampus and corpus callosum. In contrast to the SVZ, SCZ does not border on the lateral ventricle. Ultrastructural investigation of the SCZ demonstrated that it includes cells typical of the SVZ astrocytes (type B cells), migrating neuroblasts (type A cells), and type C cells (Doetsch *et al.*, 1997) as well as clustered ependymal cells (type E cells). Seri *et al.* (2006) consider the behavioral pattern of progenitor cells expressing PSA-NCAM as the main distinction between the SCZ and SVZ. PSA-NCAM$^+$ cells in the SVZ form chains oriented along the rostrocaudal axis (Doetsch and Alvarez-Buylla, 1996), while PSA-NCAM$^+$ cells in the SCZ form clusters with only a minor fraction of cells in the orientation typical of those in the SVZ. Clearly, SCZ was formed after the closure of the walls of the dorsal

lateral ventricle separating the hippocampus from the neocortex in the developing brain and reduced in the subsequent postnatal growth of the corpus callosum and hippocampal enlargement. Isolated and cultured SCZ cells can form neurospheres. Cloned neurosphere cells differentiate into oligodendrocytes, astrocytes, and neurons.

The fate of SCZ cells was studied *in vivo* using cell labeling by retroviral vector expressing acid phosphatase. After 30 days, labeled cells with morphological features of oligodendrocytes have been identified in the corpus callosum. The fate of GFP-tagged SCZ cells was studied in the normal mouse brain. Thirty days after transplantation, GFP^+ cells with immunohistochemical features of astrocytes have been identified in the corpus callosum. Thus, the migration of astrocytic precursors from the SCZ to the corpus callosum cannot be excluded *in vivo*. Seri *et al.* (2006) propose the SCZ as a source of oligodendrocytes for the corpus callosum; however, the signals of the microenvironment that can induce the transdifferentiation of migrated astrocytic precursors and their transformation into oligoglia remain unclear (Seri *et al.*, 2006).

Cells conforming to the definition of stem cells have also been found in adult brain parts where no neurogenesis is normally observed (e.g., in the neocortex) (Palmer *et al.*, 1995; Gould *et al.*, 1999). Brain injury or exposure to growth factors was proposed to activate these quiescent stem cells and initiate a latent program of neurogenesis (Palmer *et al.*, 1999). Selective degeneration of neurons in the rat cortex projecting to the thalamus induced differentiation of endogenous progenitors into mature neurons. The differentiation not only replaced affected neurons but also restored the corticothalamic projections (Magavi *et al.*, 2000). It is also possible that affected neurons were replaced with descendants of SVZ stem cells that migrated to the affected cortical region instead of the olfactory bulb under the influence of signals from degenerating cells (Goings *et al.*, 2004; Ohab *et al.*, 2006). Note, however, that the problem of the formation of new neurons in the adult cortex of animals and humans remains controversial and is not completely solved. A more recent publication addressing this problem presents data obtained on autopsy material using incorporation of intravenously administered BrdU into cortical cells in seven patients for tumor diagnosis. Immunocytochemical analysis has identified no $BrdU^+$ neurons in the neuronal structures of the neocortex and its incorporation was limited to astroglial cells, suggesting that no new neurons are generated in the adult neocortex and this process is limited to the perinatal period (Bhardwaj *et al.*, 2006).

A persistent population of multipotent stem cells proliferating throughout the life span can be found in the mammalian olfactory epithelium. The descendant progenitor cells go through several differentiation stages to replace dying olfactory receptor neurons (Roisen *et al.*, 2001; Murrell

et al., 2005; Marshall *et al.*, 2006). NSCs isolated from the human, rat, or mouse olfactory mucosa possess multipotency (Chen *et al.*, 2004). The neurospheres derived from these cells *in vitro* contained about 1000 cells expressing nestin and markers of glia (GFAP, sulfatide O4—surface marker of oligodendrocytes, and galactocerebroside [GalC]) and neurons (β-tubulin III and microtubule-associated protein MAP5). At the same time, the level of neuronal marker expression depended on the presence of various inducers of differentiation (retinoic acid, serum, nerve growth factor, and ciliary neurotrophic factor). In addition, cells of the basal layer in the mouse olfactory epithelium were reported to be capable of extraneuronal differentiation (Chen *et al.*, 2004). A cell subpopulation in the human nasal olfactory mucosa, olfactory ensheathing cells, has the properties of NSCs (Barnett *et al.*, 2000).

A source of NSCs, external germinal layer, has been found in the cerebellum. The external germinal layer persists over different time periods in the postnatal ontogeny of different animals and is commonly reduced in the adult organism. Although the population of self-renewing cells is not maintained throughout the entire life span, quiescent cells capable of proliferation after exposure to the corresponding external signals can exist (Gage *et al.*, 1995). NSCs from the postnatal cerebellum have been isolated and studied. They have the NSC marker prominin (CD133) but lack markers of neuronal and glial lineages. Such cells isolated from the postnatal cerebellum could differentiate into astrocytes, oligodendrocytes, and neurons in neurosphere culture (Lee *et al.*, 2005).

The neural crest, a transient organ of vertebrates formed along the whole embryo from the thalamencephalon to sacral somites and below, is another source of NSCs. Cephalic neural crest cells give rise to the ganglia of craniocerebral nerves as well as to the otic, vestibular, and ciliary ganglia. A fraction of cells of the cephalic neural crest generate choroid plexus stem cells. A receptor of the growth factor NTF3, p75, is an important surface marker of neural crest stem cells, while their population is not stained with antibodies against peripherin, which is expressed in Schwann cells. Neural crest stem cells were isolated from several parts of the embryonic brain, peripheral nerves, and dorsal spinal ganglia. Cultured clones of neural crest stem cells featured a pronounced heterogeneity. Migrating progenitor cells attached to the plastic surface and spread to form a monolayer (Morrison *et al.*, 1999; Stemple and Anderson, 1992). Neural crest stem cells transplanted into the embryonic brain partially differentiated into cholinergic neurons both in the brain and in the periphery (White *et al.*, 2001).

Certain cell types in the adult mammalian brain both within and outside the germinal zones also demonstrate stem cell properties in the culture. For instance, cells immunoreactive for proteoglycan NG2 and considered as oligodendrocyte progenitors can proliferate throughout the life span (Dawson *et al.*, 2000). These cells demonstrate a multipotent phenotype *in vitro*. Their descendants differentiate into electrically excitable neurons,

astrocytes, and oligodendrocytes (Belachew *et al.*, 2003). Microvascular pericytes from the mouse brain demonstrate similar multipotent properties *in vitro* (Dore-Duffy *et al.*, 2006).

3. Transcriptional Regulation of NSC Self-Renewal and Differentiation

The capacity of stem cells (both NSCs and ESCs) to transform into different cell types makes them a convenient model to study the molecular genetic events underlying self-renewal and differentiation into different lineages. During the development of the central nervous system, gene sets are induced in NSCs and associated tissue systems, which determine the fate, proliferation, self-renewal, and commitment to differentiation of stem cells. The identification of stem cells by reporter genes, cultivation, and transplantation into developing and adult experimental animals allow us to analyze the functions of gene networks at successive developmental stages (Korochkin, 2003).

The genomic control of stem cell fate has been actively studied in more recent years. In particular, the control mechanisms underlying the maintenance of the pluripotency and multipotency (i.e., the stemness) have been identified. Numerous publications reveal stemness genes in mouse and human embryonic stem cells.

3.1. Regulation of self-renewal and stemness

Identification of the mechanisms underlying the maintenance of ESC pluripotency and NSC multipotency is a key problem in current developmental biology. In cell cultures, the ESC stemness is maintained by the underlying layer of feeder cells providing them with all required signals for pluripotency. To date, we know several factors allowing feeder-free ESC culturing. The mechanisms underlying the maintenance of ESC pluripotency are different in humans and mice. For instance, leukemia inhibitory factor (LIF) signaling important for mouse ESCs (Chambers and Smith, 2004) failed to maintain the pluripotency of human ESCs (Daheron *et al.,* 2004). The responses to bone morphogenetic protein (BMP) signaling also significantly differed (Ying *et al.,* 2003b; Gerami-Naini *et al.,* 2004). Wnt signaling maintained the ESC pluripotency in both species (Sato *et al.,* 2004); however, it was not sufficient for the human ESC pluripotency without the interaction with TGF-β/activin/nodal signaling (James *et al.,* 2005).

The differences between stem cells and committed progenitors are studied at different levels. The development of methods for global gene

expression analysis made it possible to study stem cell transcriptions in order to identify the general molecular profile of stemness. However, numerous efforts failed to reveal the common fingerprint pattern for all studied ESC populations (Ivanova *et al.,* 2002; Ramalho-Santos *et al.,* 2002; Sato *et al.,* 2003; Sperger *et al.,* 2003; Bhattacharya *et al.,* 2004; Ginis *et al.,* 2004; Byrne *et al.,* 2006). Only two genes, *Oct4* and *Nanog*, were identified in all experiments irrespective of the cultivation method and animal species.

Coimmunoprecipitation experiments allowed Wang *et al.* (2006) to identify the factors directly interacting with Nanog and forming a protein network, which was proposed to mediate ESC pluripotency. Nanog was shown to maintain ESC pluripotency in mice independently of LIF/Stat3 (Mitsui *et al.,* 2003). Oct4-deficient mouse blastocyst resulting from targeted disruption of this gene had no pluripotent inner cell mass (Nichols *et al.,* 1998). During gastrulation, expression of this transcription factor decreases and is later confined to primordial germ cells (Pesce *et al.,* 1998). *Oct4* expression was also found in NSCs of adult monkeys (Davis *et al.,* 2006).

NSCs were also used to study the transcriptional profiles (Geschwind *et al.,* 2001; Easterday *et al.,* 2003; Mi *et al.,* 2005; Parker *et al.,* 2005; Gurok *et al.,* 2004). Comparison of such data for NSCs, hematopoietic stem cells (HSCs), and ESCs demonstrated an overlap in the transcription pools; however, this overlap was minor and did not exceed that between stem cells and any other cell type (Geschwind *et al.,* 2001). This result can be attributed to the heterogeneity of the initial material. Most studies use the primary culture of cells from the SVZ of the lateral ventricles forming neurospheres as NSCs, although cell heterogeneity in the neurospheres is well known (Vescovi *et al.,* 1993). Studies on the NSC clone C17.2 fulfilling a strict operational definition yields more reliable results (Parker *et al.,* 2005). This definition is identical to that used in hematopoiesis. In brief, it includes (1) multipotency, (2) the ability to populate a developing region or to repopulate a degenerated region, (3) the ability to be transplanted, and (4) self-renewal (Weissman *et al.,* 2001). C17.2 cells demonstrated a greater transcription profile overlap with HSCs and ESCs compared to differentiated cells and expressed a greater number of stem-like genes. Cells of the primary NSC culture from the SVZ cultivated in neurospheres or even C17.2 cells passaged as neurospheres differed from the operationally defined ones by a lower number of stem-like genes and were much closer to differentiated neural cells, which corresponds to the heterogeneous cell composition of neurospheres (Parker *et al.,* 2005).

The involvement of many other genes in the maintenance of ESC pluripotency has been demonstrated. These include *Foxd3* (Hanna *et al.,* 2002), *Sox2, Klf4,* and *c-Myc* (Takahashi and Yamanaka, 2006). The addition of products of the latter three genes as well as transcription factor Oct3/4

reprogrammed embryonic and adult fibroblasts to pluripotent stem cells (Takahashi and Yamanaka, 2006).

Members of the protooncogene Myc family are of interest because of their involvement in the control of proliferation and differentiation, in particular, of NSCs. Targeted deletion of N-Myc considerably reduced the proliferation of neuronal progenitors and increased neuronal differentiation in the culture of *N-Myc*-null mutant cells. At the same time, the total volume of the brain and cerebellum decreased in mutant mice twofold and eightfold, respectively (Knoepfler *et al.,* 2002).

The direct involvement of many transcription factors in the maintenance of multipotent NSCs and inhibition of differentiation into particular neural lineages has been demonstrated. One of the basic helix–loop–helix (bHLH) transcription factors Hes1 is important for NSC self-renewal and for the inhibition of neuronal differentiation of NSC progeny. This has been demonstrated in the study of brain development in *Hes1*$^{-/-}$ mice *in vivo* and their brain cell culture *in vitro* (Nakamura *et al.,* 2000). Two other bHLH factors Hes3 and Hes5 demonstrated a similar effect on NSC during embryogenesis (Hatakeyama *et al.,* 2004).

Since the *Hes* genes are targets of Notch/CSL signaling, they are also involved in the maintenance of NSC self-renewal (Hitoshi *et al.,* 2002; Yoon and Gaiano, 2005). This involvement is observed both during embryonic development and, with certain peculiarities, in the adult brain (Alexson *et al.,* 2006). A close cooperation between the Notch/CSL signaling and components of the NSC niche, β-1-integrin and epidermal growth factor receptor, has been demonstrated (Campos *et al.,* 2006).

Transcription factor PAX6 is also required to maintain NSC multipotency and proliferative activity. Developing mice with mutant PAX6 demonstrated a decreased number of radial cells in the forebrain ventricular zone and their abnormal cell cycle (Stoykova *et al.,* 1997; Estivill-Torrus *et al.,* 2002). Mice with a spontaneous PAX6 mutation had a notably decreased proliferation of GFAP^{+} early progenitor cells in the adult hippocampus (Maekawa *et al.,* 2005).

The maintenance of NSC multipotency and self-renewal is affected by many cytokines and growth factors. For instance, overexpression of LIF, a member of the interleukin 6 family, decreases neurogenesis in the SVZ and olfactory bulb, which considerably increases the pool of NSCs. At the same time, the amplification of type C cells decreases, while the amplification of GFAP^{+} NSCs (type B cells) increases. This effect was observed both *in vivo* and *in vitro* after LIF delivery by a transgenic viral vector, infusion into the lateral ventricles, or addition to culture medium (Bauer and Patterson, 2006). LIF-induced GFAP^{+} cells were phenotypically and morphologically distinct from those induced by the BMP cytokine (Bonaguidi *et al.,* 2005). In contrast to LIF-induced cells, BMP-induced ones lost the stem cell properties.

It has been known for many years that fibroblast growth factor 2 (FGF2) (Gensburger *et al.,* 1987) and EGF (Reynolds *et al.,* 1992) are mitogens and can maintain the proliferation of neural progenitors both *in vitro* and *in vivo* (Kuhn *et al.,* 1997). Still, few data are available on the target cells in the germinal zones of adult brain for these factors. For instance, FGF2 receptors were shown to localize to glial ($GFAP^+$) SVZ cells, which suggests that the mitogen effect of this factor is specific for NSCs (Chadashvili and Peterson, 2006). Zheng *et al.* (2004) demonstrated that Fgf2 knockout mice have smaller olfactory bulbs (resulting from decreased cell migration to this structure) as well as a reduced population of slow-dividing cells not expressing GFAP but occasionally expressing brain-lipid-binding protein, a molecular marker of radial glia, which likely represent an NSC subgroup.

Molofsky *et al.* (2003, 2005) demonstrated that polycomb family transcriptional repressor Bmi-1 is required to maintain NSC self-renewal. This factor also repressed the $p16^{Ink4a}$ gene coding for the cyclin-dependent kinase inhibitor. NSC self-renewal was strongly reduced in Bmi-$1^{-/-}$ mice, which led to their postnatal depletion. Upregulation of the mouse $p16^{Ink4a}$ gene in NSCs decreased their proliferation. According to Molofsky *et al.,* these findings point to a common mechanism controlling self-renewal and postnatal maintenance of different NSC types. A high level of Bmi-1 expression in progenitor cells isolated from brain tumors indicates that this gene and the associated signaling play an important role in cancer stem cell proliferation (Hemmati *et al.,* 2003).

Serine-threonine kinase Akt-1 activated by phosphatidylinositol-3-kinase (PI3K)-dependent phosphorylation is another important factor for the regulation of stem cell proliferation (Sinor and Lillien, 2004). Its overexpression increased the proportion of stem cells through the positive modulation of their survival and proliferation. Target of rapamycin (TOR) is a component of Akt-1 signaling. The proliferation inhibitor PTEN (phosphatase and tensin homolog) suppressing Akt-1 phosphorylation through the reversion of PI3K-dependent phosphorylation plays an important role in controlling stem cells and cancer stem cells, in particular (Groszer *et al.,* 2001). The proliferative activity of NSCs and cancer stem cells is also regulated by maternal embryonic leucine zipper kinase (MELK). Its overexpression enhances whereas knockdown diminishes the ability to generate neurospheres from multipotent neural progenitors (Nakano *et al.,* 2005). High MELK levels have been reported in brain tumors and cultures containing cancer stem cells (Hemmati *et al.,* 2003).

All these observations indicate that there is a complex system of signals and operating transcription factors that suppresses the differentiation of NSCs in their niche or under specific culturing conditions. A daughter cell migrated from the niche is exposed to different signals and internal factors, which commonly induce its differentiation into one or other lineage.

3.2. Neural differentiation of embryonic stem cells

The isolation, cultivation, and proliferation of ESCs descending from the inner cell mass of the blastocyst have opened up new possibilities to study the fundamental problems of developmental biology. Stem cells are a convenient experimental model to study the fine molecular genetic processes underlying cell specialization. They can also be explored *in vivo* by the isolation and analysis of the function of different gene networks during their development (Korochkin, 2003). The time pattern of gene activation controlling the development proved to coincide in postimplantation embryos and embryoid body culture (Leahy *et al.,* 1999). This gave a fresh impetus to studies of neural induction (or neuralization) and early neurogenesis.

The present classical model of early neurogenesis was initiated in 1923 by Spemann and Mangold. They demonstrated that the early embryonic ectoderm in salamander receives an induction signal from the specialized cell group of the organizer, which governs the development of neighboring ectodermal cells into neural lineages (Spemann and Mangold, 2001). Recent publications questioned the triggering of neural induction by positive signals from the organizer. The signaling molecules noggin, chordin, and follistatin proved to have a neuralizing effect (Lamb *et al.,* 1993; Hemmati-Brivanlou *et al.,* 1994; Sasai *et al.,* 1995). The neuralizing effect of these molecules is due to the inhibition of BMPs (members of the TGF-β family), which consequently inhibits neural differentiation (Piccolo *et al.,* 1996; Zimmerman *et al.,* 1996; Fainsod *et al.,* 1997). These data made it possible to propose the currently popular default model of neural induction. According to this model, all ectodermal cells have a default program of neural differentiation. This program is actively inhibited by widely expressed BMPs. The organizer (or its structural equivalent node in amniotes) secretes anti-inhibitory factors suppressing BMP signaling rather than positive inductors (Hemmati-Brivanlou and Thomsen, 1995; Hemmati-Brivanlou and Melton, 1997; Munoz-Sanjuan and Brivanlou, 2002).

The results of several subsequent studies are in conflict with the default model. For instance, experiments on chickens demonstrated that BMP4 suppression is not sufficient to trigger neural induction (Streit *et al.,* 2000; Linker and Stern, 2004). However, these experiments do not exclude incomplete suppression of BMP signaling. In addition, the positive effect of several factors on neural induction was shown. These factors include FGF and Wnt (Baker *et al.,* 1999; Streit *et al.,* 2000; Wilson *et al.,* 2000, 2001). At the same time, the mechanism underlying the effect of these factors, which can promote proliferation of committed cells rather than proper neural induction, remains unclear. Moreover, their effect can be mediated by the modulation of BMP transcription (Bainter *et al.,* 2001). These and other observations inconsistent with the default model formed the basis for

the instructive model assuming positive inducing signals triggering neural induction (Wilson and Edlund, 2001).

The default model can be tested by experiments on ESCs exposed to conditions with the minimum environmental impact and cell–cell interactions. Such an attempt was made by Tropepe *et al.* (2001) who cultivated R1 mouse ESCs at a very low density in the serum-free chemically defined medium that allowed neurosphere formation by NSCs from the germinal zones of the brain in the presence of FGF2. ESCs cultivated at a low density in this medium supplemented with LIF gave rise to spherical colonies. The proportion of sphere-forming cells was as low as 0.2 to 0.3%. After 3 days of cultivation, all cells in the colonies expressed the NSC marker nestin. The subcloned cells from the colonies gave rise to the secondary and tertiary colonies. The cells were placed on a MATRIGEL substrate in a medium containing 1% serum for 7 days. All colonies contained neurons, astrocytes, and oligodendrocytes expressing their specific markers (MAP2, βIII-tubulin, GFAP, and O4). In the course of sphere colony formation, LIF was required only to trigger differentiation, while FGF was active at all stages of stem cell colony formation. However, it remains unclear if it is a differentiation or a proliferation inducer.

Ying *et al.* (2003a) cultivated ESCs from transgenic mice expressing GFP under the Sox1 promoter (Sox1 is the earliest neuroectodermal marker) on gelatine-coated plates in a serum-free medium. The cultivation in the medium without LIF gave rise to Sox1-GFP$^+$ cells. After 4 days of cultivation in a serum-free medium supplemented with N2 and B27, more than 60% of cells expressed Sox1-GFP. After 8 days of cultivation, its expression decreased as a result of further cell differentiation into mature neural cells expressing nestin, Tau, βIII-tubulin, GFAP, CNPase, TH, and γ-aminobutyric acid (GABA). Conversely, the expression of Oct-4 decreased. Neural differentiation was suppressed in the medium supplemented with BMP4. The inhibitor of FGF receptor SU5402 had a similar effect. Ying *et al.* (2003a) concluded that the requirement for endogenous FGF to trigger neural differentiation contradicts the default model of neural induction.

The requirement for FGF in the medium to start neural induction has not been confirmed by Smukler *et al.* (2006) in experiments with a twenty-fold lower ESC density in a serum- and growth factor-free medium. Under these conditions, over 90% of cells expressed the markers of primitive NSCs nestin and Sox1 after 4 h of cultivation. Moreover, primitive NSCs appeared after a 4-h cultivation of ESCs in phosphate-buffered saline (PBS), which obviated the inducing effect of extrinsic factors. Drug inhibition of FGF and a deletion in the FGFr1 receptor gene did not interfere with rapid neural induction of ESCs.

An attempt to reproduce these results on human ESCs has failed, since these cells plated at low density spontaneously differentiated into flat fibroblast-like cells similar to extraembryonic ectodermal cells. Cell–cell

contacts are required to control ESC differentiation (Gerrard *et al.,* 2005). Nevertheless, experiments on adherent ESCs demonstrated that the addition of BMP antagonist noggin to medium blocked the formation of extraembryonic ectoderm and induced neural differentiation. This finding can indirectly confirm the default model.

However, FGF signaling plays a positive role in neural induction. Experiments on amphibians (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Hongo *et al.,* 1999; Strong *et al.,* 2000) and chickens (Rodríguez-Gallardo *et al.,* 1997; Alvarez *et al.,* 1998; Storey *et al.,* 1998) demonstrated that FGFs can induce the neural fate of ectodermal cells in the absence of other signals. Two different pathways of FGF signaling-mediated neural induction are known. One of them is direct induction independent of the BMP expression level (Wilson *et al.,* 2001), which is observed in chicken medial epiblast cells at the blastula stage. The second pathway involves an FGF-induced decrease in BMP expression during early gastrulation (Streit *et al.,* 1998; Wilson *et al.,* 2000; Wilson and Edlund, 2001). It can be inhibited by Wnt signaling observed in the chicken lateral epiblasts (Wilson *et al.,* 2000; Wilson and Edlund, 2001).

Other protein factors can be involved in neural induction (Bainter *et al.,* 2001). Many of them modulate the BMP system. The alternative model is based on calcium-dependent signaling, which involves calcium influx into potentially neural cells through L-type calcium channels. Activation of this pathway leads to neural determination, while epidermal determination occurs when this pathway is inactive (Moreau and Leclerc, 2004).

It is becoming more and more clear that neural induction is not a single event but rather a multistage process beginning at the early blastula stage (if not earlier). Each stage of this process involves various factors, whose effect is modulated in a stage-dependent manner. Understanding this pattern and application of sophisticated experimental systems can result in the future conformity of numerous studies of neural induction during early vertebrate development (Stern, 2005).

Most of the above-mentioned findings were obtained in studies on embryonic development of amphibians, fish, and birds due to the availability of the material. Studies of normal early development of mammals are limited and ESC neural induction in mammals is largely studied *in vitro*. Many mouse (Evans and Kaufman, 1981; Martin, 1981) and human (Thomson *et al*., 1998) ESCs lines are currently available. The pluripotency and unlimited self-renewal make these cells a convenient experimental model. Reprogramming of human oocytes to produce the desired differentiated ESCs is highly attractive for reparative medicine (Lanza *et al.,* 1999). Accordingly, methods for directed ESC differentiation in humans and laboratory animals are being actively developed.

This activity was primed in the mid-1990s when Yao *et al*. (1995) demonstrated that P19 embryonal carcinoma cells are capable of

differentiating into neural cells. After the induction with retinoic acid, the cells were harvested from N2 medium and placed into a medium containing bFGF and EGF, which accelerated the proliferation of induced neuronal cells. Neuronal differentiation proved to be efficiently induced by the exposure of mouse ESCs aggregated into embryonic bodies to retinoic acid and subsequent dissociation and plating (Bain *et al.,* 1995; Fraichard *et al.,* 1995). Neuronal differentiation of mouse ESCs followed a four-stage process. At the first stage, ESCs were cultured on a feeder layer; at the second stage (4 days), they were cultured aggregated into embryonic bodies; this was followed by a third stage of culturing in a medium with insulin/transferrin/selenium/fibronectin and amplification of nestin$^+$ NSCs in a medium supplemented with N2 and bFGF; the fourth stage consisted of neuronal differentiation after growth factor withdrawal from the medium (Okabe *et al.,* 1996; Lee *et al.,* 2000). The addition of sonic hedgehog, FGF8, and ascorbic acid increased the proportion of TH$^+$ dopaminergic as well as serotonergic neurons (Lee *et al.,* 2000). A similar technique for mouse ESC culturing with a different set of growth factors (FGF2 and PDGF) at the final stage allowed Brustle *et al.* (1999) to generate considerable quantities of glial cells. Retinoic acid induces differentiation of both aggregated and plated cultures of mouse ESCs into lineage-restricted neural precursors (Mujtaba *et al.,* 1999).

Human ESCs can also be induced to differentiation into a neural lineage. For instance, a human ESC line cultivated for 3 weeks without replenishing feeder cells expressed differentiation markers (Reubinoff *et al.,* 2001). Clusters of the most differentiated cells expressing nestin, PAX-6, and NCAM were mechanically separated from the feeder layer and transferred to a serum-free medium supplemented with EGF and bFGF, where they formed spherical constantly growing aggregates. For neural induction, the spheres were transferred onto polylysine–laminin-coated plates. After cultivation for 2 to 3 weeks without growth factors, the cells expressed markers of neural precursors and mature neural cells. Glial induction required the addition of PDGF, retinoic acid, EGF, and bFGF at the final stage. The cells produced expressed GFAP, O4, and other markers of glial cells (Reubinoff *et al.,* 2001). Several other protocols are available for neural induction of human ESCs involving retinoic acid, growth factors, or their combination (Schuldiner *et al.,* 2001; Guan *et al.,* 2001; Nakayama *et al.,* 2004, 2006).

There are additional factors affecting the neural fate of ESCs. In particular, the interaction of ESC with the extracellular matrix mediated by integrin receptors can determine the developmental fate of pluripotent cells (Czyz and Wobus, 2001). The impact of the extracellular matrix has been confirmed by Goetz *et al.* (2006) who demonstrated that growth substrate can specify the fate of mouse ESCs and their progeny and this effect could differ from one differentiation stage to another. Using a four-stage protocol (Okabe *et al.,* 1996), Goetz *et al.* (2006) demonstrated that ESC growth on fibronectin

and laminin-poly-L-ornithine increased the proportion of glial cells and neurons, respectively, at the third stage (generation of neural precursors). At the fourth stage, the substrate had no effect on the glia/neuron fate but influenced the neural subtype specification. The data obtained indicate a significant role of the extracellular matrix in the specification of the fate of ESCs and their progeny.

ESCs can also be induced to neural differentiation by less defined factors. For instance, mouse ESCs cultivated in a medium preconditioned with astrocytes and supplemented with mitogens (FGF2 and EGF) formed neural stem spheres (NSSs). After plating, the cells migrated from NSSs along an adhesive substrate and differentiated into mature neurons and astrocytes in the presence and absence of the same mitogens, respectively (Nakayama *et al.,* 2004, 2006). The available data on ESC differentiation into neural cells not only provide clues to identify the mechanisms of early neurogenesis but also specify a wide range of techniques to obtain cell material for future clinical application, namely, regenerative therapy.

3.3. NSC lineage determination

Recapitulation of differentiation events is observed in both ESC and NSC cultures. In the case of normal embryonic development, fetal NSCs gave rise first to neurons and later to glia. Mouse NSCs from early embryos of embryonic stage E10 to E11 cultivated for a short period of time differentiated exclusively into neurons, while those from E13 to E14 embryos largely became astrocytes (Qian *et al.,* 2000). Moreover, NSCs from early embryos gradually transformed from neurogenic to largely gliogenic in the course of long-term culturing, which points to an internal NSC program controlling the neurogenic to gliogenic switch (Sun *et al.,* 2003). These properties of NSCs allow us to study the factors of differentiation of the specification of fate.

Analysis of stem cell behavior in culture made it possible to identify the key genes and gene networks involved in their specialization and differentiation into a particular lineage. For instance, the above-mentioned BMPs were shown to trigger neuronal induction of NSCs from the SVZ at midgestation (Li *et al.,* 1998) and to induce gliogenesis in late fetal and adult NSCs (Gross *et al.,* 1996). Such opposite effects are typical of factors involved in differentiation control.

In vitro, tyrosine hydroxylase (TH) expression is the key event in dopaminergic neuron differentiation. The maximum number of TH^+ cells in culture was induced by three factors: FGF1, forskolin (a regulator of intracellular cAMP level), and protein kinase C activator (Pliego-Rivero *et al.,* 1999; Iacovitti *et al.,* 2001; Park *et al.,* 2004). This triad induced TH expression in 10 to 20% of neuroblasts. The proportion of TH^+ cells increased to 75% after their incubation for 2 weeks in a special

differentiation medium. These data demonstrate that sufficient quantities of neural cells of a desired specificity can be produced from stem cells under laboratory conditions (Iacovitti *et al.,* 2001). Such studies provide promising clues to both fundamental problems of neurogenetics and practical use of NSCs in cell-based regenerative therapy.

Stem cell differentiation can also be controlled by repetitive sequences such as micro- and minisatellites. Podgornaya *et al.* (2003) have identified proteins specifically binding tandem repeats, which determined the three-dimensional chromatin organization. At the same time, this organization governs the pattern of gene activity; hence, the state of the repetitive sequence system and its underreplication, diminution, or overreplication can play a substantial role in the specific differentiation of stem cells (Ryskov *et al.,* 2004).

3.3.1. Proneural basic Helix–Loop–Helix transcription factors

At the stage of determination, which is a key stage for stem cells, proneural genes are activated and stem cells are committed toward a neural or glial lineage. At this moment, neurogenic factors induce the expression of proneural bHLH transcription factors such as Neurogenin and Mash1. bHLH factors represent a universal mechanism of cell fate specification toward a particular lineage. This mechanism is crucial to initiate differentiation in various tissues including muscle and nerve (Weintraub, 1993; Jan and Jan, 1994). bHLH transcription factors specifically bind DNA through the basic domain and form complexes through the HLH domain. The expression of the bHLH gene exemplifies the master gene concept universal in developmental biology. Its expression triggers gene cascades governing the specialization of organs, germ layers, and individual cell types (Korochkin, 2003). This pattern is common for all animals. For instance, the *eyeless* gene controls eye development in *Drosophila*. Its expression in unusual locations induced eye development on the wings, legs, antennae, etc. (Halder *et al.,* 1995). A similar gene called Pax6 is found in vertebrates (Chow *et al.,* 1999). Its introduction into the *Drosophila* genome had the same effect as the proper host gene, which indicates the universal effect of master genes (Kumar *et al.,* 2001). The sip1 gene coding for a transcription factor of the zinc finger family involved in TGF signaling is another example of a master gene (Poliakov *et al.,* 2004).

The effect of bHLH factors involves a regulatory cascade where expressed proteins induce the expression of later ones. bHLH factors can be exemplified by Neurogenin1 and Mash1 well known in nervous system studies. These proneural bHLH proteins are master regulators of neural differentiation that coordinate expression of the neuronal genes. For instance, BMPs provide for autonomous neural induction in the peripheral nervous system (PNS) through the induction of Mash1 expression in NSCs (Shah *et al.,* 1996). Mash1 consequently induces a cascade of genes, which

eventually leads to the expression of pan-neuronal and subtype-specific markers (Lo *et al.,* 1998). Another bHLH factor, Neurogenin1, triggers the expression of other bHLH genes including *NeuroD (Neurod1),* and thus induces NSCs differentiation into other types of central nervous system and PNS neurons (Ma *et al.,* 1998). The expression of the early and late genes can occur in more or less distant time periods. In this case, the effect of the early genes is cell commitment to a particular lineage, while the effect of late genes is differentiation toward a differentiated phenotype.

This sequence of events has been described by Cau *et al.* (2002). Investigation of the olfactory system in *Mash1* knockout mice demonstrated that olfactory progenitors are not produced and the Notch signaling pathway is not activated. In *neurogenin1* mutant mice, olfactory progenitors are generated, but they express only a fraction of their normal regulatory molecules and their differentiation is blocked. In this case, *Mash1* triggers the determination, while *neurogenin1* controls one step in the ongoing sequence of events resulting in the final differentiation (Cau *et al.,* 2002).

Neurogenin1 as well as some other bHLH genes induce neuronal differentiation and simultaneously inhibit NSC differentiation into astrocytes. This is mediated by sequestering the CBP-Smad1 transcription complex away from astrocyte differentiation genes and by inhibiting the activation of STAT transcription factors that are necessary for gliogenesis (Sun *et al.,* 2001).

Oligodendrocyte lineage transcription factor Olig2 is another bHLH factor involved in NSC differentiation control (Marshall *et al.,* 2005). Its role in the differentiation of spinal oligodendrocytes and motor neurons has been demonstrated (Lu *et al.,* 2000; Takebayashi *et al.,* 2000). This factor regulates cell differentiation in the adult brain after injury. The neurogenic potential observed in NSCs from the adult brain *in vitro* is not used in brain injury *in vivo* since a considerable fraction of new cells appearing after injury differentiates into glial cells rather than into neurons (Alonso, 2005). A local expression of a retroviral vector carrying the dominant negative Olig2 gene induced endogenous neurogenesis in the area of brain injury (Buffo *et al.,* 2005).

3.3.2. Repression of neuronal genes

Numerous publications report that neuron-restrictive silencer factor (NRSF or REST) is directly involved in neuronal differentiation. This zinc finger protein binds the neuron-restrictive silencer element (NRSE or RE-1), which represses the transcription of neuronal genes in nonneuronal cells (Schoenherr *et al.,* 1995) as well as in NSCs and ESCs. The repression of neuronal promoters requires histone deacetylase (HDAC) activity (Roopra *et al.,* 2000). The REST/RE-1 interaction is mediated by many corepressors (Ballas *et al.,* 2005). The expression of neuronal genes requires the inhibition of REST activity (Ballas *et al.,* 2005). For instance, Paquette *et al.* (2000) demonstrated that constitutive expression of REST in neurons of the developing spinal cord represses two neuronal genes and

caused pathfinding errors of commissural neurons. Similar results were obtained on a neuronal cell line that expresses REST conditionally (Ballas *et al.,* 2001). Su *et al.* (2004) reported that selective upregulation of REST target genes by the recombinant REST-VP16 factor with the REST repressor domain replaced with the activator VP16 domain of herpes simplex virus transformed clonal NSCs (C17.2) into mature neurons.

Note, however, that contrary to the initial views (Chong *et al.,* 1995), REST is expressed not only in nonneuronal and stem cells but also in mature neurons. A function-dependent expression of this factor was observed in the adult hippocampus, midbrain, and pons/medulla (Palm *et al.,* 1998). At the same time, a high transcriptional level was observed for most REST target genes (Sun *et al.,* 2005). Clearly, the effect of REST is not limited to on/off switches typical of silencers. Several molecular complexes are known that can counteract REST. In addition to the above-mentioned corepressors, these can include noncoding miRNAs involved in the specification of stem cell fate. According to Kuwabara *et al.* (2004), a small noncoding dsRNA plays the key role in neuronal differentiation in the hippocampus. This molecule converts the neuronal silencer factor REST from a transcriptional repressor in undifferentiated and nonneuronal cells to a transcriptional activator during neuroblast differentiation.

In addition to the repression of neuronal genes, REST has been shown to be a negative transcription factor for miRNAs. The targets of REST include miR-124a, a well-known posttranscriptional repressor of many nonneuronal genes. During neuronal differentiation, REST releases a chromatin miR-124a loci, which derepresses both neuronal genes and miR-124a so that nonneuronal transcripts are selectively degraded. This mechanism maximizes the contrast between neuronal and nonneuronal phenotypes (Conaco *et al.,* 2006).

According to Morrison (2001), the dual functions of the transcription factors can be a general pattern in the determination of cell lineage. They can simultaneously promote differentiation toward one lineage and inhibit differentiation toward the alternative one. This introduces a problem of canceling this effect when the differentiation fate changes in ontogeny (e.g., during the previously mentioned shift from neurogenesis to gliogenesis in late embryonic development of the mouse brain). bHLH transcription factors Mash1 and Math3 are responsible for the promotion of neurogenesis and the suppression of gliogenesis during early development (Kageyama and Nakanishi, 1997). Notch signaling is the likely mechanism of suppression of neurogenesis. Activation of Notch signaling in NSCs triggers termination of neurogenesis and initiates gliogenesis even if neurogenic factors are still present (Furukawa *et al.,* 2000; Morrison *et al.,* 2000; Hojo *et al.,* 2000; Tanigaki *et al.,* 2001). Studies of retinal neural induction *in vivo* demonstrated that the activation of Notch provides an instructive signal for Müller glia generation at the expense of neuronal

differentiation (Hojo *et al.,* 2000). Notch activation also instructively induced hippocampal stem cell differentiation into astrocytes (Tanigaki *et al.,* 2001).

Morrison (2001) considered the molecular mechanism of the Notch effect on gliogenesis. Since Notch inhibits the expression of neurogenin, Mash1, and other bHLH factors, promotion of gliogenesis through the inhibition of the capacity of proneural factors to suppress gliogenesis can be expected. Notch can also be involved in a more direct mechanism to provide for the expression of glial genes. In particular, it can stimulate the expression of glial marker GFAP even with a mutation in the signal transducer and activator of the transcription (STAT3) binding site. Since the STAT3 binding site is also the site where activation of transcription by the STAT–CBP–Smad1 complex takes place, the mechanism of promotion of gliogenesis by Notch is at least partially independent of the STAT–CBP–Smad1 complex and, hence, of the capacity of proneural factors to sequester this complex. Apparently, Notch functions in many regions of the nervous system as a trigger for gliogenesis and an inhibitor of neurogenesis partially by suppressing the expression of proneuronal bHLH gene expression.

3.3.3. Epigenetic control of NSC fate

Epigenetic mechanisms underlying the specification of NSC fate are of great importance for brain development. In particular, acetylation and deacetylation by histone acetylases (HATs) and histone deacetylases (HDACs), respectively, lead to DNA modifications that switch the transcription of cell type-specific genes on and off (Hsieh and Gage, 2004). The administration of the HDAC inhibitor valproic acid to rats during the first 10 postnatal days induced hypomyelination and retained expression of progenitor markers. These data demonstrate that global modifications of nucleosomal histones are critical for the timing of oligodendrocyte differentiation and myelination in the corpus callosum (Shen *et al.,* 2005).

A sharp (twofold to fourfold) increase in mRNA levels has been shown for HDACs 5, 6, 7, and 9 during early differentiation of hippocampal progenitors in culture after mitogen withdrawal as compared to actively proliferating progenitors in the presence of mitogens. At the same time, mRNA levels for HDACs 1, 2, and 3 remained unaltered while those for HDACs 4, 8, and, 10 were undetectable. The elevated HDAC expression can be attributed to chromosomal (γ-chromatin) rearrangements induced by mitogen withdrawal (Ajamian *et al.,* 2003).

Local DNA methylation is also an important mechanism of stem cell fate specification. CpG methylation within a STAT3 binding element in the GFAP promoter blocks the transcription of this marker of early telencephalic neuroepithelial cells on embryonic day 11.5. The repression was also observed in the presence of LIF and was canceled only on embryonic

day 14.5. Hence, methylation of a cell type-specific gene promoter can be crucial for the specification of cell lineage in the brain (Takizawa *et al.,* 2001).

Studies of stem cell biology *in vitro* and *in vivo* both advanced the fundamental understanding of genomic regulation of nervous system development and gave a fresh impetus to the development of cell therapy. Understanding the basic elements of signaling underlying the pluripotency and NSC differentiation as well as neural differentiation of ESCs offers opportunities to develop new techniques and drugs to treat neurodegenerative and other diseases of the brain.

4. Neural Stem and Progenitor Cell-Based Therapy

Numerous data and techniques developed in studies of NSCs (some of them were mentioned above while others were omitted due to chapter volume limitations) stimulated the development of new trends in cell biology and medicine. In particular, this applies to the theoretical basis of the nervous system pathology as well as technical aspects of reparative medicine. The volume of published data is so great that we have to limit ourselves to schematic and largely fragmentary description of novel potential therapies.

To date, two promising trends can be identified in the medical application of our knowledge of the biology of NSCs. First, it is application of cells isolated from a particular source and propagated *in vitro* to be transplanted into patients; and second, it is application of our knowledge of stem cell biology to intensify cell repair through the mobilization of the body's pool of stem cells. Consequently, experimental publications on the first trend can be further subdivided according to sell sources and application modes.

4.1. Transplantation of stem and progenitor cells

The transplantation strategy of using stem and progenitor cells to treat neurodegenerative diseases and injuries requires the development of techniques to isolate, grow, and prepare cell material suitable to treat a particular disorder. Several sources of stem and more or less differentiated cell populations applicable to propagation and subsequent cell therapy have been identified. One of them is the fetal brain in humans and experimental animals. Dissociated cells of the fetal brain are transferred to serum-free medium with growth factors. Mainly stem cells propagate under these conditions, which enriches the cultured population in stem cells. After stem cell populations were isolated from the rodent fetal brain (Vescovi *et al.,* 1993; Palmer *et al.,* 1995; Gritti *et al.,* 1996), stem cell-rich cultures

were obtained from the human fetal brain (Flax *et al.,* 1998; Vescovi *et al.,* 1999; Carpenter *et al.,* 1999; Poltavtseva *et al.,* 2002). These cells could give rise to mature neurons and glial cells *in vitro* and *in vivo* (Snyder *et al.,* 1997; Vescovi *et al.,* 1999; Carpenter *et al.,* 1999; Fricker *et al.,* 1999; Brustle *et al.,* 1999; Aleksandrova *et al.,* 2002). Despite the selective impact of the medium, the resulting cell population is heterogeneous. This particularly applies to cultures of unattached cells in neurospheres (Revishchin *et al.,* 2001; Poltavtseva *et al.,* 2002; Suslov *et al.,* 2002; Parker *et al.,* 2005). After transplantation into the brain of experimental animals, these cells can migrate to the affected area. Committed progenitors rather than undifferentiated NSCs more commonly migrate and incorporate into the recipient cerebral tissue (Aleksandrova *et al.,* 2004; Soares and Sotelo, 2004; Revishchin *et al.,* 2005).

Many neurodegenerative diseases involve death of neurons of a particular phenotype. For instance, dopaminergic neurons of the substantia nigra degrade in Parkinson's disease, which decreases the dopamine level in the neostriatum. In these cases, the function can be restored by cells differentiated or committed toward a particular lineage (dopaminergic neurons in Parkinson's disease). Transplantation of cells from the fetal midbrain to the neostriatum in experimental Parkinson's disease ameliorated the symptoms (Olanow *et al.,* 1996). However, transplantation of fetal midbrain cells to Parkinson's patients had low clinical efficiency and induced refractory medication-independent dyskinesias (Hagell and Cenci, 2005).

The factors underlying such consequences can include the heterogeneity of the fetal midbrain cell population in the graft, which encouraged efforts to obtain highly enriched populations of dopaminergic neurons from mesencephalic progenitors (Iacovitti *et al.,* 2001). Transplantation of enriched cell populations to the striatum of parkinsonian rats led to their functional recovery (Studer *et al.,* 1998; Sawamoto *et al.,* 2001). Application of such enriched populations to treat Parkinson's disease can become efficient, although yielding sufficient material for transplantation can be a problem (Goldman and Windrem, 2006).

The problem of sufficient cell quantities for transplantation encourages the search for new cell sources. ESC lines commercially available since the 1980s can become such a source (Martin, 1981; Wobus *et al.,* 1984; Thomson *et al.,* 1998). McKay and colleagues successfully induced mouse ESCs differentiation into dopaminergic neurons (Lee *et al.,* 2000). Dopaminergic neurons generated from ESCs and transplanted into the brain of rats with the nigrostrial system destroyed by 6-hydroxydopamine restored normal function (Kim *et al.,* 2002). Similar results were obtained in experiments on transplantation of ESC-derived allogeneic dopaminergic neurons into the striatum of cynomolgus monkeys with a destroyed nigrostrial system (Takagi *et al.,* 2005). Repeated studies on ESCs have demonstrated that their differentiation can be directed toward other types of neurons

(Hayashi *et al.,* 2006; Zeng and Rao, 2007). Therapeutic application of ESC-derived neurons encounters considerable difficulties. Similar to fetal cell populations, ESC-derived cultures are heterogeneous, and even thorough lineage selection cannot remove, for example, GABAergic neurons and glial cells from the population of dopaminergic neurons required for Parkinson's treatment (Goridis and Rohrer, 2002). Tumorigenic activity of ESC cultures is well known (Bjorklund *et al.,* 2002). Both linear ESCs and ESC-derived differentiated cells demonstrate tumorigenic activity after transplantation into the animal brain (Kim *et al.,* 2002; Blyszczuk *et al.,* 2003). Thus, the outlook for using ESC-derived populations in cell therapy of neurodegenerative diseases is promising but impractical until the problems of their safety are solved.

Application of stem cells from the adult body seems equally real but also has problems. Bone marrow is the most interesting cell source. The publication of Eglitis and Mezey (1997) on the differentiation of bone marrow stem cells into both microglia and macroglia primed numerous studies of this problem. In particular, bone marrow cells transplanted into lethally irradiated mice migrate to the brain where they give rise to neurons (Brazelton *et al.,* 2000; Mezey *et al.,* 2000). These data obtained on experimental animals have been confirmed on the material obtained from female patients who had received a bone marrow transplant from male donors. The postmortem brain samples of these patients proved to have cells with the Y chromosome and neural markers (Mezey *et al.,* 2003; Weimann *et al.,* 2003). Isolated bone marrow stem cells can be induced to differentiate toward neural lineages by various factors (Woodbury *et al.,* 2000; Hermann *et al.,* 2004; Egusa *et al.,* 2005; Scintu *et al.,* 2006). At the same time, the initial marrow stromal stem cells express not only mesenchymal but also germinal, endodermal, and ectodermal genes (Woodbury *et al.,* 2002; Tremain *et al.,* 2001), which prompted the authors to propose that stromal cells are both multipotent and multidifferentiated (''multidetermined'' can be more accurate since the cells are not differentiated). Differentiating neurons descending from clonal mesenchymal stem cell (MSC) lines expressed specific neural genes β-III tubulin, tau, neurofilament-M, TOAD-64, and synaptophysin *de novo* (Woodbury *et al.,* 2000). The suppression of phenotypes discordant with the ongoing induction was due to the silencing of extraneous gene clusters rather than to the selection (Egusa *et al.,* 2005).

Therapeutic potential was also found in stem and progenitor cells of olfactory epithelium (Feron *et al.,* 2005; Marshall *et al.,* 2006). The populations of neural stem and progenitor cells found in the olfactory epithelium and proliferating throughout the life span can be used as an autologous transplantation material in injuries and degenerative disorders of the central nervous system. In addition to marrow stromal stem cells, some other nonneural stem cells proved capable of neural differentiation. These include hematopoietic stem cells of the bone marrow, which can be isolated from

peripheral blood (Hao *et al.,* 2003; Reali *et al.,* 2006), stem cells from the skeletal muscle (Schultz and Lucas, 2006), and adipose-derived stromal cells (Safford *et al.,* 2002; Zuk *et al.,* 2002; Fujimura *et al.,* 2005; Safford and Rice, 2005; Ning *et al.,* 2006).

The application of stem and progenitor cells of the adult body in cell therapy can be promising in many respects. It allows patients to be treated with their own cells, which obviates the problem of tissue incompatibility as well as the ethical problems of using abortive material and ESCs. The risk of these cells becoming malignant is much lower compared to ESCs. Note, however, that neural differentiation of mesenchymal stem cells in culture and experimental animals is continuously questioned by alternative studies (Castro *et al.,* 2002; Vallieres and Sawchenko, 2003; Vitry *et al.,* 2003; Lu *et al.,* 2004; Massengale *et al.,* 2005; Roybon *et al.,* 2006). Apparently, additional studies by many teams involving various experimental approaches are required to ultimately evaluate the potential of these cells for cell therapy.

4.2. Mobilization of internal repair potential of the brain

Replacement cell therapy consists in correcting functional defects of degenerating brain regions by attracting new cells to replace dead ones. Replacement cell therapy based on the mobilization of endogenous precursors has both advantages and disadvantages over the therapy involving exogenous cells. One of the most important advantages is the absence of foreign cells to be isolated and transplanted into the affected brain. Proliferation and neuronal differentiation in several parts of the adult brain described above allow us to use the available new neuroblasts to replace dead neurons. The ability to replace a limited population of dead neurons with endogenous cells has been demonstrated by Magavi *et al.* (2000) in mice. However, the brain capacity to replace dead neurons is not high. In addition, the normal cerebral tissue response to more or less heavy damage (e.g., in ischemic stroke or injury), follows a scenario unfavorable for functional repair. The proliferation of stem and progenitor cells increases, but a considerable fraction of new cells differentiates into glial cells rather than into neurons, which favors the formation of a glial scar incompatible with functional recovery (Alonso, 2005).

Successful mobilization of endogenous cells and correction of functional defects require not only an active progenitor proliferation and a correct differentiation pathway but also adequate migration to the affected regions. Abundant data have been obtained on cytokines and growth factors influencing the generation and migration of neuroblasts to the regions of cerebral infarction. For instance, cytokine erythropoietin playing a key role in hematopoiesis is expressed in the nervous system and its expression increases after hypoxia (Bernaudin *et al.,* 1999). Intracerebroventricular and

systemic administration of erythropoietin before and after stroke decreased the infarct volume (Bernaudin *et al.,* 1999; Wang *et al.,* 2004). Complete and conditional knockout of the erythropoietin gene considerably affected the migration of neuroblasts in embryonic development and after experimental local stroke (Tsai *et al.,* 2006). The safety and efficiency of using erythropoietin to treat poststroke patients have been confirmed in clinical trials (Ehrenreich *et al.,* 2002). A similar neuroprotective effect in behavioral tests was observed for the erythropoiesis-stimulating protein darbepoetin a (Belayev *et al.,* 2005). The infarct volume decreased after systemic administration of another hematopoietic factor granulocyte colony-stimulating factor (G-CSF) due to both its direct neuroprotective antiapoptotic effect and stimulation of neuronal differentiation of stem cells (particularly in the hippocampus) without a notable increase in the overall proliferation rate in the brain (Schneider *et al.,* 2005). The curative effect of G-CSF has been confirmed in clinical trials (Shyu *et al.,* 2006).

The studies carried out in the laboratory of S. Thomas Carmichael have demonstrated the important role of the local vascular niche formed around the insult zone in the migration of neuroblasts to the ischemic focus (Ohab *et al.,* 2006). Angiogenesis and poststroke migration of neuroblasts derived from $GFAP^+$ stem cells of the SVZ are causally linked. Blocking angiogenesis by endostatin decreased the number of migrating neuroblasts in the periinfarction zone tenfold. The chemokine SDF1 and growth factor Ang1 actively expressed after stroke control the differentiation and migration of stem cells, which is mediated by CXCR4 and Tie2 receptors, respectively (Stumm *et al.,* 2002; Imitola *et al.,* 2004; Robin *et al.,* 2006). After systemic administration, SDF1 and Ang1 enter the brain parenchyma through the blood–brain barrier broken after stroke and considerably increase the number of neuroblasts in the periinfarct zone (Ohab *et al.,* 2006). Conversely, specific inhibition of CXCR4 by the specific antagonist AMD3100 and Tie2 by antibodies against this Ang1 receptor induced the diffusion of new neuroblasts in a larger volume of the periinfarct cortex (i.e., affected the targeted migration of neuroblasts). In this case, the total number of neuroblasts did not decrease. Systemic administration of SDF1 and Ang1 accelerated the recovery of behavioral responses induced by the stroke cortex (Ohab *et al.,* 2006). Behavioral recovery was observed within the first 10 days after stroke, which indicates that the positive changes are due to the effect of cytokines and growth factors released by new cells rather than to the formation of new neuronal networks (Mi *et al.,* 2005). Monocyte chemoattractant protein-1 (MCP-1) is another example of a chemokine favoring the migration of neural progenitors to the inflammatory focus in the nervous system. Experiments on cultured hippocampal slices demonstrated that this chemokine increases the migration of neural precursors to the sites of inflammation induced by local administration of cytokines, bacterial toxin, viruses, or their proteins (Belmadani *et al.,* 2006).

The above-mentioned factors and cytokines influence not only hematopoiesis but also neurogenesis, differentiation, and neuroblast migration in the poststroke brain. Apparently, the germinal zones of the adult brain are the sources of new neurons in all these cases. However, the main degenerative processes (e.g., in Parkinson's disease), are observed far from them—in the substantia nigra. More recent studies suggest the mobilization of the proliferative potential of cells in the substantia nigra to treat the degenerative processes underlying Parkinson's disease. A population of actively dividing progenitor cells has been identified in the substantia nigra. *In situ,* it generated new glial cells; while in culture, it generated cells differentiating into neurons under particular conditions (Lie *et al.,* 2002; Hermann *et al.,* 2006). The study on nestin enhancer-controlled LacZ reporter transgenic mice as a model of Parkinson's disease demonstrated an increase in both proliferation and neurogenesis (in particular, in the number of dopaminergic neurons) in experimental mice relative to control (Shan *et al.,* 2006).

Targeted mobilization of the intrinsic reparative potential of the brain to treat the consequences of neurodegenerative diseases most closely achieves practical application among therapeutic approaches based on stem cell biology. Their application is not associated with the risk of tumors, tissue incompatibility problems, and ethical problems of using ESCs and NSCs. The only limit on their application is the low proliferative capacity of the brain.

4.3. Neural stem cells and cancer therapy

Our understanding of stem cell biology is of great practical significance for the development of new approaches to treat malignant tumors and brain tumors, in particular.

4.3.1. Stem cells as therapeutic targets

The identification of therapeutic targets is crucial for cancer therapy. Culturing of human glioblastoma multiforme cells under conditions similar to the neurosphere assay has demonstrated the presence of stem-like properties among them (Ignatova *et al.,* 2002). Similar to cells in the germinal zones of the normal brain, tumor stem cells had high proliferative potential. Under conditions allowing differentiation, they generated cells of both glial and neuronal lineages; however, some of their progeny expressed both glial and neuronal markers, which is not normally observed. Tumor stem cells differed from normal stem cells of the neurogenic zones by the expression of the *Notch* ligands, *Delta* and *Jagged*, as well as the antiapoptotic inhibitor *Survivin*. Stem-like progenitor cells forming neurospheres were also isolated from medulloblastoma. These cells expressed many genes typical of NSCs including CD133, SOX2, BMI1, and Musashi 1. After transplantation into the neonatal rat brain, tumor progenitors incorporated into the recipient

cerebral tissue, migrated away from injection site, differentiated into neurons and glia, and continued to proliferate (Hemmati *et al.,* 2003).

Angleo Vescovi and colleagues demonstrated that stem-like progenitors from the human glioblastoma forming neurospheres could generate new tumors identical to glioblastoma multiforme after transplantation into the striatum of adult immune-deficient mice. Stem-like cells could also be isolated from such tumors (Galli *et al.,* 2004). CD133-immunopositive cells isolated from the human glioblastoma or medulloblastoma demonstrated the properties of stem cells *in vitro* and high tumorigenic activity. The transplantation of 100 $CD133^+$ cells was sufficient to form a phenocopy of the original tumor in the brain of immunodeficient mice. Conversely, the injection of 100,000 $CD133^-$ cells caused no tumor. The proportion of $CD133^+$ cells in the primary glioblastoma and medulloblastoma culture varied from 3.5 to 46.3% according to flow cytometry data (Singh *et al.,* 2004).

Many established tumor cell lines include minor side populations of cells with the properties of NSCs. For instance, a side population of the C6 glioma cell line was isolated by flow sorting of Hoechst 33342 low cells. These cells can survive and expand in serum-free medium with growth factors and, under conditions permissive for differentiation, differentiate into glia and neurons. After transplantation into different organs of nude mice, they give rise to tumors containing neurons and glia. Non-side population cells could not form tumors and demonstrated no multipotency *in vitro* (Kondo *et al.,* 2004).

These data indicate that tumor stem cells should be the main target for brain tumor therapy since they have tumorigenic activity. For instance, the exposure to the differentiation factor BMP4 suppresses tumorigenic activity of human glioblastoma stem cells, reduces their proliferation, and increases the expression of neuronal markers (Piccirillo *et al.,* 2006). Studying isolated tumor stem cells opens new possibilities to understand the molecular mechanisms of tumorigenesis and can offer new approaches to cancer therapy.

4.3.2. Stem cells as a therapeutic agent against tumor

Treatment of brain tumors, particularly gliomas, is an extremely complex task due to the blood–brain barrier on the one hand and to the very high invasiveness of these tumors on the other hand. The discovery of the tropism of stem cells including NSCs for local pathologies, particularly brain tumors (Yip *et al.,* 2003), gave a new impetus to brain tumor therapy. The original study demonstrated that rat and human NSCs injected into the experimentally induced rat brain glioma not only actively spread in the tumor bed but also pursued tumor cells aggressively migrating to the neighboring brain parenchyma. After a remote injection (including injection into the opposite hemisphere), NSCs migrate toward the tumor bed. Moreover, NSCs injected into the blood also accumulated in the glioma. This work also demonstrated that NSCs expressing a therapeutic transgenic

protein (prodrug activating enzyme cytosine deaminase) can still pursue tumor cells, which allows them to be used in cancer therapy to deliver anticancer agents directly to the targets (Aboody *et al.,* 2000). Later this homing of exogenous NSCs to gliomas experimentally induced by the injection of tumor cells was extended to spontaneous tumors. Burns and Weiss (2003) demonstrated that mouse C17.2 NSCs transplanted into the brain of mice haploinsufficient for tumor suppressor genes p53 or ink4a/arf demonstrated a pronounced tropism for disseminating glioma cells in this model.

Experiments on a heterogeneous population of fetal progenitor cells from the mouse and human brain allowed Ehtesham *et al.* (2004) to demonstrate that only a specific fraction of fetal progenitors, namely early astrocytic progenitors, demonstrated tumor homing unlike uncommitted NSCs, mature astrocytes, and neuroblasts.

Tumor homing is observed in both exogenous and endogenous NSCs. Fourteen days after inoculation of G261 glioblastoma cells labeled with red fluorescent protein into the caudate putamen of transgenic mice expressing GFP under the nestin promoter, the nestin-GFP cells originated from the SVZ surrounded the induced tumor in several layers. The accumulation of endogenous precursors in the tumor improved the survival time of experimental mice. Coculturing of G261 cells with neural precursors decelerated tumor cell proliferation and induced their apoptosis (Glass *et al.,* 2005). A similar effect on N29 glioma cells inoculated into the rat brain was observed for neural progenitor cell lines HiB5 and ST14A. The life span of experimental animals increased and tumor growth was completely suppressed in 25% of cases (Staflin *et al.,* 2004). These results confirm the data obtained by Weinstein *et al.* (1990) on the transformation of C6 glioblastoma cells into nonmalignant astrocytes after coculturing with C17.2 NSCs, which was governed by a contact-mediated mechanism.

To date, several possible mechanisms underlying tumor homing of stem cells are known. One of the most important is the above-mentioned SDF1-CXCR4 chemokine axis-guided migration (Ehtesham *et al.,* 2004; Allport *et al.,* 2004). *In vitro* experiments demonstrated that the blocking of CXCR4 localized to the surface of fetal neural (astroglial) progenitors completely inhibited their tropism for the medium conditioned by tumor cells (Ehtesham *et al.,* 2004). Blocking the SDF1a ligand in the conditioned medium decreased but did not completely suppress the tumor homing in these experiments, which can indicate additional ligands of CXC chemokine receptors underlying the homing (Ehtesham *et al.,* 2004). Neural progenitors expressing surface CXCR4 migrate along the concentration gradient of the ligands of this receptor SDF1a secreted by tumor cells. Thus, astrocytoma cells expressed quantities of SDF1a depending on tumor grade (Rempel *et al.,* 2000), while glioma cells secreted CXCL12 (SDF1) (Ehtesham *et al.,* 2006). It is of interest that tumor cells use the same migration mechanism in metastasis (Zhou *et al.,* 2002; Zhang *et al.,* 2005; Kucia *et al.,* 2005).

Invasive glioma cells demonstrated from 25- to 89-fold higher expression of CXCR4 compared to noninvasive tumor cells (Ehtesham *et al.,* 2006). Apparently, this is due to tumor formation from stem cells as a result of mutations (Kucia *et al.,* 2005). The expression of CXCR4 in invasive tumor cells can explain their preferred metastasis to tissues expressing SDF1 lymph nodes, lung, liver, and bones (Kucia *et al.,* 2005).

The accumulation of NSCs injected into the blood in brain tumors is also mediated by the SDF1/CXCR4 interaction. *In vitro* experiments demonstrated that the functional blocking of SDF1a on tumor-derived endothelial cells by antibodies considerably reduced the recruitment and transendothelial migration of NSCs to the parallel plate flow chambers (Allport *et al.,* 2004). The signals attracting NSCs to the tumor bed can be released by both malignant and nonmalignant cells such as vascular endothelial or nonmalignant perivascular cells (Mapara *et al.,* 2007). Note in this context that tumor-derived endothelial cells have an inflammatory phenotype with constitutive expression of classical endothelial adhesive molecules (Allport and Weissleder, 2003). Tumor homing of NSCs can be due to the inflammatory mechanism involving the activation of the SDF1/CXCR4 pathway (Imitola, 2004).

Other CXC chemokines such as CXCL10/IP-10 and CXCL11/I-TAC are expressed in the brain during inflammation (McColl *et al.*, 2004). These chemokines can also control the migration of cells carrying their receptors (Honeth *et al.*, 2006). These experiments demonstrated the possibility of developing artificial cell vectors with tumor homing from cells initially lacking this property (Honeth *et al.*, 2006). Cells of the rat neural progenitor line HiB5 suppressing glioma development from inoculated N29 cells (Staflin *et al.*, 2004) were transfected with the gene of CXCR3, a receptor of chemokines I-TAC and IP-10. Thus, cells with no tumor homing acquired this property and demonstrated transcallosal migration toward the glioma induced in the striatum of the opposite hemisphere (Honeth *et al.*, 2006).

5. Conclusions

Knowledge about neural stem cells considerably expands our views of repair in the central and peripheral nervous systems; however, it does not contradict the main components of the current paradigm in neurobiology. Indeed, a (limited) capacity of the nervous system for regeneration was known before and it does not contradict the concept that mature cerebral neurons cannot divide *in vivo*. The concepts of the unlimited transformation of stem cells and even more of their transdifferentiation often rely on insufficient experimental data and are exaggerated. In many cases,

transdetermination of not yet differentiated multipotent cells is more apt than transdifferentiation. In addition, the nervous system is not stuffed with stem cells. There are a limited number of sites containing them; the capacity of the brain for regeneration is not high, and the rate of stem and progenitor cell proliferation declines with age. On the other hand, the discovery of neural stem cells in the adult mammalian and human brain impacted our knowledge of cerebral tissue biology and considerably revised it. This revision is far from being complete. Stem cells have been shown to be involved in more and more normal and abnormal processes in the nervous system. The data obtained suggest new approaches to treat nervous system disorders. Using stem cells as therapeutic targets opens promising avenues in neurodegenerative disorders and cancer therapy. Stem cell transplantation in patients suffering neurodegenerative and oncological disorders proved less efficient than expected from model experiments on animals. However, the development of cellular techniques yields new specific cell-targeted drugs that can become more efficient. Using stem cells as a raw material for cell vector development and their application in gene and cell therapy seem particularly promising.

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CHAPTER THREE

Mechanisms of Mitotic Spindle Assembly and Function

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Contents

Abstract

The mitotic spindle is the macromolecular machine that segregates chromosomes to two daughter cells during mitosis. The major structural elements of the spindle are microtubule polymers, whose intrinsic polarity and dynamic

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properties are critical for bipolar spindle organization and function. In most cell types, spindle microtubule nucleation occurs primarily at two centrosomes, which define the spindle poles, but microtubules can also be generated by the chromosomes and within the spindle itself. Many associated factors help organize the spindle, including molecular motors and regulators of microtubule dynamics. The past decade has provided a wealth of information on the molecular players that are critical for spindle assembly as well as a high-resolution view of the intricate movements and dynamics of the spindle microtubules and the chromosomes. In this chapter we provide a historical account of the key observations leading to current models of spindle assembly, as well as an up-to-date status report on this exciting field.

Key Words: Mitosis, Mitotic spindle, Aneuploidy, Chromosome segregation, Kinetochore, Motor protein, Ran, Anaphase

1. Introduction

During mitosis, the cell must accurately partition its replicated chromosomes into two daughter cells, a task performed by a microtubule-based machine called the mitotic spindle. At the onset of mitosis during prophase, the interphase microtubule network disassembles and the spindle sets up. While the apparent pathway of spindle assembly differs depending on the cell type (Waters and Salmon, 1997), in all cases assembled spindles share common structural features (Fig. 3.1). The slow-growing minus ends of microtubules are focused into two poles, while the faster-growing plus ends interact with chromosomes in the spindle equator, creating the typical fusiform shape at metaphase (McIntosh and Euteneuer, 1984). Interaction of microtubules with kinetochores, the specialized protein complexes located at the centromeric region of each sister chromatid, is a prerequisite for proper chromosome alignment at the metaphase plate and for segregation to opposite poles in anaphase. By signaling to the cell cortex, the spindle also functions to define the position of the cleavage plane that divides the cell into two at cytokinesis (Eggert *et al.*, 2006). Missegregation of chromosomes results in aneuploidy, which can lead to genomic instability and cancer (Weaver and Cleveland, 2006; Weaver *et al.*, 2006). Therefore, understanding the mitotic spindle machinery and how it functions has been a major focus of biomedical research. To avoid errors, the mitotic spindle has a built-in ''checkpoint'' mechanism that monitors whether all chromosomes are properly attached to the spindle before allowing the cell to proceed into anaphase (Musacchio and Hardwick, 2002). Thus, the mitotic spindle can be viewed as a sophisticated macromolecular machine that governs the process of cell division. In this chapter we summarize the current understanding of mitotic spindle assembly and function based on both recent and landmark literature.

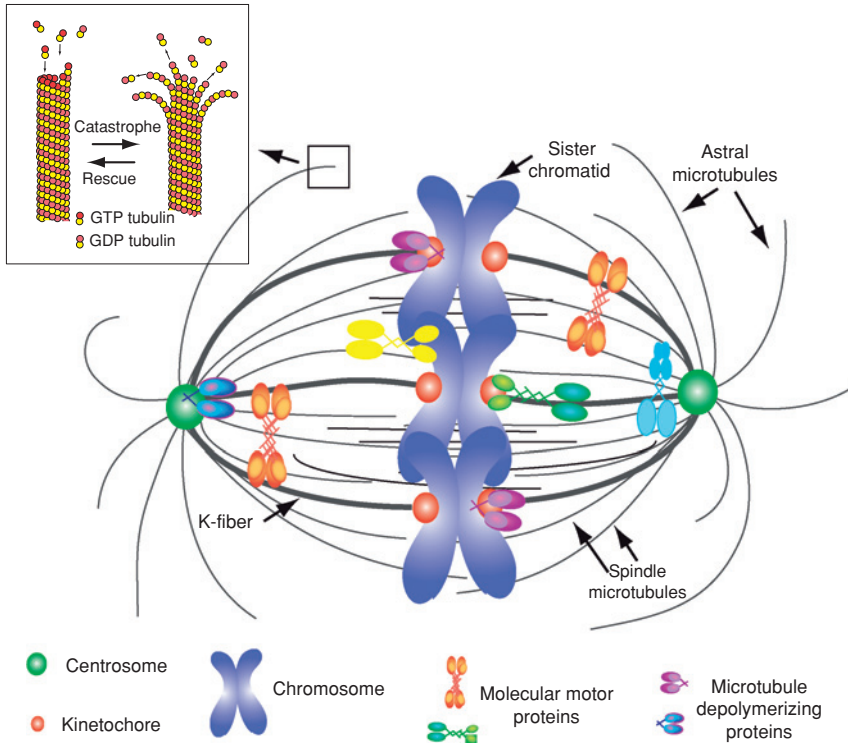

Figure 3.1 The key components of the mitotic spindle. Microtubules are shown in gray and compose the antiparallel spindle microtubules, the kinetochore microtubule bundles (K-fibers), and the astral microtubules that extend away from the spindle poles. The inset is a higher-resolution illustration of a microtubule, showing the head-to-tail configuration of the α/β-tubulin heterodimers as well as the transitions between growth and shrinkage. Duplicated chromosomes consist of two sister chromatids tightly adhered at their centromere regions, where each sister assembles a kinetochore that attaches the chromosome to spindle microtubules. Various motor proteins function in the spindle to cross-link microtubules of the spindle, move kinetochores and chromosome arms, and regulate microtubule dynamics at the plus ends (near chromosomes) and at the minus ends (near centrosomes).

2. Molecular Components of the Mitotic Spindle

2.1. Microtubules as structural and dynamic components of the spindle

The spindle consists primarily of microtubules, polarized filaments composed of α/β-tubulin heterodimers arranged in a head-to-tail configuration within protofilaments. Thirteen parallel protofilaments associate laterally to

form the hollow cylindrical microtubule structure (Nogales *et al.*, 1999). Two fundamental properties of microtubules give insight into how mitosis works: their dynamic properties and their structural polarity (Fig. 3.1). The dynamic properties of microtubules allow them to grow and shrink by addition or loss of tubulin dimers at the ends of the polymer (Desai and Mitchison, 1997). Individual microtubules switch stochastically between phases of growth and shrinkage, a property known as dynamic instability (Mitchison and Kirschner, 1984), which can be described by four parameters: the rate of growth, the rate of shrinkage, and the transition frequencies from growing to shrinking (a catastrophe) and shrinking to growing (a rescue) (Walker *et al.*, 1988). While purified microtubules can undergo dynamic instability *in vitro*, microtubules within cells are more dynamic, indicating the existence of cellular factors that regulate microtubule dynamics. These proteins include microtubule-associated proteins (MAPs), which enhance the stability of microtubules, as well as those that modify transition frequencies. Such factors are critical during mitosis when microtubule dynamics are increased relative to interphase, and undergo both temporal and spatial regulation within the spindle (Saxton *et al.*, 1984).

Microtubule polarity is another property important for spindle morphogenesis. Because tubulin subunits are asymmetric, the minus and plus ends of the microtubules have different dynamic properties (Desai and Mitchison, 1997), and a structural polarity in the microtubule lattice is created. Microtubule-based motor proteins, including dynein and a large set of kinesin-like proteins, recognize the surface lattice of microtubules, read their polarity, and move their cargo accordingly (Wittmann *et al.*, 2001). The cargoes within the spindle include chromosomes, microtubules that are sorted and organized within the structure, and other protein complexes that must be delivered to the appropriate place within the spindle to function. Different classes of motor proteins within the spindle are illustrated in Figure 3.1. The antiparallel organization and dynamic polarity of microtubules within the spindle are key features that underlie its operation.

2.2. Centrosomes: A major source of microtubule nucleation for spindle assembly

The earliest recognized spindle organizers are centrosomes, microtubule-organizing centers visualized over 100 years ago as focal sites of astral microtubule growth that define the spindle poles (Urbani and Stearns, 1999). The animal centrosome is a large ($\sim$1 μm diameter) organelle consisting of a pair of centrioles surrounded by amorphous pericentriolar material (PCM) where nucleation takes place, generating polarized microtubule arrays with their plus ends extending outward. A major component of the PCM is the specialized tubulin called γ-tubulin, which assembles into a multisubunit γ-tubulin ring complex (γ-TURC), forming lock washer

rings of 13 γ-tubulins that serve as a nucleation template for microtubule formation (Zheng *et al.*, 1995). During mitosis the nucleation capacity of the centrosomes increases due to the recruitment of more γ-TURCs and other material to the centrosome (Khodjakov and Rieder, 1999; Piehl *et al.*, 2004). This allows the spindle to rapidly increase the number of microtubules that can be used to attach to chromosomes and form the spindle structure. The centrosome is also an important site of cell cycle regulation, where kinase complexes are recruited that are essential for centrosome duplication during interphase, and other processes essential for progression through the cell cycle (Doxsey *et al.*, 2005).

2.3. Chromosomes: Active players in mitosis

Once compared to the corpse at a funeral—the reason for the proceedings but not an active participant in the event (Mazia, 1961)—chromosomes are now recognized as key players in mitosis. Although mitotic spindle assembly has been observed in the absence of chromosomes (Bucciarelli *et al.*, 2003), it is clear that chromosomes contribute to the nucleation and stabilization of microtubules during spindle assembly and harbor many important regulators of spindle dynamics. In addition, each sister chromatid of a replicated chromosome contains a kinetochore—a large macromolecular complex that constitutes the spindle microtubule interaction site crucial for chromosome movement and segregation (Kotwaliwale and Biggins, 2006). Kinetochores make end-on attachments to a subset of spindle microtubules, called kinetochore fibers (K-fibers). In budding yeast, only a single microtubule interacts with each kinetochore, whereas in vertebrates a single K-fiber consists of 20 to 30 bundled microtubules (Biggins and Walczak, 2003; Cleveland *et al.*, 2003). The kinetochore assembles at a region of the chromosome called the centromere, which also varies in complexity among organisms, from a 123-base pair "point" centromere in yeast to megabases of DNA at the primary constriction of vertebrate chromosomes (Cleveland *et al.*, 2003). Despite size differences, in all organisms the kinetochore also mediates the mitotic spindle checkpoint that monitors the attachment of chromosomes to the spindle and halts mitosis until all chromosomes are properly attached to the spindle with sisters oriented toward opposite spindle poles (Chan *et al.*, 2005). Thus, kinetochores perform multiple functions that are critical for accurate chromosome segregation, and are essential components of the spindle machine.

2.4. Proteomics and functional genomics: Generation of the complete parts list

Historically, identification of proteins that play important roles in spindle assembly has frequently resulted from genetic studies, primarily in yeast, *Drosophila*, and *Caenorhabditis elegans*. Screens could identify a mutant with a

specific defect in mitosis, and the gene responsible cloned, sequenced, and characterized. Databases were searched to identify homologous gene products and protein domains to make educated guesses on the protein's function and role in mitosis. Although a laborious process, this remains an unbiased and fruitful approach to identify key players. One drawback to genetic methods has been that proteins with additional functions outside mitosis are difficult to identify. An alternative approach has been to isolate factors based on their biochemical properties, such as association with microtubules or chromosomes. Immunocytochemical techniques have been instrumental in identifying key components of the centrosome and kinetochore. A series of autoimmune antibodies recognizing antigens at the kinetochore and underlying centromere of chromosomes was used to clone the corresponding genes, which identified many members of the centromere protein (CENP) family of centromere/kinetochore components (Earnshaw and Rothfield, 1985). Together with homology-based searches, these techniques have led to the characterization of related proteins and functional orthologs in diverse model systems, allowing a broader functional analysis of mitotic proteins.

The approaches outlined previously have dominated mitosis research until recently, as technological developments have completely revolutionized how mitosis can be analyzed using large-scale proteomic and functional genomic approaches. Protease digestion with trypsin, followed by fractionation by liquid chromatography and mass spectrometry of individual peptides, combined with growing sequence databases, has allowed efficient identification of proteins cut out of gels as well as in complex mixtures found in isolated subcellular structures. The spindle itself as well as components including the centrosome (Andersen *et al.*, 2003), mitotic chromosomes (Uchiyama *et al.*, 2005), and the midbody (Skop *et al.*, 2004), a structure that forms late in mitosis following chromosome segregation, have been isolated and analyzed (Sauer *et al.*, 2005). Some statistics of the proteins identified are summarized in Table 3.1. Together, these studies have revealed upward of 1000 proteins associated with the cell division apparatus, including hundreds of uncharacterized factors. This is a conservative estimate since not all known cell division proteins were identified, likely because biochemical treatments used to isolate the structures dissociated some components, or their low abundance made them difficult to detect. Other identified components, such as ribosomal and mitochondrial proteins, play housekeeping roles in the cell, but are nevertheless associated with the mitotic apparatus, and may also have functional roles in spindle assembly. Other proteomic studies providing information about potential mitotic factors include analyses of MAPs in HeLa and *Xenopus* egg extracts (Liska *et al.*, 2004; Mack and Compton, 2001). In addition, many spindle proteins are

Table 3.1 Proteomic screens for spindle components

Structure Analyzed	Publication	Total proteins	Known components	Uncharacterized proteins	Validated	Novel components	Special features of study
Spindle	Sauer *et al.*, 2005	795	151/795 known to localize to spindle	154 with no obvious homologies, many others uncharacterized with respect to cell division	6/17 by localization	154	Taxol used to stabilize spindle, DNAase treated
Mitotic chromosome	Uchiyama *et al.*, 2005	209, combining results from 3 different types of preparation	~65/209 proteins known to be nuclear, and large numbers of mitochondrial and ribosomal proteins	15 novel plus many known proteins with uncharacterized chromosomal functions	Many known chromosomal proteins identified	15, localization not validated	Different chromosome preparations of varying purity revealed classes of associated proteins
Centrosome	Andersen *et al.*, 2003	>500 in centrosome fraction, many contaminants	47 of 60 known interphase centrosome proteins identified	90 with only cDNA or genetic analysis	19/32 tested localized to centrosome; 41 precisely cofractionated with known centrosome components	23	Specificity tested by co-fractionation with known centrosomal proteins on sucrose gradients
Midbody	Skop *et al.*, 2002	577; 160 excluding ribosomes, mitochondria, contaminants	42/160 known to localize to midbody 57/160 known to function in cytokinesis	103 previously uncharacterized with respect to cell division	10/10 tested localized to midbody 141/160 function in cell division by RNAi depletion	5, all had mitotic phenotypes upon depletion	Mitotic functions assessed by RNAi in *C. elegans* revealed most midbody proteins function in cytokinesis

phosphorylated during mitosis, but relatively few phosphorylation sites on mitotic factors have been identified. Mass spectrometry can also be applied to this problem. An analysis of isolated HeLa spindles identified 279 novel phosphorylation sites of known spindle proteins (Nousiainen *et al.*, 2006). This phosphoproteomic analysis will be a first step toward understanding how many of the proteins of the mitotic spindle are regulated by the numerous kinases that act during mitosis (Morgan, 2007; Nigg, 2001).

The proteomic studies provide lists of potentially important mitotic factors; however, they cannot stand alone since they provide no data to indicate which factors are crucial for cell division. A complementary approach has been developed that allows large-scale phenotypic screens for defects upon protein depletion in cells by RNA interference, often combined with automated microscopy to collect large datasets. Mitotic phenotypes have been examined following functional disruption of genes on chromosome- and genome-wide scales in *C. elegans* embryos (Fraser *et al.*, 2000; Gonczy *et al.*, 2000; Sonnichsen *et al.*, 2005), in *Drosophila* cell lines (Goshima *et al.*, 2007), and in human cell culture systems (Kittler and Buchholz, 2005). More detailed screens have been performed targeting specific protein families with mitotic functions such as microtubule-based motor proteins in both *Drosophila* (Goshima and Vale, 2003) and human cell lines (Zhu *et al.*, 2005). Whereas the large scale screens bring novel factors to light, and identify those with previously unappreciated mitotic functions, more focused screens are amenable to a greater depth of analysis, and have contributed to our understanding of the particular mitotic functions of different motor proteins.

In addition, clever genetic screens in yeast have been used to identify factors important for chromosome segregation. Synthetic lethal screens isolated 211 nonessential deletion mutants that were unable to tolerate defects in kinetochore function (Measday *et al.*, 2005). A screen for factors that become essential upon increased sets of chromosomes (polyploidy) also highlighted factors affecting genomic stability and spindle function (Storchova *et al.*, 2006).

The information obtained from proteomic and functional genomic studies is a valuable resource for all in the field. However, neither the identification of factors found on the mitotic apparatus nor generation of lists of proteins required for cell division tell us exactly what each protein does, how it acts, when or where it functions, and how it is regulated! These studies therefore provide a starting point for mechanistic investigations of spindle assembly and function. In the rest of this chapter, we focus on the pathways of spindle assembly and the many mechanistic studies that have provided significant insight into the inner workings of the mitotic spindle.

3. Major Pathways of Spindle Assembly

3.1. Classic model of search and capture

In most vertebrate cells, "search and capture" appears as the predominant mechanism by which chromosomes become properly attached to and aligned on the spindle (Kirschner and Mitchison, 1986; McIntosh *et al.*, 2002) (Fig. 3.2A). In this model, centrosome-nucleated microtubules probe three-dimensional space until they are captured and stabilized by one of the sister kinetochores on a chromosome (Holy and Leibler, 1994). These chromosomes are termed "monooriented" because they are attached to a single spindle pole and oscillate back and forth but remain closely associated with one pole until the chromosome becomes bioriented through interaction with microtubules from the opposite pole. Once bioriented, chromosomes then rapidly move toward the spindle equator where they continue to oscillate. The likelihood of kinetochore capture is enhanced by increased centrosomal microtubule nucleation during mitosis. One pathway stimulating this increase is driven by the kinase Aurora A, which becomes activated in mitosis and phosphorylates the conserved centrosomal protein TACC, promoting microtubule growth (Brittle and Ohkura, 2005). In addition, the rate of microtubule turnover increases approximately 10-fold between interphase and mitosis (Desai and Mitchison, 1997). More dynamic microtubules can sample a large area because if they fail to encounter a kinetochore they rapidly depolymerize and regrow. Despite the intuitive appeal of "search and capture," mathematical modeling studies indicate that this mechanism alone is not sufficient to align all chromosomes on the mitotic spindle in a normal time frame (Wollman *et al.*, 2005). Therefore, additional mechanisms likely contribute to chromosome capture, including anisotropy of microtubule growth toward chromosomes (Dogterom *et al.*, 1996), as well as microtubule growth from chromosomes and kinetochores that would dramatically increase potential interaction sites for centrosome-generated microtubules (Khodjakov *et al.*, 2003).

3.2. Self-assembly of spindles

In contrast to the "search and capture" pathway, the"self-assembly" model posits that microtubules nucleated around chromosomes, independently of centrosomes, are sorted into an antiparallel array that generates the bipolar spindle (Lambert and Lloyd, 1994; McKim and Hawley, 1995) (Fig. 3.2B). It was originally thought that spindle self-assembly operated only in female meiosis or other systems lacking centrosomes, such as egg extract reactions containing DNA-coated beads (Heald *et al.*, 1996). However, more recent studies show that centrosome-driven and centrosome-independent

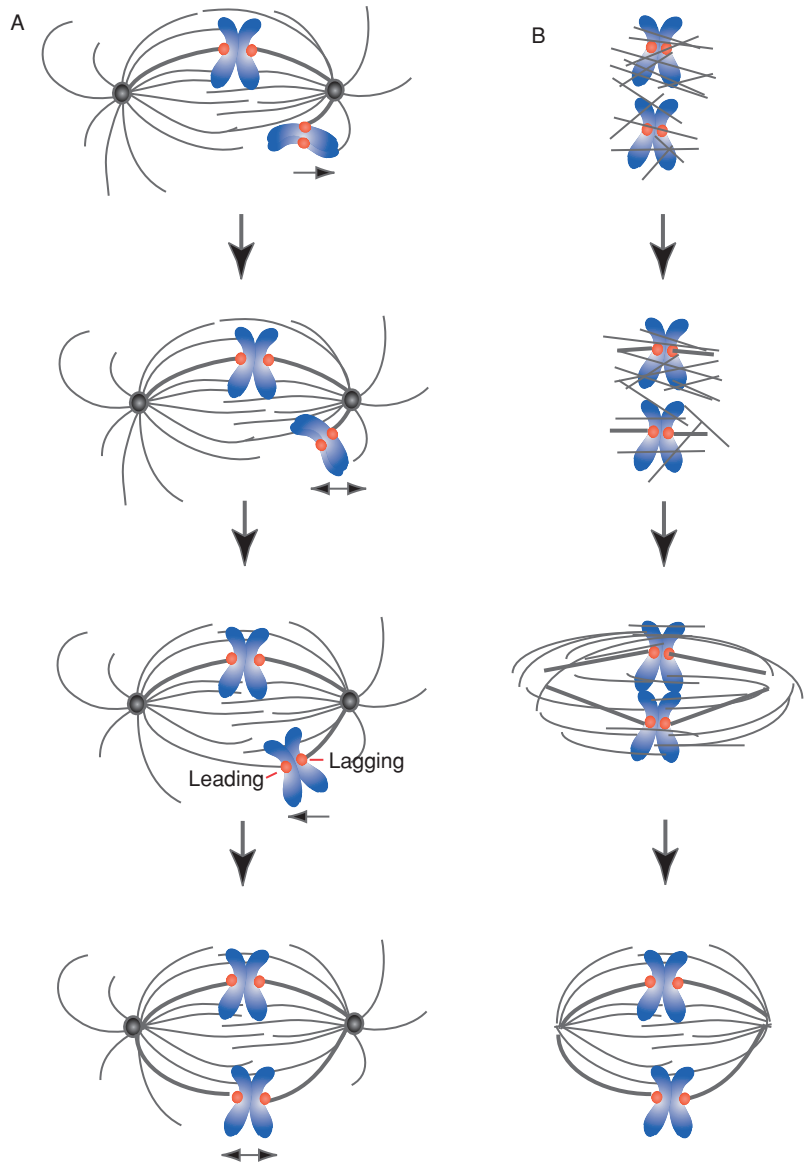

Figure 3.2 Models of spindle assembly. (A) Search and capture. A bioriented chromosome and a newly captured monooriented chromosome are depicted. In this model, one sister kinetochore attaches to a centrosomally nucleated microtubule following a random encounter driven by cycles of microtubule growth and shrinkage. The chromosome rapidly translocates toward the minus end of the microtubule and its associated pole, often by sliding along the side of the microtubule, movement that is thought to be mediated by cytoplasmic dynein. The kinetochore attachment converts to an end on association with a microtubule bundle (K-fiber). The unattached kinetochore is

mechanisms are not mutually exclusive. For example, laser ablation of centrosomes in vertebrate cells does not inhibit spindle assembly or chromosome segregation (Khodjakov *et al.*, 2000; Khodjakov and Rieder, 2001), indicating that somatic cells can self-assemble spindles when the normally dominant centrosome is absent (Heald *et al.*, 1997). Remarkably, mutant flies can be generated that lack centrosomes completely (Basto *et al.*, 2006), indicating the sufficiency of self-assembly mechanisms. How are spindle microtubules generated in these cases? One important source is within the spindle itself, where γ-tubulin complexes are recruited by factors including Nedd1/GDPWD, which contributes to both centrosome- and spindle-mediated microtubule nucleation (Haren *et al.*, 2006; Luders *et al.*, 2006).

Importantly, both ''search and capture'' and ''self-assembly'' mechanisms can lead to metaphase chromosome alignment, since forces on the chromosomes and kinetochores are balanced at the mid-plane of the spindle (Oestergren, 1951). Two unifying mechanisms appear to operate in all systems. One is the role of microtubule-based motor proteins, including cytoplasmic dynein and at least seven different kinesins, which are essential to organize microtubules into a bipolar array, even in the presence of centrosomes that provide a strong bipolar cue (Endow *et al.*, 1994; Gaglio *et al.*, 1996; Goshima *et al.*, 2005a; Goshima and Vale, 2003; Sharp *et al.*, 1999; Walczak *et al.*, 1998). The second general principle is the stabilizing force of chromosomes, due to bioriented kinetochore–microtubule interactions and the biochemical activities of chromosome arms, discussed in more detail later.

3.3. Ran as a key player in chromosome-directed spindle assembly

Chromosome-generated activities contributing to spindle self-assembly regulate both microtubule dynamics and motor proteins (Karsenti and Vernos, 2001; Mitchison and Salmon, 2001; Scholey *et al.*, 2003; Wittmann *et al.*, 2001), and are mediated at least in part by the small GTPase Ran (Dasso, 2002; Hetzer *et al.*, 2002). Addition of RanGTP to *Xenopus*

subsequently contacted by a microtubule emanating from the opposite pole, and the chromosome begins to move toward the metaphase plate (congression). The leading kinetochore is defined as the kinetochore oriented toward the direction of movement, and the lagging kinetochore is trailing. The chromosome eventually congresses to the metaphase plate and will oscillate there. (B) Microtubule self-organization. In spindle self-assembly, microtubules are nucleated in the vicinity of chromatin, and a subset may be captured and bundled into short K-fibers. Microtubules are sorted and organized by molecular motor proteins into an antiparallel array as they continue to grow. Microtubule bundling generates a bipolar axis, and the spindle poles extend and become focused.

egg extracts induced the assembly of microtubule asters and small bipolar spindles in the absence of centrosomes and chromosomes (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999). Many of the players important for normal bipolar spindle assembly in egg extracts are also required for RanGTP-dependent spindle formation, including γ-tubulin, the kinesin Eg5, and XMAP215 (Wilde *et al.*, 2001). The working model is that RanGTP exists in a gradient around chromosomes due to the chromatin association of its guanine exchange factor RCC1 and the cytoplasmic distribution of the RanGAP that promotes RanGTP hydrolysis. Chromatin-generated RanGTP then creates an environment favorable for microtubule polymerization and bipolar organization, specifically in the vicinity of chromosomes. The existence of physical RanGTP-dependent gradients has now been demonstrated both in *Xenopus* egg extracts (Caudron *et al.*, 2005; Kalab *et al.*, 2002) and in cells (Kalab *et al.*, 2006). These gradients diminish sharply as the distance from the chromosomes increases. Abolishing the RanGTP gradient by inhibiting RCC1 or introducing excess RanGTP dramatically impairs spindle assembly in *Xenopus* egg extracts, but has less severe consequences for somatic cells, indicating that the predominant centrosome-driven mechanisms in cells are less sensitive to Ran pathway perturbation than is chromatin-mediated spindle assembly (Kalab *et al.*, 2006).

The finding that Ran is involved in spindle assembly was surprising because RanGTP is best characterized as a factor regulating directionality of nucleocytoplasmic transport during interphase (Steggerda and Paschal, 2002). Investigation of the Ran-regulated nuclear transport machinery revealed that nuclear import receptors importin β and its nuclear localization sequence (NLS)-binding adaptor importin α are also involved in spindle assembly independently of their role in nuclear transport (Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001). Interphase and mitotic roles of Ran nevertheless appear to be analogous: RanGTP releases import cargoes either in the interphase nucleus or surrounding chromosomes during mitosis, some of which are spindle assembly factors (SAFs) (Dasso, 2002; Hetzer *et al.*, 2002). Ran-regulated SAFs can be divided into two categories: those that have been biochemically determined to interact directly with importins as cargo molecules, and those that function further downstream and are regulated by or in complexes with the cargoes. Altogether, proteins in the mitotic Ran pathway now number in the double digits, and it seems likely that many more remain to be discovered.

3.3.1. Mitotic importin cargoes regulated by Ran

A number of known cargo molecules are regulated by RanGTP and importins during spindle assembly (Table 3.2). For those marked with asterisks, evidence supports an interaction, but no direct binding to importins has been demonstrated with pure proteins *in vitro*. The founding

Table 3.2 RanGTP-regulated mitotic cargoes functioning in spindle assembly

Cargo	Interacting transport receptors	Protein class	Interacting spindle factors	Downstream activities	RanGTP/transport receptor-regulated activities
TPX2 (Gruss *et al.*, 2001)	Importin α/β	Spindle MAP (Wittmann *et al.*, 2000)	Xklp2 (Wittmann *et al.*, 2000), Aurora A, BRCA1/BARD1 (Joukov *et al.*, 2006), RHAMM (Groen *et al.*, 2004)	Spindle pole formation (Joukov *et al.*, 2006; Wittmann *et al.*, 2000), Aurora A activator (Tsai *et al.*, 2003), known substrates: Eg5, TPX2, TACC, HURP, BRCA1/BARD1	Microtubule nucleation (Schatz *et al.*, 2003) Aurora A interaction/activation (Trieselmann *et al.*, 2003; Tsai *et al.*, 2003)
NuMA (Nachury *et al.*, 2001; Wiese *et al.*, 2001)	Importin α/β	Spindle pole MAP (Fant *et al.*, 2004)	Dynein (Merdes *et al.*, 1996), BRCA1/BARD1 (Joukov *et al.*, 2006)	Spindle pole focusing	None identified
Maskin (TACC) (Albee *et al.*, 2006)	Importin α/β	Centrosomal MAP (O'Brien *et al.*, 2005; Peset *et al.*, 2005)	Aurora A, XMAP215	Astral microtubule growth (Gergely *et al.*, 2003)	Phosphorylation by Aurora A (Albee *et al.*, 2006)
NuSAP (Ribbeck *et al.*, 2006)	Importin α and β, Importin (Ems-McClung *et al.*, 2004)	Spindle MAP (Raemaekers *et al.*, 2003)	Chromatin, DNA	Microtubule bundling and spindle stability	Microtubule stabilization and cross-linking (Ribbeck *et al.*, 2006), NuSAP-chromatin interaction (Ribbeck *et al.*, 2007)
Rae1 (Blower *et al.*, 2005)	Importin β	mRNA export factor (Brown *et al.*, 1995), checkpoint protein (Babu *et al.*, 2003; Jeganathan *et al.*, 2005)	RNA, Maskin, NuMA (Wong *et al.*, 2006)	Spindle assembly	Microtubule polymerization (Blower *et al.*, 2005)

(*continued*)

Table 3.2 (*continued*)

Cargo	Interacting transport receptors	Protein class	Interacting spindle factors	Downstream activities	RanGTP/transport receptor-regulated activities
Xnf7 (Maresca *et al.*, 2005)	Importin α/β	MAP	Anaphase Promoting Complex (APC) (Casaletto *et al.*, 2005)	Microtubule bundling, spindle stabilization (Maresca *et al.*, 2005), APC inhibitor (Casaletto *et al.*, 2005)	None identified
XCTK2 (Ems-McClung *et al.*, 2004)	Importin α/β	Spindle kinesin motor (Walczak *et al.*, 1997)	None identified	Spindle pole formation, spindle stability (Walczak *et al.*, 1997; Walczak *et al.*, 1998)	Microtubule binding (Ems-McClung *et al.*, 2004)
Kid (Trieselmann *et al.*, 2003)	Importin α/β	Chromosomal kinesin motor (Antonio *et al.*, 2000; Funabiki *et al.*, 2000)	Chromosomes	Chromosome movements, spindle function (Levesque and Crompton 2001; Levesque *et al.*, 2003)	Microtubule binding (Trieselmann *et al.*, 2003)
HURP* (Koffa *et al.*, 2006; Sillje *et al.*, 2006)	Importin β	MAP (Koffa *et al.*, 2006; Sillje *et al.*,2006; Wong and Fang, 2006)	XMAP215, Aurora A, Eg5 (Koffa *et al.*, 2006)	Chromosome alignment, (Koffa *et al.*, 2006; Sillje *et al.*, 2006; Wong and Fang 2006)	Microtubule binding, spindle localization (Sillje *et al.*, 2006)
Lamin B* matrix (Tsai *et al.*, 2006)	Importin α/β	Nuclear intermediate filament	Eg5, XMAP215, PAR	Spindle ''matrix'' formation	Matrix stability, association of spindle assembly factors

members of Ran-regulated cargoes are NuMA and TPX2, both MAPs that function in spindle assembly and are sequestered in the nucleus during interphase, as would be expected for import receptor cargoes. NuMA interacts with dynein and is required for spindle pole organization (Merdes *et al.*, 1996), but regulation of this activity by Ran has not been established. The best-characterized cargo is TPX2. Originally identified as a protein that targets the kinesin-12 family member Xklp2 to spindles in *Xenopus* egg extracts, depletion of TPX2 caused defects in spindle pole organization and centrosome-directed spindle assembly (Wittmann *et al.*, 2000), and completely blocked microtubule growth in the absence of centrosomes (See Table 3.3 for a list of important mitotic motors.) (Gruss *et al.*, 2001). Thus, TPX2 seems to be a key mediator of the chromatin-generated microtubule-stabilizing signal. This function appears to be conserved in somatic cells, since siRNA depletion of TPX2 caused defects in spindle organization (Garrett *et al.*, 2002; Gruss *et al.*, 2002). Using an assay to distinguish microtubule nucleation from centrosomes versus chromosomes in somatic cells, Tulu and colleagues found that TPX2 knockdown completely abolished chromosome-mediated microtubule nucleation in mammalian cells (Tulu *et al.*, 2006). This is the strongest evidence to show that TPX2 is functionally important for chromatin-mediated spindle assembly. Direct regulation of at least a subset of TPX2 activities by importins has been demonstrated. A TPX2 mutant that cannot bind importin α is constitutively active in the induction of microtubule asters in *Xenopus* egg extracts, and the ability of recombinant TPX2 to induce microtubule nucleation in a reconstituted system is inhibited by importin α (Schatz *et al.*, 2003).

Other MAPs that interact directly with importins include Maskin (Albee *et al.*, 2006), a TACC family member that stimulates microtubule growth at centrosomes (Gergely *et al.*, 2003), and NuSAP, a microtubule bundling factor that likely contributes to microtubule–chromatin interactions (Ribbeck *et al.*, 2006, 2007). Another important Ran-regulated importin β cargo and microtubule binding protein has been identified as Rae1, an mRNA export factor previously characterized in yeast (Brown *et al.*, 1995), and homologs of which appear to function in the checkpoint that monitors spindle assembly (Babu *et al.*, 2003; Jeganathan *et al.*, 2005). Rae1 depletion from egg extracts or cells impaired spindle assembly and, interestingly, Rae1 exists in a large complex that requires RNA for its microtubule-stabilizing activity (Blower *et al.*, 2005). However, not all importin cargoes that bind microtubules have obvious Ran-regulated functions in spindle assembly. For example, the activity of Xnf7, a bundling MAP that contributes to spindle integrity, does not appear to be modulated by importin β despite its RanGTP-regulated interaction (Maresca *et al.*, 2005).

In addition to regulating microtubule dynamics, Ran also alters the activity of microtubule motor proteins to promote bipolar organization (Carazo-Salas *et al.*, 2001; Wilde *et al.*, 2001). One spindle motor known

Table 3.3 Molecular motors functioning in spindle assembly

Kinesin superfamily	Protein name	Organism	Localization	Proposed function
Kinesin-4	Xklp1	*Xenopus*	Chromosome arms	Chromosome positioning, chromosome-MT attachment (Antonio *et al.*, 2000; Funabiki and Murray, 2000; Levesque and Compton 2001; Tokai-Nishizumi *et al.*, 2005; Vernos *et al.*, 1995)
	Klp3A	*Drosophila*	Chromosome arms, spindle midzone	Coupling MT sliding to spindle elongation (Brust-Mascher *et al.*, 2004)
Kinesin-5	Eg5	Vertebrates	Spindle	Spindle bipolarity (Mayer *et al.*, 1999; Sawin *et al.*, 1992), Poleward MT flux (Miyamoto *et al.*, 2004; Shirasu-Hiza *et al.*, 2004)
Kinesin-6	MKLP-1	Vertebrates	Spindle midzone	Central spindle organization, MT bundling, cytokinesis (Matuliene and Kuriyama 2002; Zhu *et al.*, 2005)
Kinesin-7	CENP-E	Vertebrates	Kinetochores	Chromosome congression, kinetochore-MT attachment (Kapoor *et al.*, 2006; Putkey *et al.*, 2002; Schaar *et al.*, 1997; Wood *et al.*, 1997; Yao *et al.*, 1997)
Kinesin-10	Kid	Vertebrates	Chromosome arms	Chromosome positioning, chromosome oscillations (Antonio *et al.*, 2000; Funabiki and Murray 2000; Levesque and Compton 2001; Tokai-Nishizumi *et al.*, 2005)
Kinesin-12	XKlp2	*Xenopus*	Spindle poles	Spindle pole organization (Wittmann *et al.*, 2002)
Kinesin-13	MCAK	Vertebrates	Centromere, spindle poles	Error correction (Kline-Smith *et al.*, 2004), chromosome segregation (Maney *et al.*, 1998)
			Spindle poles	Poleward MT flux (Gaetz and Kapoor 2004; Ganem *et al.*, 2005)
	Kif2A	Vertebrates	Kinetochores	Anaphase A Pacman motility (Rogers *et al.*, 2004)
	Klp59C	*Drosophila*	Spindle poles	Anaphase A poleward MT flux (Rogers *et al.*, 2004)
	Klp10A	*Drosophila*		
Kinesin-14	XCKT2	*Xenopus*	Spindle poles	Spindle pole focusing and promotion of spindle bipolarity (Walczak *et al.*, 1997)

to be directly regulated by Ran is the kinesin-14 XCTK2, which interacts with importin α/β through an NLS in its tail domain that lies near a microtubule-binding site. Addition of importins to the XCTK2 tail *in vitro* inhibits binding to microtubules, and is relieved upon addition of RanGTP (Ems-McClung *et al.*, 2004). The chromosomally localized kinesin Kid is also a likely target of Ran regulation. A domain of the protein containing NLS sequences interacts with importins in HeLa cell extracts, and importins inhibit Kid binding to microtubules, but not to DNA (Trieselmann *et al.*, 2003). RanGTP regulation of Kid and XCTK2 motor activities *in vivo* seems likely based on the spindle defects observed upon inhibition of the Ran pathway, but has not been clearly demonstrated.

3.3.2. Downstream spindle assembly factors regulated by the Ran pathway

A theme emerging from investigation of mitotic Ran targets is that many cargoes function in complexes, indicating that the Ran pathway activates a cascade involving many components. The best characterized series of downstream effectors is the RanGTP-activated TPX2-Aurora A axis. Whereas RanGTP does not affect TPX2-dependent targeting of Xklp2 to spindle poles, it does stimulate interaction between TPX2 and Aurora A kinase, which leads to activation of the kinase and TPX2 phosphorylation (Eyers *et al.*, 2003; Tsai *et al.*, 2003). Structural and biochemical assays have elucidated the mechanism by which TPX2 activates Aurora A (Bayliss *et al.*, 2003; Eyers and Maller, 2004). While it is unclear what functional effect Aurora A phosphorylation has on TPX2, phosphorylation of other recognized substrates, including the kinesin-5 Eg5 that promotes spindle bipolarity and TACC that promotes microtubule polymerization, could be major effectors of the pathway (Giet *et al.*, 1999, 2002). Interestingly, other known substrates include the oncogene HURP (Yu *et al.*, 2005) and the tumor suppressor protein BRCA1 (Ouchi *et al.*, 2004). Evidence has emerged that the Ran pathway regulates both of these factors, which have been found to exist in complexes with known spindle regulators and importin cargoes. HURP was isolated from *Xenopus* egg extracts together with TPX2, Aurora A, XMAP215, and Eg5 (Koffa *et al.*, 2006). Interestingly, HURP is a bundling MAP that localizes to K-fibers proximal to chromosomes, and is required for proper chromosome alignment (Koffa *et al.*, 2006; Sillje *et al.*, 2006; Wong and Fang, 2006). The BRCA1/BARD1 heterodimer was found in a complex with TPX2, NuMA, and XRHAMM, another *Xenopus* protein functioning in Ran-regulated chromatin-driven microtubule polymerization and spindle pole assembly (Groen *et al.*, 2004). Depletion of BRCA1/BARD1 from either egg extracts or cells caused both chromosome alignment and spindle pole defects (Joukov *et al.*, 2006). The current model is that BRCA1/BARD1 functions in a Ran-regulated pathway that localizes TPX2 to spindle poles by downregulating XRHAMM.

Both BRCA1 and BARD1 contain E3 ubiquitin ligase domains required for their spindle activity, suggesting that in addition to modulating a kinase cascade, the Ran pathway also affects protein ubiquitination and stability. Finally, a role for a RanGTP in forming a lamin B spindle matrix has been reported (Tsai *et al.*, 2006). Eg5 and XMAP215 were found associated with the matrix, suggesting that it could link multiple SAFs. Altogether, these interesting studies implicate a network of interactions orchestrated by RanGTP that functions in all aspects of spindle assembly, including microtubule nucleation, stabilization, and organization in the spindle. However, although binding to importin α/β has been demonstrated to alter some activities, the mechanisms by which the activities of these factors are regulated by RanGTP and importins are still poorly understood.

3.3.3. Ran function at kinetochores

The best-characterized mitotic role of the RanGTPase is as a spatial signal for chromosome arms to promote microtubule polymerization and organization due to the chromatin localization of the RanGEF RCC1. However, an even more dynamic and complex picture is emerging due to the localization of other regulators of the RanGTPase cycle. Not only does the Ran pathway release spindle assembly factors from importins, but it also recruits factors to their sites of action. For example, the enzyme opposing RCC1, RanGAP, is enriched on kinetochores, where it interacts with a large nuclear pore protein called RanBP2/Nup358 (Arnaoutov and Dasso, 2003; Joseph *et al.*, 2004). The kinetochore localization of RanGAP depends on both RCC1 activity, and the nuclear export factor CRM1. CRM1 forms complexes by binding simultaneously to RanGTP and leucine-rich nuclear export (NES) motifs in proteins. By mediating such interactions with Nup358/RanGAP, CRM1 is thought to localize them to kinetochores. Disruption of these interactions leads to defective K-fibers, kinetochore misalignment, and chromosome segregation defects (Arnaoutov *et al.*, 2005). Interestingly, a role for Ran pathway components in kinetochore–microtubule interactions has also been demonstrated in yeast (Tanaka *et al.*, 2005), a system undergoing ''closed'' mitosis without nuclear envelope breakdown. In these cases, component localization, rather than gradients, is likely to be facilitating specific mitotic functions. These molecular associations at the kinetochore also impinge on cell cycle regulation. Elevation of RCC1 levels abrogates the spindle checkpoint, disrupting the kinetochore localization of known checkpoint regulators (Arnaoutov and Dasso, 2003). Crm1 and the Ran pathway have also been implicated in centrosome duplication and function, also by mediating the formation of RanGTP–CRM1–NES–protein complexes with factors such as nucleophosmin (Wang *et al.*, 2005), indicating that the centrosome might be a second site of regulated interactions that has functional consequences (Ciciarello and Lavia, 2005). Finally, Ran has additional functions at the

end of mitosis as a regulator of nuclear envelope and nuclear pore complex assembly. Interestingly, many factors, including the large Nup107–160 complex and MEL-28, are multifunctional elements contributing to both nuclear pore and kinetochore assembly (Roux and Burke, 2006). Altogether, Ran and the nuclear transport machinery perform a wide variety of important functions throughout the cell cycle.

4. Mechanisms of Chromosome Congression and Attachment

4.1. Classic model of congression

Congression is the process by which chromosomes attach to spindle microtubules and align or ''congress'' to the metaphase plate. In classic models, sister kinetochores capture dynamic microtubules from opposite poles of the spindle, become bioriented, and track toward the metaphase plate where forces acting on them become equal (Hayden *et al.*, 1990) (Fig. 3.3A). In many cell types, chromosome oscillations occur throughout the process, as the sister kinetochores alternatively ''lead'' by moving toward their respective spindle pole. As congression proceeds, additional microtubules are incorporated into the K-fiber bundle of microtubules that is embedded at the centromere (McEwen *et al.*, 1997). The mechanism of congression is thought to involve regulated dynamics of spindle microtubules as well as the action of molecular motor proteins.

A major force moving chromosomes is generated through depolymerization of the plus ends of microtubules embedded at kinetochores that nevertheless remain attached, shortening the K-fiber and pulling the chromosome poleward. In contrast, polymerizing K-fibers do not appear to push chromosomes away from the pole. The revealing experiment was to sever the connection between sister kinetochores of a bioriented chromosome with a laser beam. The sister leading toward its spindle pole continued moving, while the trailing sister stopped, indicating that the forces on each sister kinetochore are directed toward its spindle pole (Khodjakov and Rieder, 1996). Furthermore, when oscillating chromosomes are observed at high resolution, the leading sister kinetochore of a chromosome appears stretched, as if being pulled (Skibbens *et al.*, 1993).

In addition to forces acting at the kinetochore, forces on chromosome arms also contribute to chromosome congression. As a chromosome arm is severed within the spindle of a newt lung cell, the chromosome fragment is rapidly pushed toward the metaphase plate and away from the highest density of spindle microtubules, a phenomenon called the ''polar ejection force'' (Rieder *et al.*, 1986). The interpretation of this experiment has been that either the polymerization of microtubules toward the spindle center

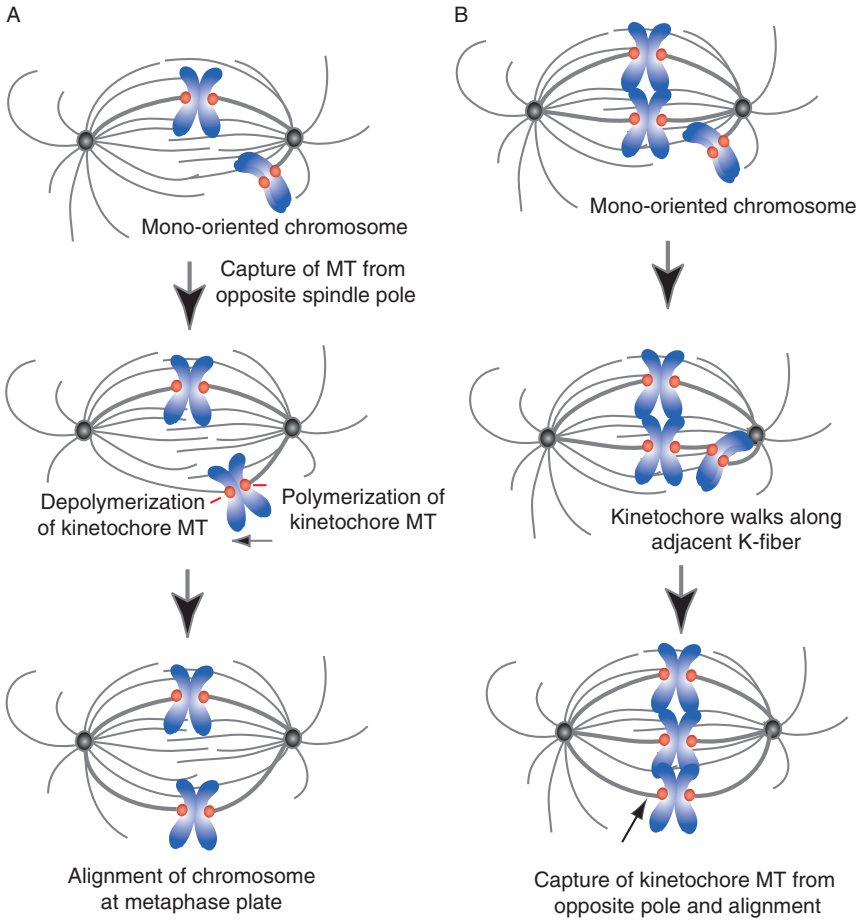

Figure 3.3 Models of chromosome congression. Both bioriented congressed chromosomes and newly captured monooriented chromosomes are depicted. (A) In the conventional model, the unattached kinetochore of a monooriented chromosome is contacted by a microtubule emanating from the opposite pole and begins to congress toward the metaphase plate. This movement is associated with net microtubule depolymerization at the leading kinetochore and microtubule polymerization at the lagging kinetochore, eventually aligning the chromosome on the metaphase plate. (B) Monooriented chromosomes can also congress to the metaphase plate by the action of molecular motors (CENP-E, not shown) at the kinetochore walking along an adjacent K-fiber toward the metaphase plate, where it encounters microtubules from the opposite pole, becoming bioriented and properly aligned. In model (A) biorientation precedes congression, whereas in model (B), congression can occur without biorientation, and may serve to facilitate bipolar attachments.

pushes the chromosome arms away from the poles, or that motor proteins on the arms slide the chromosome along the spindle microtubules toward their plus ends and the spindle equator. Indeed, motor proteins such as

kinesin-4 and kinesin-10 family members XKlp1 and Kid, respectively, have been found associated with chromosome arms. Inhibition of these kinesins results primarily in chromosome arm alignment defects (Antonio *et al.*, 2000; Funabiki and Murray, 2000; Levesque and Compton, 2001; Tokai-Nishizumi *et al.*, 2005; Vernos *et al.*, 1995). The polar ejection force has also been directly correlated with the activity of Kid using optical trap measurements in a reconstituted *in vitro* system (Brouhard and Hunt, 2005). However, another study has raised the interesting possibility that the polar ejection force does not contribute to congression per se, but rather promotes chromosome oscillations. About 80% of chromosomes in cells injected with anti-Kid antibodies congressed normally with typical movement velocities, but lacking oscillations, while the remainder were stuck at the poles (Levesque and Compton, 2001). Thus, in the process of moving arms toward the spindle equator, chromokinesins may promote kinetochore attachments that allow congression to occur. These data suggest that a combination of kinetochore and chromosome arm forces generates oscillations, but the underlying mechanism is unknown. This is perhaps not surprising given the sophisticated cytological and micromanipulation experiments required to study this phenomenon. As the functional roles of more spindle proteins are elucidated, we will be in a better position to identify key players in this process.

A fascinating feature of kinetochore behavior is that despite the complex poleward and antipoleward forces exerted on chromosomes, sister kinetochore movements are coordinated with one another. The directional instability that occurs during oscillation appears to be regulated by tension across the sisters (Skibbens *et al.*, 1993, 1995; Skibbens and Salmon, 1997; Waters *et al.*, 1996). A microtubule dynamics-based model proposes that the microtubules of the leading kinetochore's fiber are coordinately depolymerizing, while its trailing sister is associated with a fiber of polymerizing microtubules. In this scenario, oscillations are controlled by microtubule dynamics regulatory proteins at the kinetochores whose activities are readily turned on or off at a single kinetochore. However, an electron tomography study of K-fiber microtubules in cultured cells has revealed that both sisters are associated with a mixture of polymerizing and depolmerizing microtubules, with approximately two-thirds of the microtubules in the depolymerization state based on their peeling protofilament morphology (VandenBeldt *et al.*, 2006). This was surprising because it could be predicted that the fraction of polymerizing and depolymerizing microtubules would be different at each sister kinetochore. In addition, the ratio of polymerizing to depolymerizing microtubules was similar in both vertebrate PtK2 cells in which chromosomes do oscillate and in *Drosophila* S2 cells in which chromosomes do not oscillate. This study brings into question how the dynamic state of each individual microtubule within a K-fiber contributes to the overall directional movement of that kinetochore and the more fundamental issue of the physiological importance of chromosome oscillations

to spindle function. The answers to these questions require a better understanding of the molecular players that govern chromosome motility, oscillations, and kinetochore microtubule structure.

4.2. Molecular mechanisms governing chromosome congression

A major emphasis in the field now revolves around understanding which proteins contribute to the congression of chromosomes and what their underlying mechanisms are. This has actually been quite difficult to dissect because inhibition of many proteins causes a defect in chromosome alignment that may or may not reflect a direct involvement of that factor. For example, inhibition of proteins necessary for assembly of the kinetochore leads to defects in chromosome–microtubule attachment that ultimately cause defects in the congression of chromosomes (Maiato *et al.*, 2004). However, a few factors have been discovered that clearly act in chromosome congression. Most notably, loss of the kinesin-7 CENP-E leads to defects in chromosome congression in multiple systems (Schaar *et al.*, 1997; Thrower *et al.*, 1995; Wood *et al.*, 1997; Yao *et al.*, 1997; Yen *et al.*, 1991, 1992). It was initially thought that the congression defects were due to the action of CENP-E as a plus end-directed motor at the kinetochore translocating chromosomes along their individual K-fibers (Wood *et al.*, 1997). However, follow-up data pointed more toward a role of CENP-E in microtubule attachment, as inhibition of CENP-E *in vitro* impaired the ability of chromosomes to track on depolymerizing microtubules (Lombillo *et al.*, 1995a). In support of this attachment model, it was later shown that CENP-E knockout or disruption resulted in chromosomes near the poles with very few or no associated microtubules (McEwen *et al.*, 2001). However, an elegant recent study may have resolved much of this earlier debate (Fig. 3.3B). Using high-resolution imaging, Kapoor and colleagues showed that monooriented chromosomes congressed to the spindle equator by translocating their leading kinetochores laterally along already formed K-fibers of other chromosomes, and that CENP-E was required for this movement (Kapoor *et al.*, 2006). These data are exciting because they indicate that bipolar attachment of sister kinetochores is not a prerequisite for chromosome congression as previously thought (Fig. 3.3A), and that by using its plus end-directed motor activity to walk along K-fibers CENP-E drives movement of chromosomes toward the center of the spindle where interaction with spindle microtubules from the opposite pole and thus bipolar attachments are more likely to occur (Fig.3.3B).

4.3. Kinetochore–microtubule attachment

The ultimate goal of mitosis is to ensure accurate segregation of chromosomes to the two daughter cells. Defects in attachment of chromosomes to the spindle may lead to defects in chromosome congression and ultimately

to defective chromosome segregation. The number of mistakes in mitosis is reduced in part through error-correcting mechanisms that occur both prior to and after the initiation of anaphase (Cimini *et al.*, 2001, 2002, 2003, 2004). In addition, the mitotic checkpoint leads to cell cycle arrest if chromosomes are not attached and aligned properly on the mitotic spindle (Chan and Yen, 2003; Malmanche *et al.*, 2006; Musacchio and Hardwick, 2002). The mitotic checkpoint may respond to kinetochore–microtubule attachment and/or the tension generated between the sister kinetochores from their bipolar attachment to microtubules from the opposite pole, which is reflected in the physical distance between opposing kinetochores. However, these signals may not be separable since microtubule attachment is required to generate tension at the kinetochore, but tension contributes to stabilizing attachments (Pinsky and Biggins, 2005).

4.3.1. Molecular mechanisms underlying kinetochore–microtubule attachment

Our knowledge about the proteins responsible for proper kinetochore–microtubule attachment has expanded tremendously over the past decade through both genetic studies in yeast and flies and protein knockdown or inhibition in vertebrate cells in culture. In general, attachment defects can be caused by a failure in the actual attachment mechanism or in the assembly of the kinetochore. The details of the latter process have been discussed extensively in several papers and reviews and will not be focused on here (Carroll and Straight, 2006; Chan *et al.*, 2005; Liu *et al.*, 2006; Vos *et al.*, 2006).

One of the key players important for mediating kinetochore–microtubule attachment is the Ndc80 complex, which consists of Ndc80/Hec1, Nuf2, Spc24, and Spc25. This protein complex is well conserved and appears to be important for kinetochore–microtubule interactions in every organism examined to date (Kline-Smith *et al.*, 2005). The Hec1 subunit is found at the outer plate of the vertebrate kinetochore in the perfect place to mediate interactions with microtubules (DeLuca *et al.*, 2005). More recent data have provided significant new mechanistic insight into the function of this complex. Knockdown of the Ndc80 complex by RNAi results in chromosome alignment defects, reduced K-fiber stability, and poor microtubule binding as judged by electron microscopy (DeLuca *et al.*, 2002, 2005; McCleland *et al.*, 2003, 2004). Most of these defects seem to occur without perturbing kinetochore assembly, suggesting that they are indeed due to loss of microtubule attachment activities. However, a more detailed analysis using antibody microinjection and high-resolution imaging presents a slightly different picture (DeLuca *et al.*, 2006). Microinjection of an antibody that binds to the N-terminus of Ndc80 strongly stabilized microtubule–kinetochore attachments and significantly reduced K-fiber dynamics, indicating that the antibody suppressed the ability of Ndc80 to detach the kinetochore from the spindle. These results indicate a direct role

for Ndc80 in mediating attachments of microtubules to kinetochores. In addition, this attachment activity of Ndc80 is regulated by Aurora B kinase, which may represent an important mechanism to regulate proper attachments (see later).

Further support for the role of Ndc80 in mediating microtubule attachment comes from elegant biochemical studies examining the ability of this complex to bind microtubules. In *C. elegans*, the Ndc80 complex is a part of a larger complex called the KNM network, which contains KNL-1, the Mis12 complex, and the Ndc80 complex (Cheeseman *et al.*, 2004, 2006). Using an *in vitro* reconstitution approach Cheeseman and colleagues showed that both the Mis12 and the Ndc80 complexes could bind directly to microtubules *in vitro* albeit with very low affinity (Cheeseman *et al.*, 2006). However, when the full KMN complex was reconstituted, binding became very tight, providing a mechanism to not only bind microtubules but also to control the binding affinity. These data provide biochemical evidence for how the Ndc80 complex may aid in microtubule capture.

Another player critical for mediating kinetochore–microtubule attachments in yeast is the 245-kDa protein Dam1 complex, which contains 10 proteins and is essential for spindle integrity and kinetochore–microtubule associations. Dam1 complex mutants displayed significant rates of chromosome loss, indicating a role for the kinetochore (Cheeseman *et al.*, 2001; Jones *et al.*, 2001). Strikingly, the purified Dam1 complex formed ring-like structures around microtubules that moved processively along the lattice during depolymerization (Asbury *et al.*, 2006; Westermann *et al.*, 2005, 2006). This places Dam1 in the perfect position to act as the so-called collar or kinetochore sleeve, which has been hypothesized for years to be a mechanism by which kinetochores could remain attached to the dynamic ends of K-fiber microtubules (Hill, 1985). However, no orthologs of Dam1 have been identified outside of yeast, questioning whether its function is conserved. It may be that other proteins play analogous roles in other systems. Consistent with this idea, it was shown that the kinesin-13 family members could also form rings around microtubules (Moores *et al.*, 2006; Tan *et al.*, 2006). However, the nature of each of these rings appears quite distinct, and it is unclear whether the kinesin-13 tubulin complex actually forms during microtubule depolymerization and whether it slides processively as does the Dam1 complex. The identification of the Dam1 complex ortholog in vertebrate systems is clearly an important goal for future studies.

Several other types of microtubule binding proteins have also been implicated in attaching kinetochores to microtubules. A family of proteins that associates specifically with growing microtubule plus ends, called +TIPs, have emerged as important players. One member of this family called MAST/Orbit (also known as CLASP) is required to achieve proper end-on attachments of microtubules to kinetochores (Maiato *et al.*, 2003b; Walczak, 2005), where it resides and acts in part by regulating the plus end

dynamics of K-fibers (Maiato *et al.*, 2002, 2003a, 2005). In vertebrate cells, there are two CLASP orthologs that act redundantly, which may be an important mechanism to maintain proper chromosome segregation fidelity (Mimori-Kiyosue *et al.*, 2006; Pereira *et al.*, 2006). In *C. elegans*, the CENP-F like proteins HCP-1 and HCP-2 are also important for proper chromosome segregation most likely by targeting CLASP to kinetochores (Cheeseman *et al.*, 2005). *C. elegans* CLASP is specifically required for chromosome biorientation when there is a poleward directed force. These studies are consistent with the idea that CLASPs are particularly important in modulating plus end dynamics of K-fibers, which is discussed in more detail later.

CENP-F, also known as mitosin, may also be required to target additional components to the kinetochore as its knockdown by RNAi resulted in diminished levels of CENP-E, cytoplasmic dynein (Yang *et al.*, 2005), as well as several markers of the mitotic checkpoint at kinetochores (Feng *et al.*, 2006), and in some cases a complete failure in kinetochore assembly (Bomont *et al.*, 2005). CENP-F can also directly bind to microtubules (Feng *et al.*, 2006) and may play a more direct role in mediating attachments. In addition to CLASPs, other members of the +TIP tracking proteins (Lansbergen *et al.*, 2006) have also been implicated in mediating kinetochore-microtubule interactions (Dujardin *et al.*, 1998; Tanenbaum *et al.*, 2006). In particular, CLIP-170 localizes both directly to kinetochores as well as to the plus ends of growing K-fibers where it appears to affect kinetochore–microtubule attachments without affecting the dynamics there, although this has not been examined directly. Overall, these studies suggest that MAPs are critical to specifically regulate distinct subsets of microtubule dynamics during spindle assembly, particularly those that occur at the kinetochore to help mediate attachments to microtubules.

4.3.2. Molecular mechanisms required for error correction in microtubule attachments

A major goal of setting up the mitotic spindle is to ensure not only that chromosomes attach to microtubules, but also that these attachments are correct (Maiato *et al.*, 2004; McIntosh *et al.*, 2002). A proper spindle attachment, termed amphitelic, occurs when each sister kinetochore is attached to microtubules from its nearest, facing pole, such that when the cell enters anaphase and the sister chromatids disjoin, they will move to opposite poles. Monooriented chromosomes in which only one sister kinetochore is attached to the mitotic spindle occur transiently, but most chromosomes achieve proper amphitelic attachments by the end of prometaphase. However, two common types of malattachments can also form, termed syntelic and merotelic (Fig. 3.4A). In syntelic attachments, both sister kinetochores are attached to a single pole, resulting in segregation failure and two aneuploid daughters, since both sister chromatids would move to the

same pole at anaphase. In a merotelic attachment, a single kinetochore is attached to microtubules from both spindle poles (Salmon *et al.*, 2005). When anaphase ensues, the merotelic kinetochore remains in the spindle midzone and is lost, often resulting in one aneuploid daughter cell.

While most defective attachments are sensed and corrected prior to the initiation of anaphase, merotelic attachments are particularly detrimental because they are not sensed by the spindle checkpoint (Cimini *et al.*, 2001), probably because both kinetochores are attached to the spindle and some tension is generated. Molecularly, there are several factors critical for correcting malattached kinetochores. In budding yeast, the Ipl1/Aurora protein kinase is required to detect and correct monooriented attachments that result in tension defects (Biggins and Murray, 2001; Tanaka *et al.*, 2002). In vertebrates, Aurora B kinase is also critical for mediating proper attachments (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Kallio *et al.*, 2002; Murata-Hori and Wang, 2002). Aurora B exists in a complex with INCENP, survivin, and borealin/Dasra (Gassmann *et al.*, 2004; Sampath *et al.*, 2004) that together are critical for many aspects of Aurora B function (Vader *et al.*, 2006). Loss of Aurora B activity also leads to a dramatic increase in the overall stability of the K-fiber without affecting the dynamics

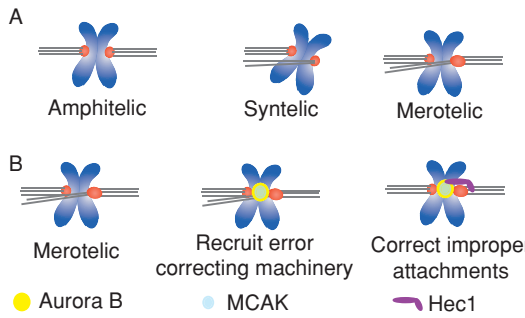

Figure 3.4 Mechanisms to correct chromosome attachment errors. (A) Types of chromosome attachments. In a proper amphitelic attachment, sister kinetochores are attached to microtubules from opposite poles. A syntelic attachment occurs when both sister kinetochores are attached to microtubules from a single pole. If anaphase ensues, both sister chromatids will move to the same daughter cell. In a merotelic attachment, one sister kinetochore is attached properly to microtubules from one spindle pole, but the other sister kinetochore is attached to microtubules from both poles. During anaphase in this scenario, one chromatid will segregate properly, and the other will be left behind at the spindle equator as a lagging chromatid. (B) Mechanisms of error correction. When an inappropriate attachment occurs, depicted in the diagram as merotelic, molecules involved in the error correction process are recruited to kinetochores and include Aurora B and mitotic centromere-associated kinesin. The error is then corrected at least in part by the action of Aurora B on MCAK and Hec1 to destabilize faulty attachments and promote amphitelic attachments. Other molecules required for this process have yet to be elucidated.

of the nonkinetochore microtubules (Cimini *et al.*, 2006; Zhang and Walczak, 2006). This suggests that Aurora B likely promotes detachment of incorrectly attached microtubules by altering dynamics of the K-fibers.

Aurora B has been shown to act through at least two major substrates to help correct errors (Fig. 3.4B). One important substrate is the microtubule destabilizing kinesin-13 mitotic centromere-associated kinesin (MCAK). Loss of MCAK at centromeres leads to an increase in malattached chromosomes with both merotelic and syntelic attachments (Kline-Smith *et al.*, 2004). MCAK is a microtubule depolymerase that resides at the inner centromere (Moore and Wordeman, 2004). It is easy to envision a model whereby Aurora B phosphorylates MCAK to stimulate its activity to depolymerize incorrectly attached microtubules. This, however, is not the case because Aurora B phosphorylation of MCAK actually inhibits its microtubule depolymerization activity (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004). In addition, Aurora B phosphorylation of MCAK changes both its localization within the centromere/kinetochore region as well as its residence time at the kinetochore, showing that the mechanism of Aurora B regulation of MCAK is quite complex (Andrews *et al.*, 2004; Lan *et al.*, 2004). Interestingly, it was shown that MCAK, in its activated form, is specifically recruited to sites of merotelic attachments (Knowlton *et al.*, 2006). This provides an attractive model in which the cell specifically recruits the error-correcting machinery to the sites of the errors.

A second key substrate of Aurora B is the Hec1 component of the Ndc80 complex (Cheeseman *et al.*, 2006; DeLuca *et al.*, 2006; Pinsky *et al.*, 2006). Loss of Hec1 not only causes defects in microtubule attachment but also causes a dramatic change in the dynamics of microtubules at the kinetochore (DeLuca *et al.*, 2006). In particular, in the absence of Hec1 there was no incorporation of tubulin into the kinetochore microtubules and no oscillations of the chromosomes. Expression of a phosphomutant form of Hec1 also resulted in aberrant microtubule attachments, suggesting that Aurora B phosphorylation of Hec1 is critical for maintaining its proper activity. Together these results are consistent with the idea that Hec1 activity is regulated to promote either attachment or detachment of kinetochores from microtubules. Such a mechanism is critical to ensure that only proper attachments are maintained and that improper attachments are eliminated.

While the previous studies provide attractive mechanisms for regulating attachment of chromosomes to the spindle, they do not represent the whole story. Kapoor and colleagues looked specifically at how Aurora B works on correcting syntellically oriented kinetochores (Lampson *et al.*, 2004). Cells were arrested with the kinesin-5 small molecule inhibitor monastrol in a monopolar configuration in which the kinetochores were attached syntelically. When monastrol was washed out in the presence of an Aurora B inhibitor, bipolar spindles assembled in which chromosomes were still malattached. After washout of the Aurora B inhibitor the syntelically

attached chromosomes rapidly moved toward the pole coupled with depolymerization of the K-fiber. Movement occurred at rates similar to that of prometaphase/metaphase chromosome movements, suggesting that it was due to end-on attachment of kinetochores. Once they reached the pole, the chromosomes began to congress toward the spindle equator. These data clearly demonstrate that Aurora B is actively involved in error correction, and causes dramatic changes in K-fiber microtubule dynamics, but it does not reveal which molecules are acting downstream of Aurora B in the correction process—an important future endeavor. In addition, Aurora B inhibition also affects the dynamics of chromatin-nucleated microtubules and spindle microtubules, suggesting that its action is not strictly limited to kinetochores, which adds to the complexity of the story (Kallio *et al.*, 2002; Sampath *et al.*, 2004).

5. Mechanisms of Chromosome Segregation

While cells utilize diverse mechanisms to ensure the proper attachment of chromosomes to the spindle, the ultimate goal of mitosis is to achieve their proper segregation, thereby distributing the genetic material equally to the two daughter cells. Anaphase can be divided into two, often overlapping phases, called anaphase A and anaphase B. During anaphase A, the separated sister chromatids move toward the spindle poles and during anaphase B, the spindle poles move apart. Both anaphase A and anaphase B promote sister chromatid separation, and the extent of anaphase A versus B varies greatly among different organisms and cell types. In budding yeast, inhibition of anaphase A movement can be compensated for by a longer duration of anaphase B (Straight et al., 1998).

5.1. Anaphase A chromosome segregation

The movement of chromatids toward the spindle poles is achieved through two major mechanisms: by coupling to microtubule depolymerization and by utilizing the activity of molecular motor proteins at the kinetochore. Early studies attempting to reconstitute chromosome motility *in vitro* concluded that both microtubule depolymerization and motor protein activity were important (Coue *et al.*, 1991; Hyman and Mitchison, 1991). This clearly is the case *in vivo*. In vertebrate cells in culture, chromatids move toward the poles as the K-fibers depolymerize mainly from their plus ends, in a movement termed "pacman," based on the video game (Gorbsky *et al.*, 1987, 1988). This observation highlights the geometric problem of how the kinetochore remains attached to a substrate (the microtubule) that is depolymerizing. The idea of a sleeve that can slide along a depolymerizing

microtubule, perhaps by the action of microtubule–kinetochore coupling proteins, is attractive (Hill, 1985) and is perhaps being fulfilled by the activity of ring structures such as the Dam1 complex described previously. Alternatively, dynamic microtubule binding proteins may be sufficient to fulfill this role. Indeed, plus end-directed kinesin motor proteins have been shown to couple the movement of either kinetochores or motor-coated beads to depolymerizing microtubules (Lombillo *et al.*, 1995b).

The movement of chromatids toward poles is not strictly achieved through depolymerization of microtubules at the kinetochores. Microtubules can also depolymerize from their minus ends, and this minus end depolymerization is correlated with the rates of chromatid movement in several systems (Brust-Mascher and Scholey, 2002; Desai *et al.*, 1998; Maddox *et al.*, 2002). Within a spindle, microtubules exhibit a treadmilling phenomenon known as microtubule "poleward flux" in which tubulin subunits incorporate into the plus end of the K-fibers, translocate toward the poles, and then dissociate from the minus ends of the microtubules (Khodjakov and Kapoor, 2005; Kwok and Kapoor, 2007; Rogers *et al.*, 2005). If K-fiber microtubule polymerization stops, continued flux could drag the attached chromatids poleward. The rate of poleward flux varies among different organisms as does the extent of chromatid movement that is correlated with flux. For example, in vertebrate cells about 70% of the chromatid movement occurs via pacman kinetochores and only ~30% of the movement occurs via flux (Mitchison and Salmon, 1992). In contrast, in *Drosophila* embryos and in spindles assembled in *Xenopus* egg extracts the situation is reversed, and the majority of chromosome movement occurs via flux (Brust-Mascher and Scholey, 2002; Desai *et al.*, 1998; Maddox *et al.*, 2002). Why different segregation mechanisms predominate in different situations is unclear, but could reflect functional redundancy that is advantageous to ensure the accurate distribution of the genetic material.

The molecular mechanisms governing anaphase A chromosome segregation are still under question, but recently much progress has been made (Fig. 3.5). Early on, the best candidate to move chromosomes poleward was cytoplasmic dynein, which is found associated with kinetochores and has poleward, minus end-directed motility (Pfarr *et al.*, 1990; Steuer *et al.*, 1990; Vallee, 1990). Inhibition of dynein activity by microinjection of antibodies into *Drosophila* embryos caused a reduction in chromatid-to-pole movement; however, there were also disruptions in spindle organization that could have had indirect effects (Sharp *et al.*, 2000). In *Drosophila* male meiosis, mutations in the dynein-associated protein ZW10 severely impaired anaphase A movement (Savoian *et al.*, 2000), providing further support for the idea that dynein drives chromosomes poleward. Consistent with the previous *in vivo* studies *in vitro* assays revealed that dynein-coated beads are able to track along depolymerizing microtubules, albeit these studies were performed with flagellar dynein (Lombillo *et al.*, 1995b).

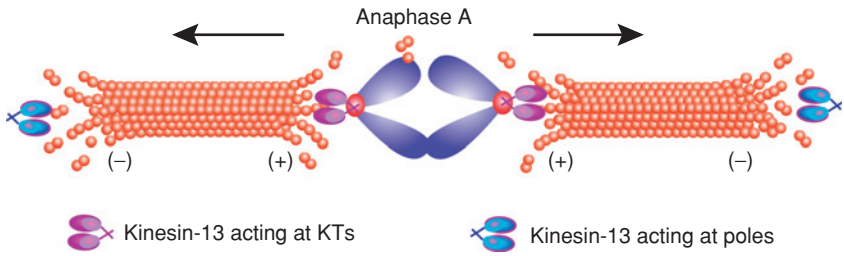

Figure 3.5 **Mechanisms of anaphase A chromosome segregation. During anaphase A, chromatids move toward the poles via depolymerization of microtubules from their kinetochores ("pacman") as well as through minus end microtubule depolymerization at the spindle poles (poleward flux). The best candidates to mediate this process are members of the kinesin-13 family of microtubule-depolymerizing kinesins.**

More recent studies on spindles assembled in *Xenopus* egg extracts provide an alternative explanation for the role of dynein in anaphase A (Gaetz and Kapoor, 2004). Disruption of dynein in the egg extracts resulted in longer spindles concomitant with a failure to deliver the microtubule depolymerizing kinesin Kif2A to the poles. In this model, dynein may contribute to chromosome segregation not by its action at kinetochores but rather by its action to deliver components of the flux machinery to spindle poles. In addition to targeting Kif2A, dynein also mediates delivery of interacting MAPs such as the cross-linking protein NuMA (Merdes *et al.*, 2000), and functions to strip checkpoint proteins from kinetochores by translocating them to the poles (Howell *et al.*, 2000). Thus dynein almost certainly acts in anaphase given its multiple spindle localizations and functions, but where and how it contributes to chromosome segregation remains unclear.

Given that chromosome movement toward poles is associated with depolymerization of microtubules from the kinetochore, it is interesting to speculate that microtubule-depolymerizing enzymes localized at the kinetochore are involved in anaphase chromosome segregation. Disruption of MCAK, a kinesin-13 family member, in vertebrate cells caused lagging chromosomes at anaphase (Maney *et al.*, 1998), but this was likely due to improperly attached kinetochores as there were no defects in the rate of chromosome-to-pole movement upon disruption of MCAK (Kline-Smith *et al.*, 2004). However, other members of the kinesin-13 family appear to be important for chromosome movement. In *Drosophila*, disruption of Klp59C in embryos resulted in a 55% decrease in the rate of chromatid-to-pole movement, suggesting that this protein is part of the pacman machinery (Rogers *et al.*, 2004). However, we have yet to uncover the vertebrate ortholog of this activity because disruption of both MCAK and Kif2a resulted in a 20% decrease in anaphase A chromatid-to-pole movement due to disruption of flux and not of the pacman machinery (Ganem *et al.*,

2005). Identifying the key players in this motility in vertebrate cells is certainly an important open question in the field.

While the molecular components that govern the pacman machinery have remained largely unknown, we are gaining a much better understanding of the components that contribute to poleward microtubule flux (Kwok and Kapoor, 2007; Mitchison, 2005; Rogers *et al.*, 2005). Microtubule flux consists of three activities: plus end polymerization, translocation of microtubules toward the poles, and minus end depolymerization. At the plus ends, CLASPs have been implicated in driving assembly of tubulin at the kinetochore, thus contributing to flux. Knockdown of CLASP in *Drosophila* S2 cells caused a complete inhibition in flux because tubulin was no longer incorporated in K-fibers, providing the first real evidence that plus end polymerization of tubulin plays an important role in driving flux (Maiato *et al.*, 2005). The major factor implicated in the translocation aspect of flux has been the kinesin-5 family member Eg5. Eg5 is a plus end director motor that can slide both parallel and antiparallel microtubules (Kapitein *et al.*, 2005). The role of Eg5 in flux, however, has been controversial. In *Xenopus* egg extracts, early studies using antibody inhibition showed that loss of Eg5 did not affect flux rates as measured by photoactivation of fluorescence (Sawin and Mitchison, 1994). In contrast, more recent studies in egg extracts using small molecule inhibitors of Eg5 showed that there was a dose-dependent inhibition of the rate of flux as measured by fluorescence speckle microscopy and cross-correlation microscopy (Miyamoto *et al.*, 2004). It is possible that the more sophisticated methods of analysis allowed detection of subtle changes in flux rates. The question of flux movement in cultured cells is a bit more complicated. Use of the same inhibitors that affect flux in extracts had only a very modest effect (30% reduction) on the flux rate in cells, suggesting that kinesin-5s do not play a major role in mediating flux in vertebrate cultured cells (Cameron *et al.*, 2006). Because the flux rate is slower in cultured cells than in extracts and constitutes only about 30% of the anaphase A chromatid motility, the cell may rely primarily on other mechanisms to drive chromosome segregation.

With regard to depolymerization at the spindle poles, it is clear in multiple systems that a member of the kinesin-13 family likely contributes. The first studies in *Drosophila* embryos showed that there was a nearly complete inhibition in the rate of flux, and chromosome movement was slowed by about 40% after inhibition of the kinesin-13 member Klp10A, consistent with the percentage of anaphase A motility that occurs via flux in this system (Rogers *et al.*, 2004). In vertebrate cells, one difficulty is that inhibition of the potentially orthologous kinesin-13 called Kif2A resulted in monopolar spindles, and flux could not be examined in those spindles (Ganem and Compton, 2004). Spindle bipolarity could be rescued by coinhibition of a second kinesin, MCAK. Under this dual inhibition, the rate of flux was substantially inhibited relative to MCAK inhibition alone,

suggesting that Kif2A drives flux (Ganem *et al.*, 2005). The rate of chromosome motility was slowed by about 25%, and there was a substantial increase in the percentage of missegregated chromosomes, which could mean that flux is utilized for error correction in vertebrate cells, but this explanation is complicated by the fact that inhibition of MCAK also increases attachment errors (Kline-Smith *et al.*, 2004).

In addition to the inhibition of Kif2A, it was shown that dynein inhibition by addition of a dominant-negative fragment of p150 dynactin called CC1 (King *et al.*, 2003; Quintyne *et al.*, 1999) caused an increase in spindle length and a decrease in the delivery of Kif2A to poles in egg extracts (Gaetz and Kapoor, 2004). In support of these findings, inhibition of dynein also resulted in longer spindles with decreased levels of flux (Shirasu-Hiza *et al.*, 2004). Overall, these studies are consistent with a model whereby major players in mediating flux include a member of the CLASP family, a motor, most likely of the kinesin-5 family, and a member of the kinesin-13 family at the poles. The exact molecule that functions in each process, how its activity is controlled, and whether there are small differences in the uses of molecules between systems remain to be addressed. One note of caution is that these minor discrepancies between systems can make it dangerous to conclude a definitive molecular function for a given protein by studies in a single organism. Rather, the different systems should be used as tools to help us understand the possible biological mechanisms underlying a particular type of chromosome or microtubule movement.

5.2. Anaphase B spindle pole separation

The driving apart of the spindle poles during anaphase B is thought to be accomplished by forces within the spindle as well as with forces on the astral microtubules where they contact the cortex (Rosenblatt, 2005). As chromosomes segregate, they leave behind "passenger proteins" at the equator, many new factors are recruited, and a microtubule structure called the central spindle forms. Motors that slide antiparallel microtubules in the central spindle are thought to make a major contribution to anaphase B. In *Drosophila* embryos, it has been proposed that the kinesin-5 Klp61F drives the sliding of antiparallel microtubules while kinesin-13 Klp10A drives depolymerization at poles and that the kinesin-4 Klp3A suppresses flux to couple sliding to spindle elongation (Brust-Mascher *et al.*, 2004; Brust-Mascher and Scholey, 2002). In addition, members of the kinesin-6 family, including MKLP1, may drive microtubule sliding through organization of the microtubule bundles in the central spindle. Inhibition of MKLP1 by antibody injection caused a block in mitotic progression and disorganized central spindles (Matuliene and Kuriyama, 2002, 2004; Nislow *et al.*, 1990, 1992). More recently, RNAi knockdown showed that the kinesin-6 proteins MKLP1 and MKLP2 are critical for central spindle

organization that is necessary for cytokinesis (Zhu *et al.*, 2005). Furthermore, laser microsurgery experiments in yeast revealed that forces for sliding of the central spindle are indeed able to drive spindle pole separation (Khodjakov *et al.*, 2004). Together, these data support the idea that motor proteins can cross-link and slide microtubules in the central spindle to drive spindle pole separation. However, it is also clear that motors organize the central spindle late in mitosis, and their inhibition gives rise to cytokinesis defects. It therefore has yet to be determined which motors are essential for antiparallel microtubule sliding during anaphase B in vertebrate cells, a clearly important area of future research.

It is also thought that interactions of microtubules with the cortex contribute to anaphase spindle pole separation (Rosenblatt, 2005). The experiments looking at how inhibition of the interaction of microtubules with the cortex have been problematic because the proteins involved, such as cytoplasmic dynein, also appear to function in the initial stages of centrosome separation at prophase (Vaisberg *et al.*, 1993). This raises the general issue that high temporal resolution is necessary to inhibit protein function in anaphase without disturbing the system earlier, which could cause secondary defects. The development of reagents such as fast-acting small molecule inhibitors that could disrupt central spindle components, or cortical microtubule interactions specifically during anaphase B is essential to address the mechanisms driving anaphase B in vertebrate cells.

6. Conclusions and Future Directions

The spindle is a huge and complex organelle. Because of conventional genetic and biochemical approaches, as well as large-scale proteomic analyses, we now have a sizable ''parts list'' for the vertebrate spindle. In addition, with the advent of RNAi technology and systems level approaches, many factors important for mitosis have been identified. More focused screens using *Drosophila* S2 cells have been used to narrow down important effectors of spindle function such as those modulating spindle length (Goshima *et al.*, 2005b). Perhaps the most complete screen is being carried out by the MitoCheck group, which is currently performing a systematic RNAi screen in HeLa cells and analyzing mitotic progression by time-lapse microscopy (Neumann *et al.*, 2006) (www.mitocheck.org). Automated analysis is used to group distinct phenotypes. Although this type of analysis seems overwhelming to those accustomed to studying the function of a single factor in detail, it will be an incredible resource for all students of mitosis to gain first-level knowledge of the critical factors. However, it is important to think of this resource as a beginning rather than an end point. Classical genetic studies emphasize why this is true. How

long does it take to go from isolation of a mutant to a true understanding of the molecular mechanisms underlying a given biological process? While we may learn that loss of a specific protein results in mitotic arrest, we will still have to answer the difficult questions of how this protein functions, when, where, and with which partners.

In the future, it will be essential for us to take the ''parts list'' and the functional genomic data and use them to converge on a better molecular understanding of mitosis. For example, what percentage of proteins in the mitotic spindle proteome gives rise to mitotic defects by RNAi? On the contrary, how many proteins that are not part of the mitotic spindle also cause mitotic defects? Are these secondary effects from a defect in another part of the cell cycle or are these effects due to actions of these proteins specifically in mitosis? Another layer of complexity involves posttranslational modifications of mitotic factors, which include phosphorylation, ubiquitination, and sumoylation. Phosphorylation events occurring during mitosis are now being characterized (Nousiainen *et al.*, 2006). However, a complete understanding requires knowledge of the kinase and opposing phosphatase, and the functional consequences of the phosphorylation event. In addition, a complete description of where within a cell and the spindle itself each protein is localized, as well its dynamic behavior, is essential. These kinds of data will elucidate the complex mechanisms underlying microtubule–kinetochore attachment, error correction, and segregation. Research in the past 100 years has uncovered a wealth of information regarding the mitotic spindle and its function. We expect that in the next century we will determine the molecular roots of the incredible process of mitosis.

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CHAPTER FOUR

Multiple Actions of Secretin in the Human Body

Ian P. Y. Lam, Francis K. Y. Siu, Jessica Y. S. Chu, *and* Billy K. C. Chow

Contents

Abstract

The discovery of secretin initiated the field of endocrinology. Over the past century, multiple gastrointestinal functions of secretin have been extensively studied, and it was discovered that the principal function of this peptide in the gastrointestinal system is to facilitate digestion and to provide protection. In view of the late identification of secretin and the secretin receptor in various tissues, including the central nervous system, the pleiotropic functions of secretin have more recently been an area of intense focus. Secretin is a classical hormone, and recent studies clearly showed secretin's involvement in neural and neuroendocrine pathways, although the neuroactivity and neural regulation of its release are yet to be elucidated. This chapter reviews our current understanding of the pleiotropic actions of secretin with a special focus on the hormonal and neural interdependent pathways that mediate these actions.

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1. Introduction

At the turn of the last century, there was a monumental breakthrough in our understanding of the coordination system of the body that eventually opened up a new era in physiological investigations. In 1902, Bayliss and Starling observed that infusion of acid in a denervated loop of jejunum of an anesthetized dog resulted in copious release from the pancreas. As intravenous infusion of acid did not produce such an effect while injection of extracts prepared from acid-stimulated mucosa did, they concluded that a chemical substance, produced in the intestinal mucosa and released into the circulation, activated pancreatic secretion (Bayliss and Starling, 1902). They named this chemical substance ''secretin'' and, afterward, any secretin-like messenger that originated from one organ and reached other organs via the circulation was termed a hormone. The importance of Bayliss and Starling's studies was not only the discovery of secretin, but that they inaugurated a totally new field in physiological studies known as endocrinology.

Secretin-producing endocrine cells are located mainly in the proximal intestinal mucosa in mouse, rat, human, dog, pig, and other mammals (Andersson *et al.*, 2000; Lam *et al.*, 2006; Straus and Yalow, 1978). Along the intestine, secretin-immunoreactivities decrease gradually from duodenum to ileum (Bryant and Bloom, 1979). Secretin was also found in other organs including the stomach, kidney, heart, lung, and several brain regions (Chey and Chang, 2003b) (Table 4.1). The physiological roles of secretin as a gastrointestinal hormone in stimulating secretions from intestine, liver, and pancreas have been well established. More recently, emerging evidence also indicated the role of secretin as a neuropeptide.

The release of secretin from the duodenum is stimulated primarily by gastric acid, and also by digested fats and proteins. Secretin release could also be activated by secretin-releasing peptides (SRPs) found in intestinal perfusate and pancreatic juice (Li *et al.*, 1990; Song *et al.*, 1999). As the release and action of SRPs are also mediated via the vagal afferent pathway (Chey and Chang, 2003a; Li *et al.*, 1995), the secretion of secretin therefore involves neural inputs. Recent findings suggested that in addition to acting on traditional endocrine tissues, secretin could also act on other target cells/tissues via neural and neuroendocrine pathways. In this chapter, we will review the expanding roles of secretin in the human body as well as the hormonal and neural pathways that mediate the physiological actions of secretin.

Table 4.1 Distribution of secretin in various tissues of the body

Tissue	Localization	Detection methods	References
Brain	Cerebellar Purkinje cells, soma and dendrites, cerebellar central nuclei	Radioimmunoassay, *in situ* hybridization, Northern blotting, immunohistochemistry	Charlton *et al.*, 1981; Lee *et al.*, 2005a; Yung *et al.*, 2001; Koves *et al.*, 2004
	Hypothalamus anterior and middle region neurons, the adjoining periventricular gray	Immunocytochemistry	Welch *et al.*, 2004; Chu *et al.*, 2006
	Cerebral cortex: external and internal pyramidal cells in the pyramidal layers of the hindlimb area of the motor cortex	Radioimmunoassay, immunohistochemistry	Charlton *et al.*, 1981; Koves *et al.*, 2004
	Brain stem: trigeminal nerve mesencephalic nucleus, medulla oblongata, superior olivary nucleus, the pons	Immunohistochemistry, quantitative real-time PCR	Koves *et al.*, 2004; Davis *et al.*, 2004
	Central amygdala	Immunohistochemistry, quantitative real-time PCR	Koves *et al.*, 2004; Tay *et al.*, 2004
	Hippocampus	Radioimmunoassay, immunohistochemistry	Charlton *et al.*, 1981; Koves *et al.*, 2004
	Area postrema, nucleus of the tractus solitarius	Quantitative real-time PCR	Tay *et al.*, 2004
	Olfactory bulb, septum, striatum, sensory ganglion	Reverse-transcriptase PCR, radioimmunoassay, immunohistochemistry	Itoh *et al.*, 1991; Charlton *et al.*, 1981; Koves *et al.*, 2004

(*continued*)

Table 4.1 (*continued*)

Tissue	Localization	Detection methods	References
	Thalamus, hypophysis	Reverse-transcriptase PCR	Itoh *et al.*, 1991
	Choroid plexus	Quantitative real-time PCR	Davis *et al.*, 2004
Stomach	Antral and corpus mucosae	Immunohistochemistry, reverse-transcriptase PCR, Southern blot analysis, immunofluorescence	Chey *et al.*, 2003
Pancreas	Developing pancreatic islet B cells	Immunofluorescence	Wheeler *et al.*, 1992
Intestine	S cells in duodenal mucosa	Immunofluorescence	Chey and Escoffery, 1976
	Ileocecum, jejunum, ileum	Northern blot analysis	Whitmore *et al.*, 2000
Reproductive system	Testis	Northern blot analysis	Whitmore *et al.*, 2000
	Principal cells of the initial segment, caput epididymis	Immunohistochemistry	Chow *et al.*, 2004
Others	Spleen	Northern blot analysis	Whitmore *et al.*, 2000; Ohta *et al.*, 1992
	Heart, lung, kidney	Reverse-transcriptase PCR	Ohta *et al.*, 1992

2. Structure and Regulation of Secretin and the Gene

2.1. Structure

Secretin was first isolated and sequenced in porcine by Jorpes and Mutt (1966; Mutt *et al.*, 1970). Afterward, this 27-amino acid peptide hormone with a molecular weight of 3055 was characterized in various mammals in the following chronological order: pig, cow, human, dog, rat, guinea pig, rabbit, and sheep (Chey and Chang, 2003b; Leiter *et al.*, 1994; Ng *et al.*, 2002). From the alignment of their amino acid sequences, mammalian secretins are found to be highly conserved (e.g., pig, cow, and sheep secretins are identical) (Chey and Chang, 2003b). Sequence comparison of secretin with other known peptides indicates that secretin belongs to a family of brain-gut peptides consisting of vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), growth hormone-releasing hormone (GHRH or GRF), peptide histidine isoleucine (PHI) or peptide histidine methionine (PHM), glucagon, glucagon-like peptide 1 (GLP-1), glucagon-like peptide 2 (GLP-2), and gastric inhibitory peptide (GIP) (Vaudry *et al.*, 2000).

Transcription and translation of the human secretin gene give rise to a 121-amino acid long precursor protein, preprosecretin, with a signal peptide (14-amino acid), an N-terminal association peptide (13-amino acid), secretin (27-amino acid), a proteolytic processing site (Gly-Lys-Arg), and a C-terminal association peptide (64-amino acid). After removal of the signal peptide by signal peptidase to generate prosecretin, by posttranslational modifications, several forms of secretins with either a C- or N-terminal extension were produced, and these peptides appeared to possess some bioactivities. For example, secretin-Gly-Lys-Arg has a higher potency and a longer half-life on stimulating pancreatic secretion when compared to that of secretin (Solomon *et al.*, 1999). The mature secretin peptide has a random coil conformation in solution, but assumes a more ordered structure in the presence of an organic environment such as phospholipids, and this ordered conformation contains two major domains, an N-terminal 6-amino acid long coil-like domain and a 18-amino acid (from Thr 7 to Gly 25) C-terminal α-helical domain. Within the coil-like domain, Asp 3 is responsible for anchoring the peptide to its receptor (Di Paolo *et al.*, 1998, 1999). The α-helical domain, on the other hand, is responsible for high affinity binding and hence biological activity (Holtmann *et al.*, 1995; Vilardaga *et al.*, 1995).

2.2. Regulation

Secretin genes from rat and porcine were cloned by Kopin and coworkers in 1990. The rat secretin gene contains four exons, with the second exon encoding the entire secretin peptide. The core promoter of the gene

contains an E-box binding site *CA*??*TG* and two GC-rich motifs. The E-box binding proteins, NeuroD/Beta2 and E2A (E12 and E47), belong to the family of basic helix–loop–helix (bHLH) transcription factors. It has been shown that the binding of NeuroD/Beta2 and E2A heterodimer to the E-box motif is essential for transcription in both rodent and human secretin genes (Lee *et al.*, 2004; Mutoh *et al.*, 1997). NeuroD/Beta2 activity is further potentiated by the coactivator p300, which is also known as cyclic AMP (cAMP) response element binding protein-binding protein or CBP (Mutoh *et al.*, 1998). It was also found that human secretin and secretin receptor genes are both regulated by an *in vivo* Sp1/Sp3 ratio and the methylation status at the CpG islands overlapping the core promoters of these genes (Lee *et al.*, 2004; Pang *et al.*, 2004).

3. Secretin's Actions in the Gastrointestinal Tract

3.1. Stomach

Secretin's activity in the stomach originates not only from duodenal S cells, but also from secretin-producing cells localized in the gastric antrum and corpus mucosae (Chey *et al.*, 1983, 2003; Chey and Escoffery, 1976). In the stomach, specific binding sites for secretin were identified in the fundic membrane and the smooth muscle layer of the forestomach isolated from rat (Gespach *et al.*, 1981; Steiner *et al.*, 1993). The binding of ^{125}I-labeled secretin to the forestomach membrane decreased significantly after vagal ligation, vagotomy, and perivagal colchicine treatment. It is therefore believed that the vagus nerve is responsible for modulating the capacity of these binding sites (Kwon *et al.*, 1999) and, thus, potentially modulating secretin's actions in the stomach.

In humans, gastric acid secretion stimulated by intravenous administration of pentagastrin was inhibited by secretin in a physiological range (You and Chey, 1987), and hence secretin is a potential enterogastrone. This inhibitory effect was also observed in rats (Shiratori *et al.*, 1992) and dogs (Gerber and Payne, 1996). Shiratori *et al.* (1992) demonstrated that intraduodenal administration of oleic acid emulsion increased plasma secretin levels, which is accompanied by the inhibition of gastric acid release, and such an effect was blocked by intravenous infusion of rabbit antisecretin antiserum. Consistently, secretin dose dependently inhibited the postprandial gastric acid output, gastric emptying, and gastrin response, and again these effects could be reversed by immunoneutralization using antisecretin antibodies (Jin *et al.*, 1994). Thus, these data suggested that endogenous secretin could regulate postprandial gastric acid secretion, although gastric

lipase secretion was not influenced by secretin (Olsen *et al.*, 1998). In summary, secretin-mediated gastric acid regulation depends on the interactions of the peptide with lipid messengers, hormones, and neural pathways.

The inhibitory action by secretin, or the secretin-releasing agent plaunotol, on gastric acid release in isolated, vascularly perfused rat stomach was abolished by intravenous administration of a prostaglandin synthesis inhibitor, indomethacin, in anesthetized and conscious rats (Chung *et al.*, 1994; Rhee *et al.*, 1991; Shimizu *et al.*, 1995; Shiratori *et al.*, 1993a,b) and in humans (Taylor *et al.*, 1994). This blocking action could be reversed by subsequent administration of prostaglandin E_2 (Rhee *et al.*, 1991), suggesting that indomethacin could also abolish oleic acid-induced inhibition of gastric acid output in rats; however, Taylor *et al.* (1994) demonstrated opposite effects in humans. Despite the controversy, endogenous prostaglandin is believed to be, at least partially, a mediator of secretin-induced inhibition of gastric acid release. The findings that plaunotol could increase secretin's concentration in plasma and endogenous prostaglandin levels in gastric mucosa provided the rationale for using it as an antiulcer agent (Takeuchi *et al.*, 1991).

In addition, secretin significantly increased endogenous somatostatin in isolated, perfused rat (Chung *et al.*, 1994) and dog stomach (Gerber and Payne, 1996). Immunoneutralization of somatostatin by rabbit antisomatostatin could abolish secretin-induced inhibition of gastric acid release (Chung *et al.*, 1994; Shimizu *et al.*, 1995) as well as basal and pentagastrin-stimulated acid output in conscious rats (Shimizu *et al.*, 1995). Furthermore, somatostatin-induced inhibition of gastric acid release was not influenced by the application of indomethacin (Shimizu *et al.*, 1995). Therefore, these studies strongly suggested that the inhibitory action of secretin on gastric acid secretion is mediated by the local release of somatostatin. Similarly, it was also suggested that the inhibitory effects of PACAP-27 on gastric acid release was mediated via secretin, somatostatin, and prostaglandin E_2 in rats (Li *et al.*, 2000).

Apart from hormonal control, the inhibition of gastric acid secretion by duodenal acidification is also mediated by neural pathways (Brooks *et al.*, 1971; Code and Watkinson, 1955; Orloff *et al.*, 1992). In conscious rats Li *et al.* (1998) demonstrated that bilateral vagotomy and subdiaphragmatic perivagal (PV) application of capsaicin (Cap), but not periceliac ganglionic (PCG) treatment of Cap, abolished the secretin-mediated inhibition of pentagastrin-stimulated acid secretion. Whereas in anesthetized rats, the suppression of pentagastrin-stimulated acid release by duodenal acidification was reversed by rabbit antisecretin antiserum or PV, but not PCG, application of Cap. These data indicated, for the first time, that secretin-induced inhibition of pentagastrin-stimulated acid secretion is mediated by the vagal afferent pathway, but not the splanchnic afferent pathway, in rats. This could be explained by modulating the secretin binding sites in the stomach

by the vagal pathway (Kwon *et al.*, 1999). These studies showed the interdependence of secretin and neural pathways in controlling secretions in the stomach.

Several early studies showed that secretin was the mediator for inhibiting gastric motility. It was demonstrated in humans and dogs that secretin reduced the contraction force of the antrum and delayed the process of gastric emptying (Chey *et al.*, 1970; Dinoso *et al.*, 1969; Keinke *et al.*, 1987; Vagne and Andre, 1971). More recently, Lu and Owyang (1995, 1999) demonstrated that intragastric pressure was diminished by intravenous application of secretin or duodenal acidification. As secretin release is induced by duodenal acidification (Schaffalitzky de Muckadell *et al.*, 1979; Schaffalitzky de Muckadell and Fahrenkrug, 1978), secretin therefore protects the duodenum from excessive acid by a negative feedback inhibition on both acid release and gastric motility. Interestingly, secretin levels in the duodenums of nonobese diabetic mice were higher when compared to those of controls, an observation that could be correlated with the slower gastric emptying observed in the nonobese diabetic mice (El Salhy and Spangeus, 2002). In an *in vivo* rat model, bilateral vagotomy, PV, and gastroduodenal application of Cap diminished secretin's actions on gastric motility, suggesting that secretin partially functioned via afferent pathways originating from the gastroduodenal mucosa (Lu and Owyang, 1995). However, another study showed that the application of antisecretin antibody had no effect on acid-induced inhibition of gastric motility (Raybould and Holzer, 1993). This discrepancy was explained later by Lu and Owyang (1999) using various doses of acid. They observed that when acid was applied at a low rate, gastric relaxation was mediated by endogenous secretin via a vagal afferent pathway, while at a high rate of acid infusion, reduced gastric motility was mediated by secretin and other pathways, such as cholecystokinin (CCK). In addition, it was also suggested that secretin's action on gastric motility could be mediated by local release of somatostatin and prostaglandin in the gastric antrum (Zhou and Wang, 1990).

Secretin protects the gastric mucosa by stimulating mucus secretion, which forms a protective gel layer on the mucosal surface (Tani *et al.*, 1997). In cultured gastric epithelial cells, secretin stimulated cAMP accumulation and induced calcium-sensitive mucus secretion (Tani *et al.*, 2002), suggesting the involvement of cAMP and calcium in carrying out the actions of secretin. Moreover, secretin may protect the mucosal layer by regulating the paracellular permeability of the epithelial cells. In canine gastric epithelial cell cultures, basolateral application of secretin increased transepithelial resistance in a concentration-dependent manner (Chen *et al.*, 2002). Apart from these actions in the stomach, secretin also stimulates pepsinogen secretion, and potentiates secretion induced by carbamylcholine, cholecystokinin, and carbachol *in vitro* (Raufman *et al.*, 1983; Sanders *et al.*, 1983; Sutliff *et al.*, 1986; Tanaka and Tani, 1995).

3.2. Pancreas

The pancreas is an exocrine and endocrine organ. The exocrine element consists of principal cells (ductal and centroacinar cells) and *acini*, while the endocrine portion is the pancreatic islets. Principal cells are responsible for bicarbonate and water secretion. Acinar cells secrete chloride-rich fluid and digestive enzymes, which are stored in the zymogen granules and released in response to secretagogue stimulation. Collectively, pancreatic exocrine secretion is needed for digestion of carbohydrate, protein, and fat in the proximal small intestine and is under both neural and hormonal controls.

Secretin is the main hormonal regulator in the release of pancreatic bicarbonate-rich fluid and other electrolytes. Binding of secretin to its receptors on the basolateral membrane of the principal cells provoked intracellular cAMP (Konturek *et al.*, 2003), which opened and increased the number of chloride channels in the apical membrane (Gray *et al.*, 1988). The efflux of chloride ions was coupled to the apical bicarbonate/chloride anion exchanger (AE) resulting in bicarbonate secretion into the pancreatic juice. Recently, the cAMP responsive cystic fibrosis transmembrane conductance regulator (CFTR) on the luminal membrane was identified as the chloride channel responsible for chloride efflux (Raeder, 1992), while conductance of this channel function was not required for the activation of AE (Lee *et al.*, 1999a,b). Nevertheless, the expression of CFTR indeed was essential for activating AE *in vitro* and *in vivo* (Lee *et al.*, 1999a,b).

In addition, secretin induces bicarbonate secretion via its synergistic effects with CCK, demonstrated in human, dog, and rat (Chey *et al.*, 1984; Moriyoshi *et al.*, 1991; You *et al.*, 1983). In dogs with chronic pancreatic fistulas, euglycemic-hyperinsulinemic clamp inhibited secretin-induced bicarbonate secretion (Howard-McNatt *et al.*, 2002). This inhibitory effect of exogenous insulin was reversed by bethanechol, a parasympathomimetic choline ester that selectively stimulates muscarinic receptors, suggesting that insulin inhibits secretin-stimulated bicarbonate output via a cholinergic mechanism.

Along with bicarbonate release, secretin induces proton secretion into the interstitial fluid in the microdissected pancreatic duct from pigs (Villanger *et al.*, 1995). Proton secretion, which may be involved in epithelial pH homeostasis, can be estimated by the ability of intracellular pH recovery after acid loading. The effect of secretin is blocked by bafilomycin A1, suggesting that proton transport depends on vacuolar H^+-adenosine triphosphatase.

Regarding chloride secretion from acinar cells, it was believed that the secretion was regulated by the basolateral potassium channels activated by cAMP agonists. The efflux of potassium created a more negative membrane potential driving chloride release. In rat acinar cells, it was demonstrated that a voltage-dependent potassium channel current, slowly activating

potassium channel currents (I_{Ks}), could be stimulated by secretin via the cAMP-phosphokinase A (PKA) pathway (Kim *et al.*, 2001). Although the effects of secretin at physiological doses were not potent, these effects were significantly potentiated by carbachol. It was then hypothesized that costimulation by secretin and vagus could augment chloride secretion by modulating $I_{Ks.}$ In addition, secretin, via cAMP, could stimulate amylase secretion in pancreatic acini (Collen *et al.*, 1982; Gardner and Jackson, 1977; Gardner and Jensen, 1986; Trimble *et al.*, 1986). Aside from regulating pancreatic exocrine secretion, secretin also modulates the pancreatic flow by inhibiting the sphincter of Oddi of the pancreatic duct in dog, human, guinea pig, and Australian possum (Al Jiffry *et al.*, 2001; Carr-Locke *et al.*, 1985; Cox *et al.*, 1989; Geenen *et al.*, 1980; Lin, 1975; Toouli and Watts, 1972).

On the other hand, secretin had no effect on sodium/bicarbonate cotransporter activity (Novak and Christoffersen, 2001) or on water channel aquaporin-1 (AQP1) expression and localization (Furuya *et al.*, 2002), which are responsible for transporting bicarbonate and water, respectively, across the ductal basolateral membrane. Furthermore, infusion of secretin in physiological doses exhibited no effects on endocrine secretion of the two most important pancreatic hormones, insulin and glucagon (Ferrer *et al.*, 2001). Although these findings contradicted earlier studies in mouse and human, the researchers argued that the high doses of secretin used in previous studies could not represent a physiological function of secretin. However, it should also be noted that the responses to secretin may vary in different species (Konturek *et al.*, 2003). For instance, in human, dog, and cat, the flow volume and bicarbonate content in pancreatic secretion are relatively low at basal conditions but increased dramatically in response to secretin, while in rat, spontaneous pancreatic secretion is higher and hence secretin causes only a moderate change in pancreatic flow.

3.3. Liver

Liver cells consist of hepatocytes and cholangiocytes. *In situ* ^{125}I-labeled secretin receptor autoradiographic studies using liver sections showed that secretin receptor expression was limited in cholangiocytes in rats and humans (Farouk *et al.*, 1992; Korner *et al.*, 2006). Cholangiocyte purification followed by membrane separation confirmed secretin receptor expression on the basolateral membrane of medium and large cholangiocytes in rats (Alpini *et al.*, 1994; 1996; Farouk *et al.*, 1993), on which the receptor density could increase fivefold after bile duct ligation (BDL) (Tietz *et al.*, 2001). Cholangiocytes line the bile duct, which was thought to be merely a passage for bile delivery, but now is recognized as an extensive surface for bidirectional exchange of materials for regulating bile volume and its composition. In the intrahepatic cycling of bile acids, cholangiocytes take up the bile acids secreted by hepatocytes via an apical

sodium-dependent bile salt transporter (ASBT) and release bile acids into the periductular capillary plexus across the basolateral membrane. Meanwhile, cholangiocytes modify bile composition by chloride channels (e.g., CFTR) and the chloride/bicarbonate exchanger (anion exchanger 2, AE2) (Kullak-Ublick *et al.*, 2004).

Bayliss and Starling (1902) were again the first to demonstrate that secretin could stimulate bile flow in dogs. Since then, numerous *in vitro* and *in vivo* studies have provided evidence to indicate that secretin stimulates bicarbonate-rich bile secretion and bile flow in biliary epithelium instead of hepatocytes (Kanno *et al.*, 2001) (Fig. 4.1). Such an effect, however, was observed only in rats with cholangiocyte proliferation induced by BDL, cirrhosis, and α-naphthylisothiocyanate (ANIT) feeding (Kanno *et al.*, 2001), whereas the same effect could be inhibited by gastrin and γ-interferon in BDL rats and cirrhotic mice, respectively (Alpini *et al.*, 1997; Glaser *et al.*, 1997). Only recently was the importance of secretin in bile regulation in normal rats with a maintained bile acid pool by continuous infusion of taurocholate demonstrated (Banales *et al.*, 2006). Interestingly, secretin-stimulated choleresis represents 30% of the basal flow in humans and 10% in rats (Alpini *et al.*, 1989; Tavoloni, 1987). Secretin's actions are mediated via its receptors on the basolateral membrane of cholangiocytes. This leads to an increase in intracellular cAMP, which probably, via the PKA/Src/MEK/ERK1/2 pathway (Francis *et al.*, 2004), stimulates the opening of CFTR chloride channels. The efflux of chloride ions depolarizes the cell and subsequently activates the sodium/bicarbonate symport to import bicarbonate ions. As a result of the chemical gradient across the membrane, bicarbonate enters the bile via the chloride/bicarbonate anion exchanger located on the luminal membrane of cholangiocytes. AE2 has been shown to be the major chloride/bicarbonate exchanger in normal rat cholangiocytes *in vitro* (Banales *et al.*, 2006).

Aside from anions, many studies have also shown that secretin could regulate water movement in cholangiocytes. Cholangiocytes transport water mainly via the water channel AQP1, which is present in the plasma membrane as well as the intracellular vesicles (Marinelli and LaRusso, 1997; Nielsen *et al.*, 1993; Roberts *et al.*, 1994). The introduction of AQP1 to the apical membrane by secretin-induced exocytosis increases the membrane osmotic permeability (Kato *et al.*, 1992; Marinelli *et al.*, 1997, 1999; Tietz *et al.*, 2003). Such intracellular vesicle trafficking is microtubule dependent and can therefore be inhibited by colchicine (Kato *et al.*, 1992; Marinelli *et al.*, 1997). Interestingly, AQP1-containing vesicles are also enriched with CFTR and AE2 (Tietz *et al.*, 2003), thus allowing simultaneous cotranslocation of these transporters onto the apical membrane. The passive water movement through AQP1 could be facilitated by the transmembrane anion gradients created by CFTR and AE2. In addition, secretin also causes colchicine-sensitive translocation of ASBT from the intracellular membrane

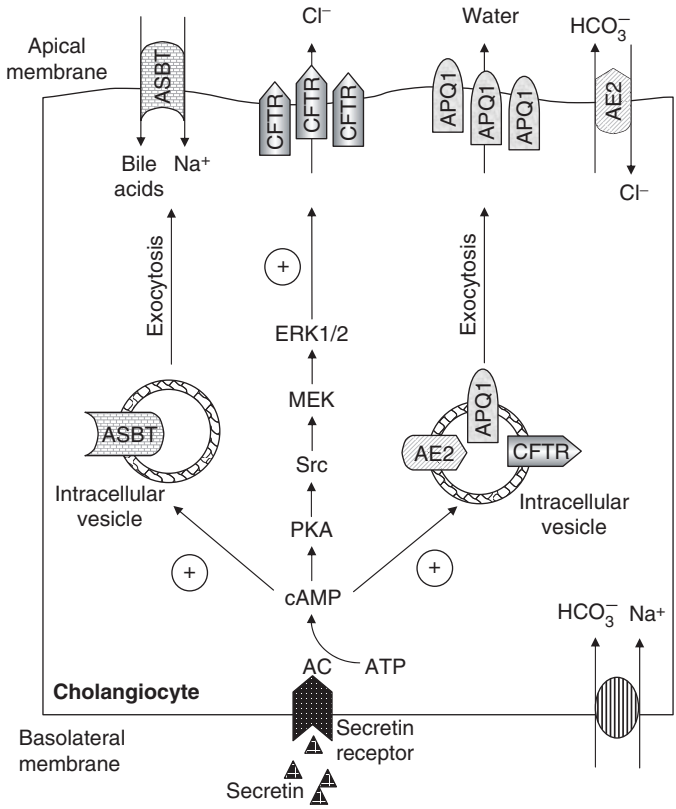

Figure 4.1 A current model of secretin-stimulated bile secretion in rat cholangiocyte. Secretin triggers cyclic AMP (cAMP) production by adenylyl cyclase (AC) on the basolaterally located secretin receptor. Through the cAMP/PKA/Src/MEK/ERK1/2 pathway, secretin stimulates the opening of the cystic fibrosis transmembrane conductance regulator (CFTR) causing the efflux of chloride ions and subsequently the introduction of bicarbonate ions via the sodium/bicarbonate symport. Intracellular bicarbonate is then secreted into the bile through the anion exchanger 2 (AE2) on the apical membrane. Secretin also causes the insertion of aquaporin 1 (AQP1) onto the luminal membrane to facilitate water movement by stimulating exocytosis of the intracellular vesicle, which also contains the cystic fibrosis transmembrane conductance regulator and anion exchanger 2. To modulate the bile acid composition in the bile, secretin leads to relocation of an apical sodium-dependent bile salt transporter (ASBT) from the intracellular vesicle to the apical membrane for sodium-dependent bile acid transport.

to the cholangiocyte apical membrane for bile acid transport in rats (Alpini *et al.*, 2005). Consistent with its control of the pancreatic duct sphincter of Oddi, secretin can relax the bile duct sphincter of Oddi to facilitate the flow of bile into the duodenum (Al Jiffry *et al.*, 2001).

Similar to the situations in BDL and carbon tetrachloride-induced cirrhosis (Alpini *et al.*, 1997; Tietz *et al.*, 2001), feeding rats with taurocholate (TC) and taurolithocholate (TLC) resulted in upregulation of cholangiocyte proliferation and secretin receptor expression (Alpini *et al.*, 1999). The change in proliferation was associated with the cAMP/PKA/Src/MEK/ERK1/2 pathway (Francis *et al.*, 2004). Moreover, in cholestatic rat models, with high doses of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), and taurodeoxycholic acid (TDCA), secretin significantly increased bile flow and bile acid excretion (Fukumoto *et al.*, 1994, 2002). Thus, these studies revealed the potential role of secretin in regulating the proliferation of cholangiocytes and the excretion of bile acids under high doses of bile acids via the cAMP signaling pathway. The exact function and mechanism of secretin in response to cholestatic condition, however, need further clarification.

3.4. Intestine

Small intestinal cells immunoreactive for secretin were detected in various mammalian species including cat, dog, pig, guinea pig, rat, monkey, human (Straus and Yalow, 1978), lesser mouse deer (Agungpiryono *et al.*, 1994), babirusa (Agungpriyono *et al.*, 2000), carabao (Baltazar *et al.*, 1998), cow, and calf (Kitamura *et al.*, 1985). It has also been demonstrated that secretin levels in mice duodenal extracts were higher in 24- and 12-month-old than 1-month-old mice (El Salhy and Sandstrom, 1999). Secretin immunoreactive cells in duodenum were significantly more abundant in 24-month-old mice compared to 3-month-old mice (Sandstrom and El Salhy, 2000). Data from studies in pig agreed with these reports that the volume occupied by secretin-immunoreactive epithelial cells increased in the jejunum after birth (Van Ginneken and Weyns, 2004). These intestinal differences can correlate with the demand on secretin in an age-related manner or intestinal dysfunction that occurred at an advanced age.

Acid is the most potent stimulant for secretin release. Infusion of acid (pH 1.5, 3.8 mmol/h) in pig duodenum significantly increased secretin levels in the portal vein and also bicarbonate secretion by the duodenal mucosa (Glad *et al.*, 1996). The stimulatory effect of duodenal acidification could be enhanced or reduced by inhibiting or stimulating duodenal mucosal bicarbonate secretion using indomethacin or misoprostol, respectively (Ainsworth *et al.*, 1994). Over decades, the role of secretin in activating duodenal bicarbonate release is unclear, as infusion of secretin in pig (Ainsworth *et al.*, 1991), guinea pig (Reimer *et al.*, 1996), and humans (Wolosin *et al.*, 1989) produced no significant effects, while studies in rats indicated a role of secretin in stimulating bicarbonate secretion in the proximal duodenum (Isenberg *et al.*, 1984). In rats, secretin at doses as low as 15 ng/kg/h augmented bicarbonate and protein output (Kirkegaard *et al.*, 1984).

The inconsistency observed by different research groups may be the result of different segments of duodenum studied and the inability to distinguish the contribution of duodenal epithelium and Brunner's gland in earlier studies.

The main function of Brunner's glands is to produce a bicarbonate-containing alkaline secretion. The amount of epidermal growth factor (EGF) released from the Brunner's gland was also increased by secretin, but reduced by somatostatin (Olsen *et al.*, 1994). Moore and colleagues (2000) developed an *in vitro* model in guinea pig to study the effects of various agents on the secretion of isolated Brunner's glands that are devoid of epithelial cells. They demonstrated the secretory products were transported from acinar cells to the lumen during luminal dilation; therefore, the luminal diameter was used as an index to monitor secretion. Videomicroscopic recordings indicated secretin, gastrin, and vasoactive intestinal peptide (VIP) could all lead to the dilation of glandular acini in similar potencies. Previous studies have provided evidence to support a physiological role of secretin in stimulating Brunner's glands, although we should be extremely cautious when analyzing these data as secretin could also activate duodenal epithelial secretion. In summary, secretin's action on Brunner's glands is more potent and has a higher preference than the epithelial cells, which may be evoked only to ensure that an adequate physicochemical barrier is present under certain conditions.

In addition, several studies in the 1970s suggested that secretin could inhibit the contraction of the small intestine in dogs and humans (Leiter *et al.*, 1994). Petzold *et al.* (1991) used electromyographs to monitor gut motility, and in the same study, they demonstrated that secretin could relax the duodenums of dogs and rats. *In vitro* studies using strips of longitudinal smooth muscle of rat distal colon and distal ileum also showed a concentration-dependent relaxation upon secretin incubation (Andersson *et al.*, 2000).

4. Secretin's Actions in Other Tissues

4.1. Brain

4.1.1. Cerebral cortex

Expression of secretin in the cerebral cortex was first identified by radioimmunoassay in rats (Charlton *et al.*, 1981). Subsequent studies by immunohistochemical staining further localized secretin peptide in the motor cortex and pyramidal cells of the cerebral cortex (Koves *et al.*, 2004). Intracerebroventricular (icv) injection of secretin activates c-fos expression in the prefrontal cerebral cortex and olfactory cerebral cortex and reduces c-fos expression in the motor and parietal cerebral cortex (Welch *et al.*, 2003).

Welch *et al.* (2003) suggested that secretin-induced c-fos in the prefrontal cortex could modulate behavior, emotional expression, and stress response to homeostatic challenges via the hypothalamus. Thus, secretin might be important in social recognition and early environmental conditioning of neonatal behavior (Welch *et al.*, 2003). For the parietal cortex, despite the controversy concerning secretin's therapeutic actions in treating autism, it was proposed that this could be a putative site for secretin to modulate motor activities of autistic children (Welch *et al.*, 2003).

4.1.2. Cerebellum

The cellular actions of secretin in the brain have been best studied in the cerebellum. In 1981, radioimmunoassay was used to demonstrate the expression of secretin in the cerebellum (Charlton *et al.*, 1981). Several studies using *in situ* hybridization and immunohistochemistry techniques confirmed that secretin is expressed in the prenatal cerebellum of mouse (Siu *et al.*, 2005) and in the Purkinje neurons of adult human and rat (Koves *et al.*, 2004; Lee *et al.*, 2005a; Yung *et al.*, 2001) . By using whole cell patch-clamp recording, it was proposed that secretin was released from the postsynaptic Purkinje cells to increase the frequency of miniature inhibitory postsynaptic currents (mIPSCs) initiated from the presynaptic basket cell (Yung *et al.*, 2001) (Fig. 4.2). The hypothesis is that depolarization of Purkinje cells triggers the release of secretin from the somatodendritic regions of the neuron in a voltage-gated calcium channel-dependent manner (Lee *et al.*, 2005b). The released secretin may act in three ways: (1) it binds to secretin receptors on the same or nearby Purkinje cells, but the function of this is still unknown; (2) it acts as a retrograde messenger and binds onto secretin receptors located on the presynaptic basket cell to release GABA in a cAMP-dependent manner; this hyperpolarizing inhibitory signal is received by $GABA_A$ receptors on the postsynaptic Purkinje cell (Ng *et al.*, 2002); and (3) it binds to secretin receptors located on an unknown glutamate source to trigger the release of glutamate, resulting in the activation of AMPA receptors on basket cells to potentiate secretin's effect on GABA release (Lee *et al.*, 2005b).

4.1.3. Amygdala

The expression of secretin in the amygdala is mainly localized in the amygdaloid complex as revealed by immunohistochemical stainings (Koves *et al.*, 2004), but when secretin is injected icv, intravenously (iv), or intraperitoneally (ip), c-fos expression in the amygdala is activated (Goulet *et al.*, 2003; Welch *et al.*, 2003; H. Yang *et al.*, 2004). Intraduodenal infusion of HCl and electrical stimulation of the amygdala could both significantly increase pancreatic secretion through the elevated levels of plasma secretin; however, the later effect was abolished by bilateral vagotomy (Jo *et al.*, 1994). In addition, systemic administration of secretin could also

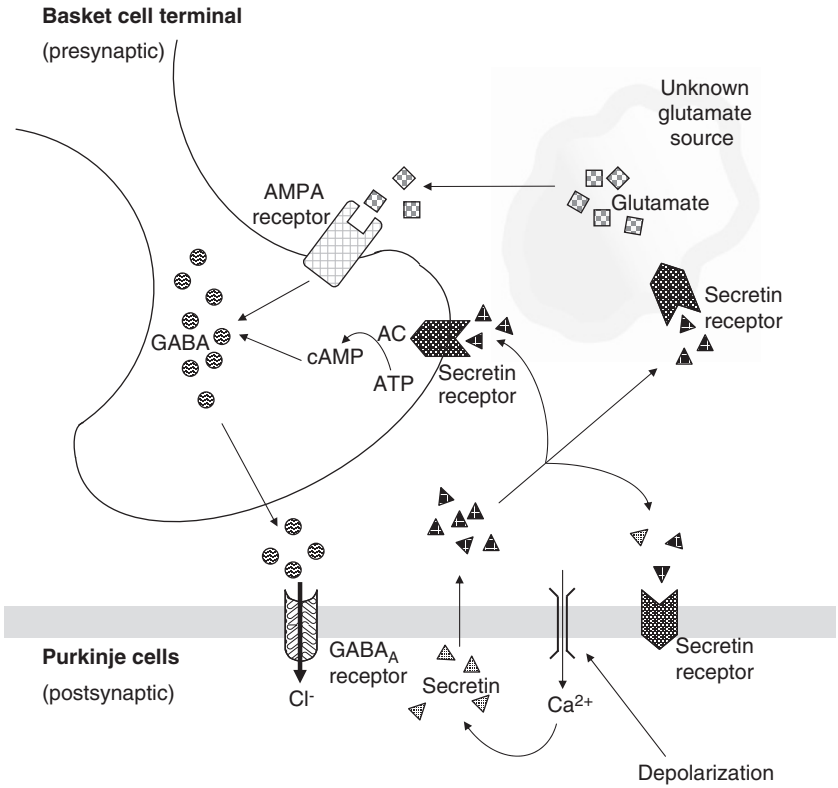

Figure 4.2 A proposed schematic model of the action of secretin in the cerebellum. Upon depolarization, secretin is voltage-gated calcium channel dependently released from the somatodendritic area of the Purkinje neuron. The released secretin acts on an unknown glutamate source triggering the release of glutamate and acts on the presynaptic basket cell facilitating GABA release to the postsynaptic area. The GABA-facilitating effect is potentiated by the glutamate from an unknown source. The inhibitory signal from the basket cell stabilizes the excited Purkinje neuron. The Purkinje released secretin could also act on the Purkinje neuron itself, but the function of that is still unclear. (Adapted from Yung *et al.*, 2006.)

lower the magnitude of the fear potentiated startle (emotional behavior), which could be abolished by subdiaphragmatic vagotomy (Myers *et al.*, 2004). This agrees with the hypothesis that secretin could potentially be important in social recognition and early environmental conditioning of neonatal behavior (Welch *et al.*, 2003).

4.1.4. Hippocampus

Secretin expression in the hippocampus was confirmed by immunohistochemistry stainings and radioimmunoassays (Charlton *et al.*, 1981; Koves *et al.*, 2004), while the peptide itself in the hippocampus could activate

cAMP production (Karelson *et al.*, 1995). With information from the secretin-receptor-deficient mouse, it was found that the loss of secretin's action in the hippocampus led to impairment of synaptic transmission in Schaffer collateral synapses, alteration of density of CA1 apical dendrites in the spine, and abnormal behavior in the reversal water maze. All these suggested the importance of secretin in social and cognitive functions (Nishijima *et al.*, 2006).

4.1.5. Other brain areas

By immunohistochemistry and/or radioimmunoassay techniques, secretin's expression in various regions of the brain, including the olfactory bulb, septum, striatum, brain stem, and sensory ganglion (Charlton *et al.*, 1981; Koves *et al.*, 2004), was found. Infusion of secretin, both icv and ip, could activate c-fos expression in the area postrema, lateral septal complex, ventral periaqueductal, lateral tegmentum, arcuate nucleus, and nucleus of the solitary tract (NTS) (Goulet *et al.*, 2003; Welch *et al.*, 2003; H. Yang *et al.*, 2004). In the NTS, other than c-fos, secretin could also activate tyrosine hydroxylase and depolarize the NTS neuron via a nonselective cationic conductance (NSCC) (B. Yang *et al.*, 2004; H. Yang *et al.*, 2004). When considering both the NTS and lateral tegmentum together, these areas form a visceral sensorimotor reflex circuit that regulates vagal release of secretin-releasing peptide and the adrenergic influence on intestinal secretin-producing S cells (Welch *et al.*, 2003). In addition, secretin could stimulate cAMP production in the superior cervical ganglion (SCG) (Ip *et al.*, 1985), which innervates the iris, submaxillary gland, pineal gland, and right cardiac ventricle. Hence, it is possible that secretin could regulate those organs via the SCG (Ip *et al.*, 1985; Schwarzschild and Zigmond, 1989).

4.2. Hypothalamus–pituitary–kidney axis

4.2.1. Hypothalamic–pituitary axis

With the preponderance of evidence that within the central nervous system (CNS), the hypothalamus and hypophysis have higher concentrations of secretin (Chang *et al.,* 1985; Itoh *et al.,* 1991; Nussdorfer *et al.,* 2000; O'Donohue *et al.*, 1981; Samson *et al.*, 1984), these two connected areas have more recently became one of the most intriguing sites for studying new physiological roles of secretin. In the hypothalamus, secretin induces cAMP formation and elevates the activity of tyrosine hydroxylase (TH) for the biosynthesis of catecholamine (Babu *et al.*, 1983; Karelson *et al.*, 1995). As TH and secretin were both found to be actively expressed in the hypothalamic neurosecretory neurons that coordinate major neuroendocrine and behavioral mechanisms in response to homeostatic challenges (Chu *et al.,* 2006; Kontostavlaki *et al.*, 2006), it was postulated that secretin could act as a central stress regulatory neuropeptide. In this context, plasma

secretin levels were shown to be elevated in various conditions of stress, such as severe dynamic exercise and restraint stress/hypoxia (Bell *et al.*, 1984; Oektedalen *et al.*, 1982). Consistent with these findings, we have shown an increase in plasma secretin levels in response to water deprivation as a stressor (Chu *et al.*, 2007).

The role of secretin in regulating the central stress axis at the level of the hypothalamus has been suggested by various studies. It was found that secretin could function by interacting with other systems in the hypothalamus, such as the secretin/angiotensin and secretin/dopamine systems (Fuxe *et al.*, 1979; Ruggiero *et al.*, 2003; Walker *et al.*, 1999; Welch *et al.*, 2004). Additionally, it could modulate the hypothalamic–pituitary–adrenal (HPA) axis output by increasing noradrenalin and dopamine turnover in the hypothalamus and median eminence (Fuxe *et al.*, 1979). This could probably lead to an increase in blood adrenocorticotropic hormone (ACTH), as observed upon prolonged subcutaneous injection of secretin (Malendowicz *et al.*, 1997).

In addition to the HPA axis, secretin was also believed to act through the hypothalamic–pituitary–kidney axis to modulate dehydration-related stress. We have shown that secretin, as a neuropeptide, is localized in the releasable pools within the paraventricular and supraoptic nuclei, since its release is TTX sensitive, and is dependent on the L-, N-, and P-type high voltage-activated calcium channels (Chu *et al.*, 2006). Since catecholamines could modulate vasopressin release (Dudas *et al.*, 2006), it is possible that secretin could activate the catecholaminergic inputs into the neurosecretory neurons to trigger vasopressin release in hyperosmolality.

Apart from functioning as a stress-related neuropeptide, secretin could also be involved in appetite control. H. Yang *et al.* (2004) demonstrated that intraperitoneal administration of secretin, at 40 or 100 μg/kg, induced a dose-related increase in the number of Fos-positive neurons in the arcuate nucleus, the hypothalamic appetite control center. This suggested that secretin could have a role in starving and/or postprandial satiety. Since secretin suppressed both basal and insulin-stimulated lipogenesis (Ng, 1990), the putative relationships between secretin and satiety, as well as energy homeostasis as a whole, is worthy of future investigations.

In the pituitary, scanty data are available on the function of secretin. Nevertheless, it was reported that the peptide could modulate the release of prolactin from the anterior pituitary. As suggested by Babu *et al.* (1983), a low dose of secretin (1000 ng) inhibited prolactin release via the hypothalamic dopaminergic system. However, secretin at a higher dose (5000 ng) resulted in an enhancement of prolactin release.

4.2.2. Kidney

The renal function in early studies with impure preparations of secretin suggested a diuretic action of the peptide, opposing the antidiuretic effects of Vasopressin (Vp) and SS (Dragstedt and Owen, 1931; Londong *et al.*, 1987).

Subsequent studies by Barbezat *et al.* (1972), Baron *et al.* (1958), Viteri *et al.* (1975), and Waldum *et al.* (1980) consistently indicated the diuretic activity of secretin while Charlton *et al.* (1986) showed an anti-diuretic function of this peptide. Findings of the former groups suggested that secretin could increase renal excretion of water, bicarbonate, sodium, potassium, and calcium in normal human subjects and dogs, resulting in a significant rise of urinary pH. The latter group, however, showed that iv injection of secretin decreases urine output through activating adenylate cyclase in the outer medulla of the kidney, thus mimicking the effect of Vp in this region. In addition, the group also showed that the antidiuretic effect of secretin is as potent as Vp in homozygous Vp-deficient Brattleboro rats. In agreement with this, we have colocalized the secretin receptor with the aquaporin-2 (AQP2) water channel in the cuboidal epithelial cells of the renal collecting duct (Chu *et al.*, 2007). Direct stimulation of renal medullary tubules with secretin could induce trafficking of AQP2 onto the apical membrane, presumably promoting water reabsorption in these cells. Consistently, secretin receptor-knockout mice exhibited mild polydipsia and polyuria phenotypes, indicating a role of secretin in regulating body water homeostasis (Chu *et al.*, 2007).

Lastly, secretin could also increase renal blood flow (Fadem *et al.*, 1982; Lameire *et al.*, 1980), peritubular capillary and interstitial hydrostatic pressures (Mertz *et al.*, 1983), single-nephron glomerular filtration rate, and glomerular plasma flow (Marchand *et al.*, 1986). Therefore, secretin could have a broad spectrum of biological functions within the renal system.

4.3. Additional other tissues

Despite extensive investigations of secretin in the gastrointestinal tract, data regarding the function of secretin in the esophagus are scattered. In anesthetized dogs, intraduodenal acid infusion caused a transient increase in the plasma secretin level, which correlates with changes in the lower esophageal sphincter pressure (LESP) (Hongo *et al.*, 1980). Miyata *et al.* (1991) studied the effect of iv administration of secretin in esophageal achalasia patients with higher LESP than normal individuals. Compared to controls, secretin produced a longer-lasting effect on lower esophageal sphincter relaxation in esophageal achalasia patients. However, more studies are required to draw any conclusions on secretin's bioactivity in the esophagus.

In addition to the gastrointestinal tract, secretin bioactivity was also detected in other organs. Davis *et al.* (2004) conducted a study to examine secretin's expression and potential functions in the human respiratory tract. It was found that real-time polymerase chain reaction (PCR), *in situ* hybridization, and immunohistochemical staining all demonstrated the presence of the secretin receptor in lung tertiary bronchus, primarily on the basolateral membrane of the epithelial cells. They also showed that secretin could

dose dependently induce chloride efflux in human cultured tertiary bronchial cells and this effect was abolished by the application of the nonselective chloride channel blocker, NPPB. However, these studies did not show the involvement of CFTR or other chloride channels in epithelial chloride trafficking. Finally, secretin receptor expression was also detected in bronchial smooth muscle, together with the information that secretin could dose-dependently relax smooth muscle, suggesting the potential role of secretin in bronchodilation (Davis *et al.*, 2004).

In the heart, secretin was shown to exert certain actions on cardiac contraction. Secretin caused an increase in cAMP on crude membrane preparations from rat atria (Robberecht *et al.*, 1984). Correlating with the increased cAMP, the spontaneous and electrically stimulated contraction force of the right atrium was enhanced by the peptide. Consistently, in *in vitro* studies using rat ventricular cardiomyocyte suspension, secretin increased intracellular cAMP levels and stimulated contractile response in a concentration-dependent manner (Bell and McDermott, 1994). In addition to the positive inotropic effect, secretin also increased cardiac output (Gunnes *et al.*, 1983, 1989). Systolic and diastolic blood pressures were slightly increased by the administration of secretin in normal and diabetic rats (Sitniewska and Wisniewska, 1999). Furthermore, secretin reversed the positive effect of L-NAME (N[G]-nitro-L-arginine-methyl ester hydrochloride) on atria permeability in normal rats, and the negative effect of that in diabetic rats (Sitniewska and Wisniewska, 2001). Regarding modulation of ion trafficking, secretin potentiates calcium and barium currents (I_{Ba}) through voltage-dependent L-type calcium channels in adult rat ventricular myocytes (Tiaho and Nerbonne, 1996). Collectively, secretin may contribute to cardiac output and distribution of blood flow in the body.

5. Summary and Future Prospects

As early as the second century, the presence of a ''vital chemical'' in the circulation that regulates bodily functions was proposed. However, the concept of hormones took 18 more centuries to develop, inaugurated by Bayliss and Starling who discovered secretin. The principal function of secretin as a gastrointestinal hormone is now firmly established. Generally, the functions of secretin in the gastrointestinal tract are to provide protection against gastric acid and to optimize conditions for enzymatic digestion in the small intestine via neural and neuroendocrine pathways. In the stomach, secretin is an enterogastrone that inhibits gastric acid, gastrin, gastric emptying, and motility, in order to prevent dumping of gastric acid into the duodenum. To optimize intraduodenal pH, secretin stimulates the production and secretion of bicarbonate-rich fluid in the pancreas and

small intestine, bile from the liver, as well as the flow of these fluids via the sphincter of Oddi.

Only recently have the extensive distribution of secretin in the human body and the pleiotropic actions exerted by this peptide been fully appreciated. However, there are still many important questions to answer (e.g., the neuroactivities of secretin and its effect on social and cognitive behaviors are yet to be defined). Future studies utilizing novel tools such as secretin- and secretin receptor-deficient mice should provide a better understanding of its physiological roles, particularly those roles that have not been adequately addressed in the past, of endogenously produced and released secretin. Our recent study showing that secretin is involved in water homeostasis is a good example of the demonstration that novel functions of secretin could be discovered by these animal models. Although it is sometimes difficult to extrapolate the functions of secretin to putative therapeutic treatments of human disorders, future investigations should provide insight into the role of secretin in conditions such as pancreatic disorder, autism, stroke, diabetes, and SIADH. Potentially, our understanding of secretin may eventually help in the effective management of some human diseases in the future.

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CHAPTER FIVE

Biology of the Striated Muscle Dystrophin–Glycoprotein Complex

James M. Ervasti *and* Kevin J. Sonnemann

Contents

Abstract

Since its first description in 1990, the dystrophin–glycoprotein complex has emerged as a critical nexus for human muscular dystrophies arising from defects in a variety of distinct genes. Studies in mammals widely support a primary role for the dystrophin–glycoprotein complex in mechanical stabilization of the plasma membrane in striated muscle and provide hints for secondary functions in organizing molecules involved in cellular signaling. Studies in model organisms confirm the importance of the dystrophin–glycoprotein complex for muscle cell viability and have provided new leads toward a full understanding of its secondary roles in muscle biology.

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1. Introduction

Dystrophin is the largest protein isoform expressed from the gene defective in Duchenne muscular dystrophy (Hoffman *et al.*, 1987; Koenig *et al.*, 1988), a lethal muscle-wasting disease that afflicts 1 in 3500 live-born males (Engel, 1986). Based on sequence homology, dystrophin is divided into four distinct domains (Koenig *et al.*, 1988). The amino-terminal 250 residues encode a pair of calponin homology (CH) modules common to several proteins that bind filamentous actin. Adjacent to the amino-terminal domain, more than 2800 amino acids encode 24 homologous triple helical repeats and four hinge domains (Koenig and Kunkel, 1990) that are thought to confer flexibility and elasticity. A third domain of ~400 residues is more complex, encoding a WW module (Bork and Sudol, 1994), two EF hand modules (Koenig *et al.*, 1988), and two ZZ modules in series (Ponting *et al.*, 1996). Finally, the carboxy-terminal ~240 amino acids are unique to dystrophin and its related proteins (Tinsley *et al.*, 1992; Wagner *et al.*, 1993). In total, the four domains of dystrophin are encoded by 3685 amino acids with a molecular weight of 427 kDa.

In skeletal muscle, dystrophin was isolated as part of a large, tightly associated oligomeric complex of proteins synonymously referred to as the dystrophin–glycoprotein complex or dystrophin-associated protein complex (Ervasti *et al.*, 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). Investigations into the biological function of the dystrophin–glycoprotein complex suggest it plays an important mechanical function in stabilizing the plasma membrane (the sarcolemma) against stresses imposed during muscle contraction or stretch. The dystrophin–glycoprotein complex has also garnered attention as a putative cellular signaling complex. Here, we review the data supporting current views on the biological function(s) of the dystrophin–glycoprotein complex in striated muscle.

2. Composition of the Core Dystrophin–Glycoprotein Complex

In addition to dystrophin, the core dystrophin–glycoprotein complex contains nine protein subunits encoded by eight different genes. Other proteins include dystroglycans, sarcoglycans, sarcospan, dystrobrevins, and syntrophins. The constituents of the core dystrophin–glycoprotein complex

remain associated in stoichiometric amounts even after multiple purification steps under moderately stringent buffer conditions (Ervasti *et al.*, 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). However, several biochemical perturbations further resolve the dystrophin–glycoprotein complex into three subcomplexes (Ervasti and Campbell, 1991; Ervasti *et al.*, 1991; Butler *et al.*, 1992; Yoshida *et al.*, 1994; Kramarcy *et al.*, 1994), each of which will be discussed in greater detail in the following.

2.1. Dystroglycan complex

The dystroglycan subcomplex consists of α- and β-dystroglycan, which are encoded as a single polypeptide from one highly conserved gene (Ibraghimov-Beskrovnaya *et al.*, 1992) that undergoes posttranslational proteolytic cleavage to yield the two tightly (but noncovalently) associated subunits (Ibraghimov-Beskrovnaya *et al.*, 1992; Gee *et al.*, 1993; Smalheiser and Kim, 1995; Deyst *et al.*, 1995; Holt *et al.*, 2000; Esapa *et al.*, 2003; Jayasinha *et al.*, 2003). α-Dystroglycan is an extensively glycosylated extracellular protein (Ervasti and Campbell, 1991, 1993; Gee *et al.*, 1993; Smalheiser and Kim, 1995; Ervasti *et al.*, 1997) consisting of two globular domains connected by an extensible stalk such that α-dystroglycan appears dumbbell shaped when viewed by electron microscopy (Brancaccio *et al.*, 1995, 1997; Bozic *et al.*, 2004; Kunz *et al.*, 2004). Although the chemical makeup of the sugar moieties remains poorly understood, the glycoepitopes on α-dystroglycan mediate binding to components of the extracellular matrix and proper glycosylation is essential to dystroglycan function (Ervasti and Campbell, 1993; Grewal *et al.*, 2001; Michele *et al.*, 2002; Kanagawa *et al.*, 2004; Barresi *et al.*, 2004). β-Dystroglycan is a single-pass transmembrane protein with a largely unstructured amino-terminal extracellular domain that binds to the carboxy-terminal globular domain of α-dystroglycan (Di *et al.*, 1999; Boffi *et al.*, 2001) and a 121-residue carboxy-terminal cytoplasmic domain that binds directly to the WW, EF, and ZZ modules in dystrophin (Suzuki *et al.*, 1992; Jung *et al.*, 1995; Rosa *et al.*, 1996; Rentschler *et al.*, 1999; Chung and Campanelli, 1999; Huang *et al.*, 2000; Ishikawa-Sakurai *et al.*, 2004).

2.2. Sarcoglycan complex

The sarcoglycan/sarcospan subcomplex is composed of α-, β, γ-, and δ-sarcoglycan isoforms, each encoded by a separate gene (Roberds *et al.*, 1993; Lim *et al.*, 1995; Noguchi *et al.*, 1995; Nigro *et al.*, 1996), and sarcospan (Crosbie *et al.*, 1999). All sarcoglycans are single-pass transmembrane glycoproteins with long extracellular domains and relatively short cytoplasmic domains (Roberds *et al.*, 1993; Lim *et al.*, 1995; Noguchi *et al.*, 1995; Nigro *et al.*, 1996). α-Sarcoglycan differs from β-, γ-, and δ-sarcoglycan

in that its amino terminus is oriented extracellularly (Roberds *et al.*, 1993). Sarcospan encodes four transmembrane-spanning segments homologous to the tetraspanin family of proteins (Crosbie *et al.*, 1997), which are thought to mediate interactions between transmembrane proteins. Beyond the four sarcoglycan isoforms initially characterized as a subcomplex of the dystrophin–glycoprotein complex in striated muscle, two additional sarcoglycan genes have been identified. ϵ-Sarcoglycan is most similar in sequence homology and membrane topology to α-sarcoglycan (Ettinger *et al.*, 1997; McNally *et al.*, 1998); it can compensate for the absence of α-sarcoglycan in skeletal muscle (Imamura *et al.*, 2005), but also forms part of a high-molecular-weight complex in muscle that is distinct from the dystrophin–glycoprotein complex (Durbeej *et al.*, 2000). ζ-Sarcoglycan is most homologous to δ- and γ-sarcoglycan (Wheeler *et al.*, 2002), and, accordingly, expression studies in heterologous cells suggest that ζ-sarcoglycan can substitute for γ-sarcoglycan in the sarcoglycan complex (Shiga *et al.*, 2006).

2.3. Dystrobrevin/syntrophin complex

Several studies indicate that dystrophin directly interacts with syntrophins (Butler *et al.*, 1992; Kramarcy *et al.*, 1994; Dwyer and Froehner, 1995; Ahn and Kunkel, 1995; Suzuki *et al.*, 1994, 1995; Yang *et al.*, 1995b; Peters *et al.*, 1997a) and dystrobrevins (Butler *et al.*, 1992; Suzuki *et al.*, 1994; Dwyer and Froehner, 1995; Sadoulet-Puccio *et al.*, 1997; Nawrotzki *et al.*, 1998; Peters *et al.*, 1998), two families of cytoplasmic proteins encoded by multiple genes expressed in a tissue-specific manner (Yang *et al.*, 1994; Ahn *et al.*, 1994, 1996; Adams *et al.*, 1995; Piluso *et al.*, 2000). All syntrophins share a common modular structure consisting of one domain unique to syntrophins, one PDZ domain, and two pleckstrin homology domains that suggest syntrophins function as adaptor proteins involved in anchoring cell signaling molecules to the plasma membrane (Adams *et al.*, 1995; Piluso *et al.*, 2000). The syntrophin unique domain and carboxy-terminal pleckstrin homology domain interact with the extreme carboxy terminus of dystrophin (Ahn and Kunkel, 1995; Suzuki *et al.*, 1995). Of the five known syntrophins, all except γ1 are expressed in skeletal muscle (Peters *et al.*, 1997a; Piluso *et al.*, 2000), but β1 and β2 syntrophins show differences in fiber-type distribution or cellular location that argue for distinct functions (Peters *et al.*, 1997a). Dystrobrevins are so named because they share significant sequence homology with the carboxy-terminal domains of dystrophin (Wagner *et al.*, 1993; Sadoulet-Puccio *et al.*, 1996). Two dystrobrevin genes encode multiple isoforms expressed in a wide array of tissues with α-dystrobrevins expressed predominantly in skeletal muscle (Wagner *et al.*, 1993; Yoshida *et al.*, 1995; Sadoulet-Puccio *et al.*, 1996; Peters *et al.*, 1997b; Blake *et al.*, 1996, 1998; Puca *et al.*, 1998; Holzfeind

et al., 1999). α-Dystrobrevin-1 localizes to the neuromuscular junction while α-dystrobrevin-2 is distributed uniformly throughout the sarcolemma (Peters *et al.*, 1998; Nawrotzki *et al.*, 1998). Pairs of coiled-coil motifs present in α-dystrobrevin and the dystrophin carboxy terminus are responsible for their binding interaction (Sadoulet-Puccio *et al.*, 1997; Peters *et al.*, 1998). In addition to their independent interactions with dystrophin, syntrophins and dystrobrevins directly bind one another (Butler *et al.*, 1992; Dwyer and Froehner, 1995), suggesting that two syntrophin molecules associate with each dystrophin–glycoprotein complex through independent interactions with dystrophin and dystrobrevin. Finally, α-dystrobrevins interact with the sarcoglycan complex (Yoshida *et al.*, 2000), suggesting a role in coupling dystrophin to the glycoprotein complex.

3. Function in Mammals

3.1. Mechanical stabilization and force transmission

While detectable beneath the entire sarcolemma of normal skeletal muscle, dystrophin is particularly concentrated in three subcellular structures implicated in the transmission of contractile force from myofibrils to extracellular elements of muscle tissue. Dystrophin immunostaining is enriched at myotendinous junctions (Fig. 5.1A; Samitt and Bonilla, 1990), and intrafascicular fiber terminations (Paul *et al.*, 2002), which are also referred to as myomuscular junctions (Bassett *et al.*, 2003). Myotendinous and myomuscular junctions are the sites of attachment between the ends of muscle fibers and tendons or serially arranged muscle fibers, respectively, and which

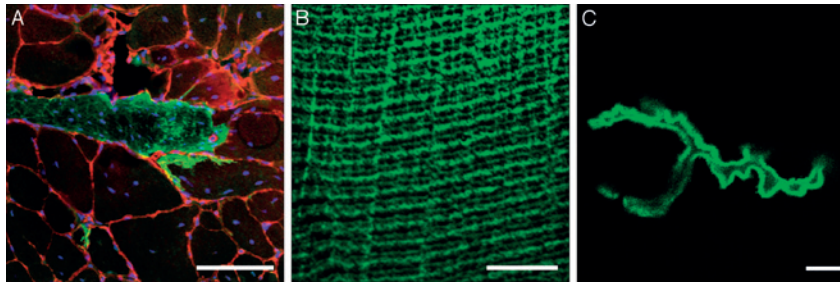

Figure 5.1 Sites of dystrophin–glycoprotein complex enrichment in skeletal muscle. (A) The myotendinous junction identified by immunostaining for tenascin C (green) with individual muscle fibers delineated by immunostaining for laminin (red) and myonuclei (blue) labeled with TO-PRO-3 iodide. Scale bar = 50 μm. (B) Dystrophin immunoreactivity detected at costameres. Scale bar = 10 μm. (C) Neuromuscular junction detected with α-bungarotoxin. Scale bar = 10 μm.

ultimately transmit the force of muscle fiber contraction to bone. The sarcolemma is highly folded at the myotendinous junction, presumably to increase the surface area contact between muscle fiber ends and tendons. The myotendinous junctions of dystrophin-deficient *mdx* mice are less folded than in wild-type muscle, but also show defective lateral association of terminating sarcomeric thin filaments with the sarcolemma (Tidball and Law, 1991; Law and Tidball, 1993). The dystrophin homologue utrophin is also enriched at myotendinous junctions (Khurana *et al.*, 1991) and mice deficient in both dystrophin and utrophin exhibit more marked reduction in sarcolemmal folding at the myotendinous junction compared to dystrophin-deficient *mdx* mice (Deconinck *et al.*, 1997b).

At nonjunctional regions of the sarcolemma, dystrophin immunostaining displays a rectilinear pattern (Fig. 5.1B) with a major transverse element aligning to myofibrillar Z disks and finer strands aligning with M lines or the long axis of the myofiber (Porter *et al.*, 1992; Straub *et al.*, 1992). The rectilinear lattice is commonly called a costamere, coined from the rib-like appearance of its major Z disk element (Pardo *et al.*, 1983). Clever experiments on carefully dissected bundles of frog myofibers and isolated cardiac myocytes adhered to flexible substrata suggested that costameres physically transmit myofibrillar force across the sarcolemma to the extracellular matrix and adjacent muscle cells (Street, 1983; Danowski *et al.*, 1992). Electron microscopy studies suggested that costameres may also coordinate folding of the sarcolemma during muscle contraction (Street, 1983; Shear and Bloch, 1985). The costameric lattice is disorganized in dystrophin-deficient muscle (Minetti *et al.*, 1992, 1994; Porter *et al.*, 1992; Ehmer *et al.*, 1997; Williams and Bloch, 1999). Costameric disruption is accompanied by greatly increased sarcolemmal fragility/permeability (Mokri and Engel, 1975; Menke and Jockusch, 1991, 1995) resulting in dramatically increased movement of membrane-impermeant molecules across the sarcolemma (Fig. 5.2A; Engel, 1986; Weller *et al.*, 1990; Cox *et al.*, 1993; Clarke *et al.*, 1993; Menke and Jockusch, 1991, 1995; Matsuda *et al.*, 1995; Tinsley *et al.*, 1996; Straub *et al.*, 1997; Vilquin *et al.*, 1998; Harper *et al.*, 2002; Barton *et al.*, 2002; Bansal *et al.*, 2003). Both sarcolemmal permeability and necrosis of dystrophin-deficient muscle are exacerbated by physical exercise and improved by muscle immobilization (Karpati and Carpenter, 1986; Weller *et al.*, 1990; Mizuno, 1992; Clarke *et al.*, 1993; Vilquin *et al.*, 1998; Mokhtarian *et al.*, 1999; Bansal *et al.*, 2003). Thus, enrichments of dystrophin in three structural elements of muscle are important for muscle function, as these structures are perturbed when dystrophin is absent.

Studies using noninvasive assays that measure the pulling force or grip strength of intact alert animals have demonstrated that dystrophin-deficient *mdx* mice are weak (Carlson and Makiejus, 1990; Tinsley *et al.*, 1998; Connolly *et al.*, 2001; Sonnemann *et al.*, 2006). Because muscle-specific expression of the dystrophin homologue utrophin in *mdx* mice restored

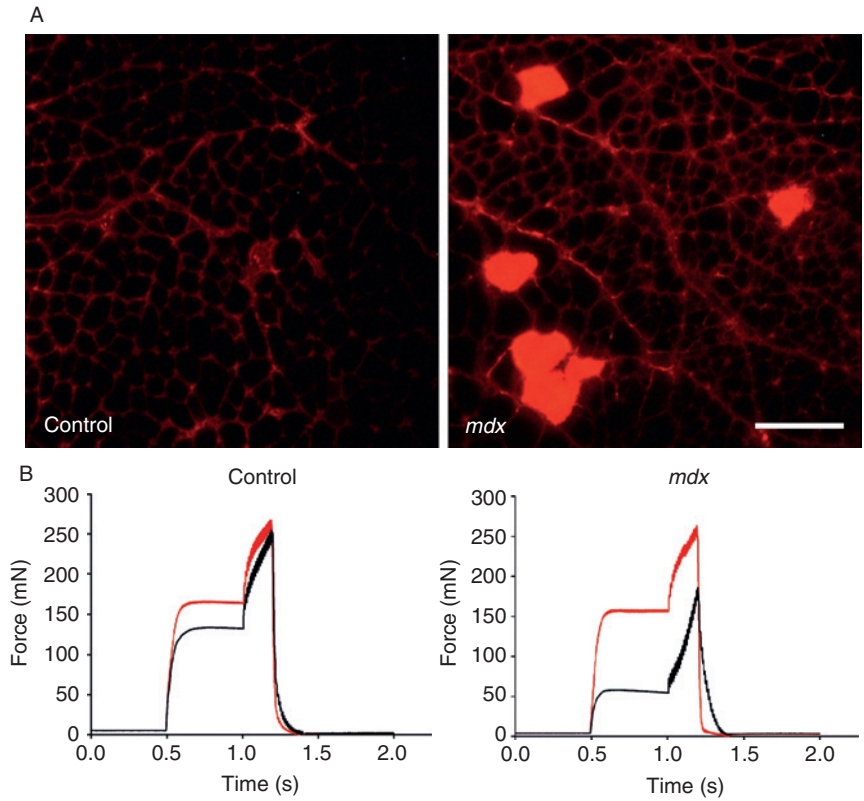

Figure 5.2 Dystrophin-deficient muscle exhibits increased sarcolemmal permeability and contraction-induced injury. (A) Infiltration of membrane-impermeant Evans blue dye (red) into muscle fibers of dystrophin-deficient *mdx* muscle but not wild-type control muscle. Scale bar = 100 μm. (B) Tetanic force is significantly depressed from the first (red) to the fifth (black) eccentric contraction in extensor digitorum longus muscles from dystrophin-deficient *mdx* mice compared to control.

muscle strength to normal values (Tinsley *et al.*, 1998), it is likely that the primary cause of weakness is due to the absence of dystrophin from skeletal muscle and not from a nervous tissue defect. Numerous *ex vivo* studies confirm that dystrophin-deficient muscle is weak, particularly when force output is normalized against muscle cross-sectional area (Coulton *et al.*, 1988; Kometani *et al.*, 1990; Stedman *et al.*, 1991; Sacco *et al.*, 1992; Quinlan *et al.*, 1992; Cox *et al.*, 1993; Pastoret and Sebille, 1993; Tinsley *et al.*, 1998; Deconinck *et al.*, 1997c, 1998; Bobet *et al.*, 1998; Stevens and Faulkner, 2000; Lynch *et al.*, 2001; DelloRusso *et al.*, 2001; Harper *et al.*, 2002; Barton *et al.*, 2002). Although the molecular basis for muscle weakness associated with dystrophin deficiency has been elusive, it seems not to involve a defect in sarcomeric force production (Lynch *et al.*, 2000; Lowe

et al., 2006) but may be due in part to defective force transmission at the myotendinous junction (Deconinck *et al.*, 1997b). While excitation–contraction coupling is not altered, calcium release from the sarcoplasmic reticulum is significantly decreased in *mdx* myofibers, which also likely contributes to the observed force deficit (Woods *et al.*, 2004, 2005).

In addition to decreased normalized force production, dystrophin-deficient muscle is hypersensitive to lengthening (eccentric) contractions (Fig. 5.2B). When *mdx* muscle is forcibly lengthened during tetanic stimulation, force production is immediately and dramatically reduced (Petrof *et al.*, 1993; Moens *et al.*, 1993; Brooks, 1998; DelloRusso *et al.*, 2001). Consistent with a role in sarcolemmal stability, the drop in force generated by intact dystrophin-deficient muscle after experiencing eccentric contraction is well correlated with increased sarcolemmal permeability (Petrof *et al.*, 1993; Moens *et al.*, 1993; Deconinck *et al.*, 1996, 1997c, 1998; Brooks, 1998; Tinsley *et al.*, 1998; DelloRusso *et al.*, 2001; Harper *et al.*, 2002; Barton *et al.*, 2002). In contrast, permeabilized dystrophin-deficient myofibers are neither weaker nor more sensitive to eccentric contraction compared to controls (Lynch *et al.*, 2000).

Knockout of the dystroglycan or sarcoglycan subcomplexes also causes muscular dystrophy that is accompanied by defects in sarcolemmal integrity (Duclos *et al.*, 1998; Araishi *et al.*, 1999; Coral-Vazquez *et al.*, 1999; Cote *et al.*, 1999; Hack *et al.*, 1998, 2000; Durbeej *et al.*, 2000; Straub *et al.*, 2000; Cohn *et al.*, 2002; Sasaoka *et al.*, 2003). Genetic ablation of individual sarcoglycan genes results in a progressive muscular dystrophy phenotype associated with loss of expression of the other three sarcoglycan proteins and sarcospan (Duclos *et al.*, 1998; Araishi *et al.*, 1999; Durbeej *et al.*, 2000; Coral-Vazquez *et al.*, 1999; Hack *et al.*, 1998, 2000; Sasaoka *et al.*, 2003) while knockout of sarcospan caused no apparent muscle phenotype or effect on sarcoglycan complex expression (Lebakken *et al.*, 2000). Because the biochemical stability of the dystrophin–glycoprotein complex is greatly impaired in sarcoglycan-deficient muscle (Duclos *et al.*, 1998; Araishi *et al.*, 1999; Durbeej *et al.*, 2000), the sarcoglycan complex is thought to play a role in stabilizing the linkages formed by β-dystroglycan with α-dystroglycan and dystrophin.

Dystrophin is also enriched in costameres and intercalated disks of cardiac muscle (Kaprielian *et al.*, 2000). Like skeletal muscle, dystrophin-deficient cardiac myocytes are abnormally vulnerable to mechanical stress-induced injury and contractile failure (Danialou *et al.*, 2001; Kamogawa *et al.*, 2001; Yasuda *et al.*, 2005). Interestingly, treatment with chemical-based membrane sealants was shown to correct the cardiac defects associated with dystrophin deficiency in mice (Yasuda *et al.*, 2005). Finally, coxsackie B virus infection results in dilated cardiomyopathy and a virally expressed protease specifically cleaves dystrophin (Badorff *et al.*, 1999, 2000) and is sufficient to induce dilated cardiomyopathy (Xiong *et al.*, 2007).

When taken together, the previous studies indicate that one primary function of the dystrophin–glycoprotein complex is to stabilize muscle cells, and particularly the sarcolemma, against mechanical forces transduced through costameres during muscle contraction or stretch.

While the impressive biochemical stability of the dystrophin–glycoprotein complex combined with the loss of sarcolemmal integrity when it is absent or defective strongly supports a role in mechanical stabilization, the complex does not function in isolation but instead through collaboration with several other cellular constituents. *In vitro* studies demonstrated that spectrin repeat 2 of the large dystrophin rod domain binds strongly to phospholipids, which may modify the physical properties of the sarcolemmal lipid bilayer and/or associated proteins (DeWolf *et al.*, 1997; Le *et al.*, 2003). In support of a role for phospholipid binding *in vivo*, truncated dystrophin transgenes lacking spectrin repeats 2 and 3 were less effective in rescuing the phenotype of dystrophin-deficient *mdx* mice compared to constructs containing these repeats, although substitution of hinge 2 with hinge 3 also complicated the interpretation (Harper *et al.*, 2002).

Several studies have demonstrated that dystrophin purified from a variety of tissues can bind actin filaments *in vitro* with submicromolar affinity (Ervasti and Campbell, 1993; Fabbrizio *et al.*, 1993; Senter *et al.*, 1993; Lebart *et al.*, 1995; Rybakova *et al.*, 1996). *In vivo*, dystrophin interacts with actin filaments composed of the nonmuscle γ-actin isoform (Rybakova *et al.*, 2000; Ursitti *et al.*, 2004), which concentrates primarily at the sarcolemma and particularly within costameres (Craig and Pardo, 1983; Rybakova *et al.*, 2000; Hanft *et al.*, 2006; Sonnemann *et al.*, 2006). Studies aimed at identifying the actin-binding sites within dystrophin have confirmed an important contribution by the amino-terminal, tandem calponin homology domain (Hemmings *et al.*, 1992; Way *et al.*, 1992; Fabbrizio *et al.*, 1993; Corrado *et al.*, 1994; Jarrett and Foster, 1995; Rybakova *et al.*, 1996), but also identified a second actin-binding domain encoded by a cluster of basic spectrin repeats located in the middle rod domain of dystrophin (Rybakova *et al.*, 1996; Rybakova and Ervasti, 1997; Amann *et al.*, 1998, 1999). Although either actin-binding domain is sufficient to physically anchor costameric actin filaments to the sarcolemma (Warner *et al.*, 2002; Hanft *et al.*, 2006), the amino-terminal actin-binding domain appears to be more important from a functional perspective (Warner *et al.*, 2002; Harper *et al.*, 2002). α-Syntrophin also binds actin filaments *in vitro* (Iwata *et al.*, 1998, 2004), which suggests it may contribute to the actin-binding activity of the dystrophin–glycoprotein complex (Rybakova *et al.*, 1996). However, more recent quantitative comparisons of the actin-binding properties of full-length recombinant dystrophin with those measured for the dystrophin–glycoprotein complex indicate that dystrophin alone can account for all actin-binding activity of the complex (Rybakova

et al., 2006). Through direct interaction with the sarcoglycan complex (Thompson *et al.*, 2000), filaminC forms yet another linkage between the dystrophin–glycoprotein complex and the actin cytoskeleton. Like dystrophin, filamins contain an amino-terminal tandem calponin homology actin-binding domain but encode 24 immunoglobulin G (IgG) motifs rather than spectrin-type repeats (Stossel *et al.*, 2001). The phenotype of mice ablated for filaminC expression indicates that filaminC plays a crucial role in maintaining muscle structure (Dalkilic *et al.*, 2006).

In addition to interactions with the actin cytoskeleton, multiple studies have demonstrated an association of the dystrophin–glycoprotein complex with the intermediate filament cytoskeleton through several distinct pathways. First, two-hybrid screens and subsequent experiments identified desmuslin/synemin (Mizuno *et al.*, 2001) and syncoilin (Newey *et al.*, 2001; Poon *et al.*, 2002) as two proteins that couple α-dystrobrevin of the dystrophin–glycoprotein complex with the intermediate filament desmin. More recent biochemical studies have demonstrated the ability of desmuslin/synemin to directly bind sequences within the middle rod and WW/EF/ZZ domains of dystrophin (Bhosle *et al.*, 2006). Dystrophin also associates with cytokeratins 8 and 19 at costameres through a direct interaction of keratin 19 with the amino-terminal, tandem calponin homology domain of dystrophin (O'Neill *et al.*, 2002; Ursitti *et al.*, 2004; Stone *et al.*, 2005). Most recently, an isoform of the giant cytolinker plectin has been localized to the sarcolemma and costameres of skeletal muscle and was demonstrated to directly bind dystrophin and β-dystroglycan (Rezniczek *et al.*, 2007), which provides yet another mechanical linkage between the dystrophin–glycoprotein complex, the actin cytoskeleton, and the intermediate filament lattice. In conclusion, dystrophin, β-dystroglycan, α-dystrobrevin, and sarcoglycans all appear to couple the dystrophin–glycoprotein complex to other structural elements, which provides additional support for an essential structural/mechanical role in striated muscle.

On the external surface of the sarcolemma, components of the dystrophin–glycoprotein complex have been shown to interact with several constituents of the extracellular matrix. Through its incompletely characterized carbohydrate epitopes (Section 2.1), α-dystroglycan has been shown to interact with laminins (Ibraghimov-Beskrovnaya *et al.*, 1992; Ervasti and Campbell, 1993), agrins (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994), and perlecan (Talts *et al.*, 1999; Peng *et al.*, 1999; Kanagawa *et al.*, 2005), which all bind α-dystroglycan through a conserved G-domain motif (Gee *et al.*, 1993; Hohenester *et al.*, 1999). Laminin-2 is the predominant laminin isoform expressed in striated muscle (Patton *et al.*, 1997) and mutations leading to laminin-2 deficiency cause forms of congenital muscular dystrophy (Xu *et al.*, 1994; Sunada *et al.*, 1994; Helbling-Leclerc *et al.*, 1995). Unlike dystrophies caused by defects in the dystrophin–glycoprotein complex, laminin-2-deficient muscular dystrophy

is not associated with compromised sarcolemmal integrity (Straub *et al.*, 1997), but instead involves muscle cell apoptosis in its pathomechanism (Girgenrath *et al.*, 2004; Dominov *et al.*, 2005). While dystroglycan binding to agrin and perlecan has been most extensively studied at the neuromuscular junction (Section 3.2), it bears noting that transgenic expression of agrin minigenes (Moll *et al.*, 2001) or agrin/perlecan chimeras (Meinen *et al.*, 2007) can rescue the dystrophic phenotype of laminin-2-deficient muscle. These results suggest that dystroglycan plays an important role in anchoring muscle cells to the extracellular matrix. While strong, the mechanical linkages between the basement membrane, the dystrophin–glycoprotein complex, and the costameric cytoskeleton are not static and unchanging as denervation causes rapid reorientation of costameres and the laminin-2 matrix from a transverse to a longitudinal pattern (Bezakova and Lomo, 2001). Moreover, it appears that agrin isoforms secreted by muscle cells are important for the maintenance of costamere organization because the transverse to a longitudinal reorientation after denervation was prevented by exogenous application of muscle agrin (Bezakova and Lomo, 2001). More recently, experiments demonstrated that biglycan, a small proteoglycan, binds to α-dystroglycan (Bowe *et al.*, 2000) and α- and γ-sarcoglycans (Rafii *et al.*, 2006). Biglycan null mice display a mild dystrophic phenotype and a subpopulation of muscle fibers shows evidence of impaired sarcolemmal integrity (Mercado *et al.*, 2006), suggesting it may stabilize links between the dystroglycan and sarcoglycan complexes and between the sarcoglycan complex and the extracellular matrix. However, the loss of α-dystrobrevin isoforms in biglycan null muscle may have also contributed to the dystrophic phenotype (Mercado *et al.*, 2006).

Finally, a structural/mechanical role for the dystrophin–glycoprotein complex is supported by examining how muscle responds to the loss of dystrophin. Several structural proteins of costameres, myotendinous and/or myomuscular junctions are upregulated in *mdx* muscle including talin and vinculin (Law *et al.*, 1994), $\alpha_7\beta_1$ integrin (Vachon *et al.*, 1997; Hodges *et al.*, 1997), plectin (Schroder *et al.*, 1997; Rezniczek *et al.*, 2007), filaminC (Thompson *et al.*, 2000), biglycan (Bowe *et al.*, 2000), dysbindin (Benson *et al.*, 2001), syncoilin (Newey *et al.*, 2001), and cytoplasmic γ-actin (Hanft *et al.*, 2006). While targeted to the neuromuscular and myotendinous junctions in adult muscle (Khurana *et al.*, 1991; Ohlendieck *et al.*, 1991), utrophin expression is increased in dystrophin-deficient muscle (Matsumura *et al.*, 1992; Porter *et al.*, 1998) and is redirected to costameres (Williams and Bloch, 1999; Rybakova *et al.*, 2000, 2002). These data suggest that dystrophin-deficient muscle responds to mechanical instability through compensatory remodeling of the cytoskeleton. Moreover, transgenic overexpression of α_7 integrin (Burkin *et al.*, 2001) or utrophin (Tinsley *et al.*, 1998) results in partial to complete rescue of the dystrophic phenotype. In summary, the proteins upregulated in response to dystrophin deficiency

are capable of compensating for the missing physical linkage between the sarcolemma and myofibrillar apparatus and thus support a mechanical function for the dystrophin–glycoprotein complex.

3.2. Organization and stabilization of the neuromuscular junction

The dystrophin–glycoprotein complex is also enriched at the motor end plate of the neuromuscular junction (Fig. 5.1C). However, differences in molecular composition and subcellular distribution suggest its function at this important site of nerve/muscle communication may vary as well. A dystrophin–glycoprotein complex with molecular composition very similar to that expressed throughout the extrasynaptic sarcolemma and costameres is localized to the deep troughs of the junctional folds (Byers *et al.*, 1991; Sealock *et al.*, 1991; Peters *et al.*, 1998). At the crests of junctional folds, dystrophin is replaced by its close homologue utrophin and the longer α-dystrobrevin-1 isoform replaces the shorter α-dystrobrevin-2 isoform that predominates in the extrasynaptic dystrophin–glycoprotein complex (Peters *et al.*, 1998). As noted earlier, β_2-syntrophin is exclusively localized to the neuromuscular junction (Peters *et al.*, 1997a).

Interest in the role of the dystrophin/utrophin–glycoprotein complex in neuromuscular synaptogenesis was piqued by reports from several laboratories that α-dystroglycan bound with high affinity to agrin (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994), an interaction that induces high-density clustering of acetylcholine receptors at the motor end plate (Gautam *et al.*, 1996). Furthermore, β-dystroglycan was shown to directly bind rapsyn (Cartaud *et al.*, 1998; Bartoli *et al.*, 2001), a cytoplasmic protein that is required for acetylcholine receptor clustering in muscle (Apel *et al.*, 1997). However, agrin can induce acetylcholine receptor clustering in dystroglycan null myotubes (Grady *et al.*, 2000), leaving the physiological relevance of agrin binding to α-dystroglycan at the neuromuscular junction unclear. Perlecan binding to α-dystroglycan, on the other hand, has been implicated in anchoring acetylcholinesterase to the neuromuscular junction (Peng *et al.*, 1999; Arikawa-Hirasawa *et al.*, 2002). Genetic ablation of dystrophin (Lyons and Slater, 1991; Grady *et al.*, 1997b), utrophin (Grady *et al.*, 1997a; Deconinck *et al.*, 1997a), α-syntrophin (Adams *et al.*, 2000) or β_1-syntrophin (Adams *et al.*, 2004), α-dystrobrevin (Grady *et al.*, 2000), or dystroglycan (Cote *et al.*, 1999) all caused morphological abnormalities in the neuromuscular junction. Similarly, the density of acetylcholine receptors at the neuromuscular junction is significantly decreased in mice lacking α-dystrobrevin (Grady *et al.*, 2000; Akaaboune *et al.*, 2002) or α-syntrophin (Adams *et al.*, 2000), suggesting that the dystrophin/utrophin–glycoprotein complex contributes to the long-term stability of functionally important elements in the motor end plate.

While α-dystrobrevin and dystroglycan null animals present with muscular dystrophy (Grady *et al.*, 1999; Cote *et al.*, 1999; Cohn *et al.*, 2002), utrophin and syntrophin null animals are phenotypically normal (Grady *et al.*, 1997a; Deconinck *et al.*, 1997a), and dystrophic α-dystrobrevin null mice can be rescued by transgenic expression of α-dystrobrevin-2 without restoring neuromuscular junction morphology (Grady *et al.*, 2003). These data suggest that morphological defects in the neuromuscular junction do not contribute significantly to pathologies associated with absence or abnormality in the dystrophin–glycoprotein complex.

3.3. Cellular signaling

Through syntrophins, the dystrophin–glycoprotein complex is thought to anchor a variety of signaling molecules near their sites of action. Neuronal nitric oxide synthase (nNOS) was first shown to copurify with the dystrophin–glycoprotein complex (Brenman *et al.*, 1995) through a direct interaction with α-syntrophin (Adams *et al.*, 2001). Syntrophins have since been found to bind a variety of channels (Gee *et al.*, 1998; Adams *et al.*, 2001; Vandebrouck *et al.*, 2007), kinases (Lumeng *et al.*, 1999; Abramovici *et al.*, 2003), and kinase substrates (Luo *et al.*, 2005). Because nNOS localization to the sarcolemma and enzymatic activity were disrupted in dystrophin-deficient *mdx* mice (Brenman *et al.*, 1995), it was hypothesized that aberrant nNOS regulation may importantly contribute to the muscle degeneration accompanying dystrophinopathy. However, genetic ablation of nNOS did not induce a muscular phenotype in mice (Huang *et al.*, 1993). Furthermore, transgenic expression of a truncated dystrophin (Harper *et al.*, 2002) or full-length utrophin (Tinsley *et al.*, 1998) fully reversed muscular dystrophy in *mdx* mice without restoring sarcolemmal nNOS (Judge *et al.*, 2006; Yokota *et al.*, 2006). Finally, mice knocked out for α-syntrophin (Kameya *et al.*, 1999; Adams *et al.*, 2000), β_2-syntrophin (Adams *et al.*, 2004), or both genes (Adams *et al.*, 2004) exhibited no evidence of muscle disease. Thus, it appears that the loss of signal-molecule anchoring function plays no primary role in causing dystrophin-deficient muscular dystrophy, although it may contribute to secondary disease features such as impaired vascular perfusion during muscle contraction (Sander *et al.*, 2000; Thomas *et al.*, 1998, 2003), altered muscle regeneration (Anderson, 2000; Hosaka *et al.*, 2002), inflammation (Wehling *et al.*, 2001), or oxidative stress (Dudley *et al.*, 2006).

The dystrophin–glycoprotein complex is also hypothesized to directly regulate the mitogen-activated protein (MAP) kinase and AKT signaling pathways in skeletal muscle (Rando, 2001; Batchelor and Winder, 2006). Early studies in support of this hypothesis documented *in vitro* binding of β-dystroglycan (Yang *et al.*, 1995a) and syntrophin (Oak *et al.*, 2001) to Grb2, an adaptor protein containing Src homology 2 and 3 domains that

couples receptor tyrosine kinases to MAP kinase cascades via small GTPase family members. More recent *in vitro* experiments have led one group to propose that laminin binding to α-dystroglycan induces assembly of a syntrophin–Grb2–Sos1–Rac1–Pak1–JNK complex initiated by tyrosine phosphorylation of syntrophin and resulting in JNK activation (Oak *et al.*, 2003; Zhou *et al.*, 2006). Another group has reported that β-dystroglycan can directly bind either MEK or ERK alone *in vitro*, leading the authors to hypothesize that it serves a scaffold function important for MAP kinase signaling (Spence *et al.*, 2004). The relevance of both stories (Zhou *et al.*, 2006; Spence *et al.*, 2004) to the *in vivo* function of the dystrophin–glycoprotein complex remains to be demonstrated. In neither case have the signaling molecules actually been shown to interact with the fully assembled dystrophin–glycoprotein complex expressed in striated muscle. As noted previously, mice null for α-syntrophin (Kameya *et al.*, 1999; Adams *et al.*, 2000), β_2-syntrophin (Adams *et al.*, 2004), or both genes (Adams *et al.*, 2004) exhibited no muscular dystrophy. Furthermore, the dystrophic phenotype of *mdx* mice was not improved by transgenic overexpression of dystroglycan (Hoyte *et al.*, 2004), Dp71 (Cox *et al.*, 1994; Greenberg *et al.*, 1994), or Dp116 (Judge *et al.*, 2006) even though the dystrophin–glycoprotein complex constituents necessary for signaling were restored to the sarcolemma.

To investigate a potential role for the dystrophin–glycoprotein complex in MAP kinase signaling *in vivo*, a number of groups have compared MAP kinase activity in muscle from wild-type and dystrophic mice lacking either dystrophin or γ-sarcoglycan (Kolodziejczyk *et al.*, 2001; Kumar *et al.*, 2004; Lang *et al.*, 2004; Nakamura *et al.*, 2001, 2002, 2005; Griffin *et al.*, 2005; Barton, 2006). Results have differed dramatically across studies even when the same MAP kinase was evaluated in the same animal model. Three studies found no difference in activated ERK1/2 of resting *mdx* muscle (Kolodziejczyk *et al.*, 2001; Nakamura *et al.*, 2005; Kumar *et al.*, 2004) while three others reported increased ERK1/2 activation in *mdx* muscle (Nakamura *et al.*, 2001, 2002; Barton, 2006). The activity of p38 in *mdx* muscle was not altered in four studies (Kolodziejczyk *et al.*, 2001; Kumar *et al.*, 2004; Nakamura *et al.*, 2005; Lang *et al.*, 2004), but was elevated in two others (Nakamura *et al.*, 2001, 2002). JNK1 was elevated in *mdx* in one study (Kolodziejczyk *et al.*, 2001) but was not found to be different from control in four others (Nakamura *et al.*, 2001, 2002, 2005; Kumar *et al.*, 2004). Even after taking into account the variation in animal ages and muscles analyzed, it is difficult to draw any firm conclusions over how defects in the dystrophin–glycoprotein complex may affect MAP kinase signaling. The results of several studies further suggest that a mechanotransduction pathway impinging on MAP kinase activation may be perturbed in muscle expressing a defective dystrophin–glycoprotein complex (Nakamura *et al.*, 2005; Kumar *et al.*, 2004; Griffin *et al.*, 2005; Barton, 2006), yet even

some of these data appear contradictory. ERK1/2 became more activated in stretched/exercised *mdx* muscle in two studies (Kumar *et al.*, 2004; Nakamura *et al.*, 2005), but activity was reduced by eccentric contraction in a third (Barton, 2006). Stretch had opposite effects on ERK1/2 activity when compared across two studies of γ-sarcoglycan null muscle (Griffin *et al.*, 2005; Barton, 2006). Finally, the activity of p38 was significantly enhanced in exercised *mdx* muscle (Nakamura *et al.*, 2005) but not in *mdx* muscle that was acutely stretched (Kumar *et al.*, 2004).

Toward a role for the dystrophin–glycoprotein complex in regulating Akt signaling in skeletal muscle, one group reported that disruption of laminin binding to α-dystroglycan induced apoptosis in cultured myotubes accompanied by decreased Akt activity (Langenbach and Rando, 2002). It was suggested that loss of dystrophin–glycoprotein complex function may impair cell survival signaling through the Akt pathway with enhanced apoptosis contributing to dystrophic pathogenesis. However, two new studies have paradoxically reported elevated Akt activity in dystrophin-deficient *mdx* muscle (Dogra *et al.*, 2006; Peter and Crosbie, 2006). Moreover, transgenic overexpression of the antiapoptosis proteins ARC (Abmayr *et al.*, 2004) and BCL2 (Dominov *et al.*, 2005) failed to alleviate dystrophy in *mdx* muscle. Thus, it remains to be demonstrated that the dystrophin–glycoprotein complex directly regulates a signal transduction pathway or that its disruption directly alters muscle cell signaling in a manner that contributes substantively to the pathologies observed in dystrophic mammalian muscle. Alternatively, aberrant signaling may be an adaptive response by dystrophin-deficient muscle attempting to maintain homeostasis, or more interestingly, may be an adverse consequence of cytoskeletal remodeling (Rezniczek *et al.*, 2007).

4. Function in Model Organisms

4.1. Caenorhabditis elegans

Of three popular model organisms interrogated, the function of the dystrophin–glycoprotein complex is best understood in the nematode worm, *Caenorhabditis elegans*. The *C. elegans* genome encodes genes for dystrophin/utrophin (*dys-1)*, dystroglycan (*dgn-1)*, sarcoglycans, dystrobrevins (*dyb-1)*, and syntrophins (*stn-1)* (Bessou *et al.*, 1998; Gieseler *et al.*, 1999, 2001; Grisoni *et al.*, 2002, 2003). Mutations or RNAi that target each gene similarly cause a mild phenotype characterized by exaggerated head bending, hyperactivity, and hypercontractility (Bessou *et al.*, 1998; Gieseler *et al.*, 1999, 2001; Grisoni *et al.*, 2002, 2003). Mutations in *dys-1, dyb-1,* and *stn-1* also show increased sensitivity to acetylcholine and the acetylcholinesterase inhibitor aldicarb, which suggested that the motility phenotypes

were due to altered cholinergic signaling (Bessou *et al.*, 1998; Gieseler *et al.*, 1999, 2001; Grisoni *et al.*, 2003). In support of this hypothesis, the *snf-6* gene encodes a novel acetylcholine transporter localized to the motor end plate of the *C. elegans* neuromuscular junction, snf-6 binds to stn-1, and *snf-6* mutations phenocopy *dys-1, dyb-1*, and *stn-1* mutants (Kim *et al.*, 2004). Ablation of *dys-1* (Gieseler *et al.*, 2000), *dyb-1* (Gieseler *et al.*, 2001), or *snf-6* (Kim *et al.*, 2004) combined with a mildly affected mutant of *MyoD* (*hlh-1)* causes a more severe, muscle degeneration phenotype consistent with the more severely affected *mdx/MyoD*$^{-/-}$ double knockout mice (Megeney *et al.*, 1999). Furthermore, microarray comparisons between wild-type and *dys-1* mutants revealed altered transcript profiles (Towers *et al.*, 2006) comparable to the differences reported for patients with Duchenne muscular dystrophy (Chen *et al.*, 2000). Interestingly, mutations that slightly impair contractility at several steps downstream of nerve–muscle communication can suppress muscle degeneration in *dys-1/hlh-1* double mutants (Mariol *et al.*, 2007). Thus, it appears that the dystrophin–glycoprotein complex in *C. elegans* primarily functions to localize a protein (snf-6) important for termination of neuromuscular transmission and when disrupted, hypercontractility can initiate muscle degeneration on a sensitized background. Finally, the phenotype of *dyc-1* mutants resembles that of *dys-1* mutants and dyc-1 overexpression partially suppresses the *dys-1* phenotype (Gieseler *et al.*, 2000). *dyc-1* is homologous with mammalian CAPON, which interacts with neuronal nitric oxide synthase through its PDZ domain (Gieseler *et al.*, 2000) and is upregulated in dystrophin-deficient *mdx* muscle (Segalat *et al.*, 2005). Thus, studies in *C. elegans* may help explain why defects in the dystrophin–glycoprotein complex cause perturbations in the neuromuscular junction without apparent functional consequence in mammals (Section 3.2).

4.2. Drosophila

As in *C. elegans*, the *Drosophila* genome encodes homologues for dystrophin, dystroglycan, dystrobrevin, sarcoglycans, and syntrophin (Roberts and Bobrow, 1998; Neuman *et al.*, 2001; Greener and Roberts, 2000). Consistent with studies in mammals (Section 3) and worms (Section 4.1), genetic and RNAi-mediated knockdown of dystrophin or dystroglycan in *Drosophila* causes decreased mobility and age-dependent muscle degeneration (Shcherbata *et al.*, 2007). Also like *C. elegans, Drosophila* mutants lacking a large dystrophin isoform localized to the neuromuscular junction show enhanced neuromuscular transmission, but through elevated release of neurotransmitter from presynaptic sites (van der Plas *et al.*, 2006) rather than impaired postsynaptic uptake as found in the worm (Kim *et al.*, 2004). Interestingly, a hypercontraction-induced myopathy has been described in

Drosophila with mutations in the myosin heavy chain (*mhc*) locus (Montana and Littleton, 2004). While not directly relevant to dystrophin–glycoprotein complex function, comparison of transcript expression profiles of *Drosophila mhc* mutants with those from mammalian dystrophies suggested that compensatory cytoskeletal remodeling may be a common response to muscle disease that is conserved across species (Montana and Littleton, 2006).

4.3. Zebrafish

The small vertebrate zebrafish (*Danio rerio*) has emerged as a model system to study the function of the dystrophin–glycoprotein complex. Orthologs for dystrophin, dystroglycan, dystrobrevin, and sarcoglycans have been identified in zebrafish through a variety of approaches (Parsons *et al.*, 2002; Guyon *et al.*, 2003; Bassett *et al.*, 2003; Jin *et al.*, 2007; Steffen *et al.*, 2007). Ablation of dystrophin or dystroglycan results in loss of stable muscle attachments analogous to the myotendinous or myomuscular junction in mammals, impaired muscle integrity, and necrosis (Parsons *et al.*, 2002; Bassett *et al.*, 2003). A more recent study further identified an important role for dystroglycan in the formation of distributed neuromuscular synapses as opposed to the focal neuromuscular junctions almost exclusively studied in mammals (Lefebvre *et al.*, 2007). Most interestingly, a zebrafish mutant in the nicotinic acetylcholine receptor (*sofa potato*) was shown to suppress myopathy associated with dystrophin deficiency but did not suppress the myopathy caused by dystroglycan ablation (Etard *et al.*, 2005), similar to conclusions that emerged from a study of the zebrafish *candyfloss* mutant, a model of laminin-2-deficient muscular dystrophy (Hall *et al.*, 2007). These studies suggest that decreased contractility can reduce mechanically induced injury in dystrophin-deficient muscle, but they also suggest that the absence of dystrophin and dystroglycan induces different mechanisms of pathogenesis. In summary, the zebrafish is emerging as the model organism that may best recapitulate the functional deficits associated with dystrophin–glycoprotein complex abnormalities in mammals (Section 3), and that may also provide the most insight into poorly understood functions of the complex.

5. Concluding Remarks

In comparing the studies in mammals (Section 3) with model organisms (Section 4), we notice two distinct but interrelated pathways to muscle degeneration associated with defects in the dystrophin–glycoprotein complex. First, the cytoskeletal framework of muscle may be compromised to the point that it cannot protect muscle cells against the normal forces generated within. In the second pathway, defects lead to excessive force

production that appears to overwhelm an otherwise normal cytoskeletal support structure. In both cases, there are indications that dystrophic muscle attempts to compensate through cytoskeletal remodeling. It also seems likely that the dystrophin–glycoprotein complex plays multiple roles in mammalian muscle but that some model organisms may employ the complex for fewer, and perhaps only one function. In conclusion, comparing and contrasting results of dystrophin–glycoprotein complex studies across a wide range of organisms promises not only to shed light on its versatility, but also on secondary functions that are difficult to address solely in mammals.

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CHAPTER SIX

Providing Unique Insight into Cell Biology via Atomic Force Microscopy

Victor Shahin* *and* Nelson P. Barrera†

Contents

Abstract

The invention of atomic force microscopy (AFM) some two decades ago opened up new realms for our perception of cell biology. AFM produces three-dimensional images of biological surfaces at atomic resolution in physiologically relevant environments. Beyond this one-of-a-kind capability, AFM can be applied to cell biology for a variety of investigations, such as to recognize single molecules at work and study their function and structure. This admirable technique is also being widely applied to measure forces, study characteristic surface properties such as adhesion, and detect mechanical responses, for example, volume and elasticity changes of cells to various physiological and pathophysiological stimuli. In more recent years, AFM has become the most rapidly developing imaging technique. In this chapter, the AFM capabilities and the usefulness of its broad application to cell biology are highlighted, with the emphasis on structural and functional investigations into a number of biological samples focusing on cells, membranes, and single molecules.

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1. Introduction

The invention of atomic force microscopy (AFM) in 1986 has revolutionized the field of biomedical sciences. AFM is a member of a family of microscopy instruments, commonly known as scanning probe microscopes (SPM). As reviewed by Hoh and Hansma (1992), these techniques pass a probe in close proximity to a sample surface. The latter is next scanned by the probe, following parallel lines, measuring local interactions, and collecting spatially resolved information on surface properties, such as tunneling current, physical topography, and ion conductance or temperature, and registering the values for each position both horizontally (x, y) and vertically (z).

The first member of SPM, the scanning tunneling microscope (STM) (Binnig *et al.*, 1982), developed by G. Binnig *et al.* in 1982, provided information at atomic resolution on various structures. These structures, however, had to be electrically conducting (Hansma *et al.,* 1988; Zasadzinski *et al.,* 1990). Thus, the application of STM in biomedical science remained limited to conducting samples. Although attempts have been made to obtain structural information on nonconducting biological specimens, the mechanisms of image generation and their interpretation have remained unclear. Fortunately, the desire of biologists for a powerful SPM applicable on nonconducting biological specimens was cut short. It took Binnig, Quate, and Gerber only 4 years to develop an appropriate microscope for biologists, AFM, through the adaptation of one of the scanning probe microscopes (Binnig *et al.,* 1986). At the other end of the scale, a scan may cover a distance of over 100 μm in the x and y directions and up to 115 μm in the z direction. This is an enormous range. It can truly be said that the development of AFM was a major achievement, for it has had profound effects on many areas of science and engineering. Not only is AFM applicable to nonconducting biological specimens, but it can also operate in a fluid environment. This unique property provides the opportunity to observe biological and physiological processes in real time at molecular and often atomic resolution.

In contrast to what happened to the majority of the microscopy and several other experimental techniques, no sooner was AFM developed than it started to be applied by biologists. Only 2 years after its invention, the first work in which AFM was used for the study of biological samples was published (Worcester *et al.,* 1988). However, the lack of reproducibility

of the presented results and the refutation of the conclusions of these initial studies raised serious doubts concerning the applicability of AFM to the study of biological specimens. Fortunately, these doubts were soon abandoned when reliable DNA images, obtained using AFM, were first published in 1992 (Bustamante *et al.,* 1992).

Since then and led by ongoing improvements both at the instrumental and sample preparation levels, application of AFM to diverse biological specimens has gathered momentum and has not yet reached a steady state. Evidence for the usefulness of using AFM for structural and functional investigations of a large number of diverse biological specimens comes from the steadily growing number of studies imaging samples that have been previously characterized by alternative techniques (Arakawa *et al.,* 1992; Barrera *et al.,* 2005a; Geisse *et al.,* 2004; Hansma *et al.,* 2003; Henderson *et al.,* 1996; Hoh *et al.,* 1991; Malkin *et al.,* 2002, 2004; Oberleithner *et al.,* 1994, 1996, 1997, 2004, 2006; Radmacher *et al.,* 1994b; Rotsch *et al.,* 1997; Shahin *et al.,* 2001, 2006). Many of these specimens, from cells to individual molecules, have been structurally and functionally investigated in near physiological environments (Hillebrand *et al.,* 2006; Lawrence *et al.,* 2003; Oberleithner *et al.,* 2004, 2006; Schafer *et al.,* 2002). Examples to date include large molecules such as DNA, bacteria, viruses, proteins, native membranes, supported lipid bilayers, and live cells. Dynamic biological processes, key surface and material properties (e.g., adhesion and elasticity), and various chemical forces on biological specimens have been successfully studied (Oberleithner *et al.,* 2006; Radmacher *et al.,* 1994a, 1996; Radmacher, 1997; Shahin *et al.,* 2006). Another emerging application of AFM to biology is focused on molecular recognition and is gaining steadily growing interest (Hinterdorfer and Dufrene, 2006). The aim of this chapter is to emphasize the usefulness of broad AFM applications to biology.

2. AFM: Principle of Operation and Operation Modes

AFM was invented by Binnig, Quate, and Greber in 1985 (Binnig *et al.,* 1986). The atomic force microscope uses a diminutive, extremely sharp tip (on the order of a few nanometers) to scan a sample surface. The tip is mounted at the very end of a flexible, microscale cantilever (typically silicon or silicon nitride) (Fig. 6.1A, left). In the conventional AFM operation mode, the so-called contact mode (Fig. 6.1A, top right), the tip is brought into close proximity to the sample surface pressing the latter with a small loading force. The tip is next raster scanned over the sample surface, either by moving the sample beneath the tip or by moving the tip over the

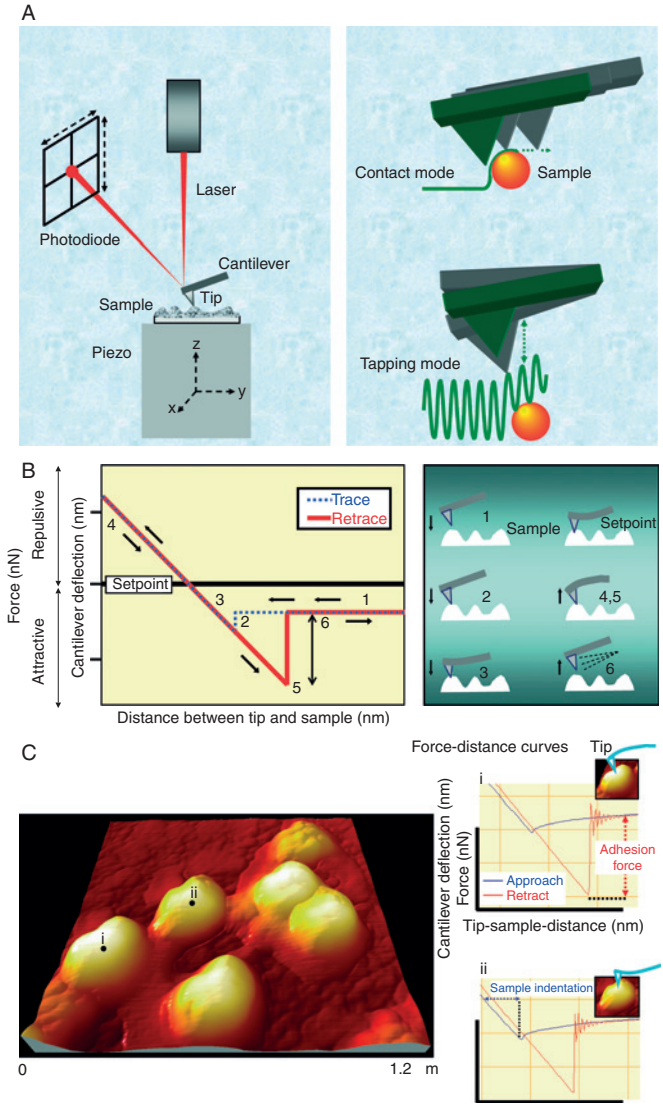

Figure 6.1 (A) Schematics of atomic force microscopy principle (left) and two frequently applied atomic force microscopy operation modes (right), contact and tapping. (B) Schematic force–distance curve describing a single trace–retrace (approach–retract) cycle of the atomic force microscopy tip, which is continuously repeated during scanning. (C) Force–distance curves performed on herpes simplex virus type I capsids. On either scanned point (indicated by black circles), the adhesion forces between the capsid surface and the scanning atomic force microscopy tip can be directly derived from the corresponding force–distance curves (top right). The same force–distance curves quantify the force necessary to indent the capsid for a given distance (bottom right), thus, enabling measurement of the capsid elasticity as described in detail previously (Radmacher *et al.*, 1996). (Figure 6.1C is modified from Shahin *et al.*, 2006.)

sample. The movement is controlled by piezoelectric drivers (servo-system) in either a horizontal (*x, y*) or vertical (*z*) dimension (Fig. 6.1A). When the tip encounters the sample surface, various forces between the tip and the sample lead to a deflection of the cantilever (Fig. 6.1B and C) according to Hook's law. These forces are measured by the amount of deflection of the cantilever. The magnitude of the deflection is captured by a laser beam (focused onto the backside of the cantilever surface right above the underlying tip). The laser beam reflects off the cantilever, the angular direction of which changes as the tip undergoes deflections. The reflected beams are captured and converted into electrical signals by a position-sensitive photodetector (photodiode). The optical lever amplifies the deflection signal up to 1000-fold, so that even deflections of <1 nm can be measured (Marti *et al.,* 1988). By calculating the difference signal in the photodiode (quadrants), the amount of deflection can be correlated with a height, and because the flexible cantilever obeys Hook's law for small displacements, the interaction force between the tip and the sample can be determined. Hence, it is the variation of the point of incidence of the reflected beam on the photodiode that measures any minimal bending or twisting of the cantilever and, thus, the interaction of the tip with the sample. A plot of the laser deflection versus the tip position on the sample surface provides the resolution of the hills and valleys that constitute the topography of the surface, and a three-dimensional visualization of the surface topography is obtained (Fig. 6.1C).

The atomic force microscope can be operated in various modes. The most commonly used AFM imaging mode is the previously described *contact mode,* which is believed (Fotiadis *et al.,* 2002) to provide the highest spatial resolution among all of the AFM operation modes so far available. The contact mode is most useful for hard surfaces, providing extraordinary high-resolution images. However, a tip in contact with a surface is subject to contamination from removable material on the surface. An excessive, particularly lateral, force in the contact mode can also damage the surface or erode the sharpness of the probe tip. An alternative approach is to reduce the frictional forces produced as the tip moves across the sample. This has resulted in the development of two alternative AFM modes, the tapping mode (Hansma *et al.,* 1994; Zhong *et al.,* 1993) and the magnetically activated oscillating mode (MAC) (Han *et al.,* 1996). Both are frequently used and have in common that the AFM cantilever is oscillated vertically while scanning an object (Fig. 6.1A, bottom right).

In these modes, the cantilever is oscillated at near its resonance frequency (often hundreds of kilohertz) with amplitudes ranging between 20 and 100 nm. The oscillation amplitude, phase, and resonance frequency are modified by tip–sample interaction forces; these changes in oscillation with respect to the external reference oscillation provide information about the sample's characteristics and make it possible to obtain a pseudo-three-dimensional

image of the scanned surface. In these modes, the tip is positioned slightly above the surface of the sample so that it taps the surface for only a very small fraction of its oscillation period. This is still contact with the sample in the sense defined earlier, but the very short time over which this contact occurs means that lateral forces are virtually eliminated. The advantage of tapping the surface is improved lateral resolution on soft samples. For poorly adsorbed specimens on a substrate surface the advantage is clearly seen (Dryden *et al.,* 1999; Ellis *et al.,* 1999b; Geisse *et al.*, 2004; Hansma *et al.*, 2003).

Other methods for obtaining image contrast are also possible with the tapping mode, such as phase imaging. Phase imaging is a powerful extension of the tapping mode. More recently, there has been much interest in phase imaging. In the phase imaging mode, the phase shift of the oscillating cantilever relative to the driving signal is measured (Bhushan and Qi, 2003). This phase shift can be correlated with specific material properties that affect the tip–sample interaction (Bhushan and Qi, 2003; Kasai *et al.,* 2004). Thus, by mapping the phase of the cantilever oscillation during the tapping mode scan, phase imaging goes beyond simple topographic mapping to obtain key information on the surface being scanned, namely variations in composition, adhesion, friction, elasticity, and numerous other properties. Phase imaging is used simultaneously with the tapping mode, so that sample topography and key material/surface properties of the same sample can be imaged and mapped, respectively, at one time.

Another quite interesting and increasingly applied imaging mode is the force–volume mode (Radmacher *et al.*, 1996; Radmacher, 1997). This mode is a combination of surface force measurements and contact mode imaging. During scanning the tip records a force curve in every scanned point of the surface and displays the result as an ordinary two-dimensional image (Fig. 6.2) (Ellis *et al.,* 1999a; Shahin *et al.,* 2005b). Chemical mapping of the surface is possible if, for example, adhesion or electrostatic force is displayed as the image. The force curves are available in every point for display.

3. AFM Can Measure Forces and Elasticity

Several forces are manifest between the tip and sample surface before and after contact. These forces are measured by collecting a force curve, a so-called force–distance curve (Fig. 6.1B), that is a plot of cantilever deflection as a function of sample position along the z-axis (the z-piezo position toward or away from the probe tip). It assumes a simple relationship, in accordance with Hooke's law, between the force (F) and the cantilever deflection (d_c): $F = -kXd_c$ where k is the spring constant of the cantilever.

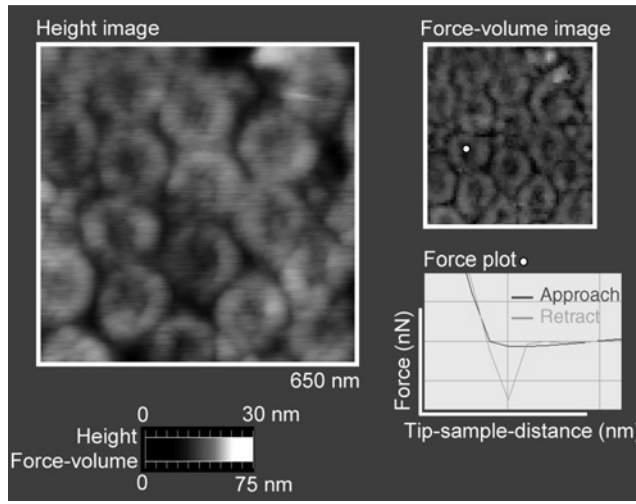

Figure 6.2 Height (top left) and corresponding force volume (top right) images of a *Xenopus laevis* oocyte's nuclear envelope. The force volume image maps adhesion properties of the nuclear envelope and provides information on its elasticity. The adhesion is displayed as color-coded (bottom left), dark being more adhesive than bright. The rectangular window in the lower right displays the force curves as they are collected point by point (exemplarily indicated by a white circle) in real time. (Modified from Shahin *et al.*, 2005b.)

The basic force–distance curves can be understood by considering the example of a cantilever in air approaching a hard, incompressible surface such as glass or mica. As the cantilever approaches the surface, initially the forces are too small to provide a measurable deflection of the cantilever, and the cantilever remains in its undisturbed position. At some point, the attractive forces, usually van der Waals (but also capillary forces when imaged in air), overcome the cantilever spring constant and the tip jumps into contact with the surface. Once the tip is in contact with the sample, it remains on the surface as the separation between the base and the sample decreases further, causing a deflection of the tip and an increase in the repulsive contact force. As the cantilever is retracted from the surface, the tip often remains in contact with the surface due to some adhesion and the cantilever is deflected downward. At some point the force from the cantilever will be enough to overcome the adhesion, and the tip will break free. In liquid, there may not be an obvious snap to contact in the approach curves. Both the adhesive and repulsive forces can be easily derived from the recorded force–distance curves (Fig. 6.1C, right) as previously described in detail (Shahin *et al.*, 2005b). These forces are surface characteristic. Their measurement using AFM can, thus, provide additional crucial information on the surface properties (Shahin *et al.*, 2006). Additionally, force–distance

curves can also be used to determine the elasticity of the scanned sample, and extensive technical details have been published previously (Hoh and Schoenenberger, 1994; Oberleithner *et al.*, 2006).

In principle, the atomic force microscope is used as a mechanical sensor to measure the elastic modulus. The AFM tip is pressed against the sample so that the latter is indented. At the same time, the AFM cantilever that serves as a soft spring is distorted. Force–distance curves quantify the force (N) necessary to indent the membrane for a given distance (m). The elastic (Young's) modulus can be estimated using the Hertz model that describes the indentation of elastic materials, such as cells and multilayer films (Radmacher *et al.*, 1996), and is defined as follows: $F = \delta^2 \times (2/\pi) \times [E/(1 - v^2)] \times \tan(\alpha)$, where F is the applied force (calculated from the known/measurable spring constant multiplied by the measured cantilever deflection), E is the elastic modulus (kPa), v is the Poisson's ratio (known), α is the opening angle of the AFM tip (known), and δ is the indentation depth (measurable).

4. Advantages of AFM

AFM has several advantages over other high-resolution imaging techniques. It provides a true three-dimensional surface profile. Additionally, samples viewed by AFM do not require any special treatment (such as metal/carbon coatings) that would irreversibly change or damage the sample. While some of the other high-resolution imaging techniques, such as electron microscopy, need an expensive vacuum environment for proper operation, most AFM modes can work perfectly well in ambient air or even a liquid environment. Currently, structural information at a molecular resolution (under special circumstances) is obtained from other microscopic techniques, particularly electron microscopy, electron and X-ray diffractions, nuclear magnetic resonance, and infrared spectroscopy. Indeed, molecular functions can be studied with various biochemical, electrophysiological, and molecular biological techniques. However, it is difficult to combine both structural and functional studies with these techniques. Moreover, these techniques provide little information on the surface of biomolecules, the very sites of molecular interaction. This is where AFM excels: it obtains topographical information as to the surfaces of biological molecules, that is, it images the surfaces where most of the regulatory biochemical and other signals are directed. Indeed, other microscopic techniques can also view surfaces, for example, the scanning electron microscope. The atomic force microscope, however, differs from the scanning electron microscope in that it can image living cells and molecules in an aqueous environment at comparable and often greater resolution.

This makes it possible to study biological macromolecules and even living organisms. The development of atomic force microscopes with a high speed at which good quality successive images can be recorded is of key importance.

A new AFM apparatus, recently described (Ando *et al.,* 2001), generates movies with 80-msec frames, enabling tracing of faster biological events. Finally, the fact that AFM can be combined with other techniques makes it even more attractive for all-round investigations on biological specimens. Some results were obtained by AFM coupled with fluorescence techniques (Mathur *et al.,* 2000), optic microscopy (Vesenka *et al.,* 1995), scanning ion conductance microscopy (Proksch *et al.,* 1996), or scanning electrochemical microscopy (Macpherson and Unwin, 2000).

5. Application of AFM to Biology

AFM is an admirable approach by means of which cells and subcellular structures can be imaged at a resolution far exceeding that of optical microscopes. It also has developed into a highly qualified tool for studying surface properties of key importance, such as adhesion and elasticity. A wide range of studies indicated that AFM as an approach by which single molecules can be identified, manipulated, and functionally investigated, facts that open new and unique perspectives toward understanding the biological properties of single molecules. In principle, using AFM can make it possible to perform whole-cell-to-molecule experiments in a physiological environment, where dynamic changes in the molecular structure and function of channels, receptors, and other macromolecules can be observed. In addition, AFM can be an important tool for the growth and developmental studies of native unstained cells and processes (e.g., nerve growth and synapse formation) (McNally and Borgens, 2004; Quist *et al.,* 2000; Weissmuller *et al.,* 2000).

5.1. Cells

It did not take more than 5 to 7 years after the invention of AFM before a variety of fixed and dried cells had been imaged successfully (Butt *et al.,* 1990; Gould *et al.,* 1990). Red blood cells (Fig. 6.3) and bacteria dried onto a glass cover slip were some of the first cells to be examined (Gould *et al.*, 1990). Step by step, a huge number of other cell types was imaged with AFM, at admirable resolution, and under physiological (aqueous) conditions. Initially, AFM studies of cells dealt predominantly with exploring their structure and the achievement of topography images at a resolution that was at the time unprecedented; these often focused on how to improve

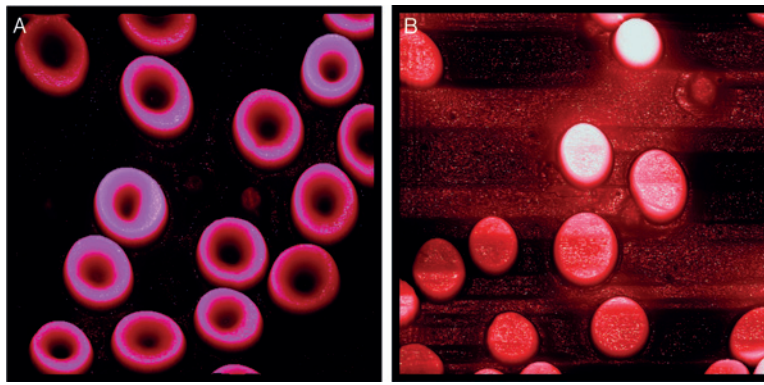

Figure 6.3 Atomic force microscopy images (50 μm $\times$ 50 μm) of normal (A, donut-shaped) and abnormal (B, sphere-shaped) red blood cells. Sphere-shaped red blood cells, termed spherocytes, are observed in spherocytosis, an autohemolytic anemia.

the image resolution even further by optimizing the sample preparation. In intact cells such as living glial cells and platelets, AFM has revealed actin and other cytoskeletal filaments (Chang *et al.,* 1993; Henderson *et al.,* 1992).

Henderson *et al.* (1992) have imaged glial cells showing F-actin under the surface of the plasma membrane at a resolution down to 20 nm. At somewhat greater resolution live cultured adult atrial cells were imaged (Kordylewski *et al.,* 1994), and the increased resolution allowed for visualization of the cytoskeletal elements including muscle sacromeres and cross bridges.

Barbee *et al.* (1994) have imaged live cultured bovine aortic endothelial cells that were subjected to a flow-induced shear stress, and could visualize a significant reorganization of the cell morphology and cytoarchitecture. Astonishingly, they also showed that by increasing the imaging force, it is possible to truly distinguish the images obtained of the external surface from those of the intracellular structures.

Horber *et al.* (1992) imaged monkey kidney cells at a resolution of $\sim$10 nm. When these cells were transfected with vaccine virus, the cell surface morphology changed significantly, and real-time extrusions of proteins and viruses were observed.

The few studies mentioned previously and the many others cited therein have been followed by far more studies, and it seems that the application of AFM for topographic investigations on biological systems is still a subject of major interest and will long remain so. Moreover, topographic investigations are gradually becoming far more sophisticated and versatile, for instance, aiming at monitoring structural changes taking place during cell death and cell growth, thereby opening up new perspectives that contribute to our understanding of profound physiological and pathophysiological

processes. For example, Yunxu *et al.* (2006) developed the magnetic AC (MAC) mode AFM to image the three-dimensional (3D) ultrastructure of living hippocampal neurons under physiological conditions. Initially, the soma, the dendrites, and the growth cones of hippocampal neurons were imaged. The imaging force was adjusted to a small value for long-term observation. The neural spines were damaged when the tip produced a large force; the spines regenerated after the force was reduced. Subsequently, they explored the relationship between structural changes in hippocampal neurons and Alzheimer's disease by employing the new imaging technique. Time-lapse image acquisition (10 min intervals) showed that the growth cone collapsed after the addition of amyloid peptide fragment β (25 to 35), which is thought to initiate Alzheimer's disease. In addition, they found substantial changes in the mechanical properties and in the volume of individual growth cones. Yunxu *et al.* (2006) have therefore concluded that the MAC mode AFM may be a powerful tool for observing long-term structural changes in living neural cells under physiological conditions.

McNally and Borgens (2004) have used AFM to three-dimensionally image living and dying neurons, and to study at the nanoscale their morphological responses to damage, nano/micropuncture to the membrane, intentionally inflicted upon the neurons by the scanning AFM tip. This experimental study not only provided unreported neurobiology and neurotrauma, but also emphasized the unique versatility of AFM. Not only does AFM yield extraordinary high resolution as shown in a large number of studies, but it can also be used to physically manipulate cells and study their mechanical response to various stimuli. Investigating (by AFM) the mechanical response/behavior of cells in turn has been a subject of intense investigation for years. AFM has been widely applied to investigate whole-cell mechanical behavior. An understanding of whole-cell mechanical behavior can provide insight into profound cellular responses to physiological mechanical loading and diseases in which such responses are altered. This key aspect of cellular mechanical behavior (e.g., change in volume and elasticity), however, has gained little interest despite being of major importance. This lack of interest was confounded by the lack of an appropriate technical approach, but interest grew as AFM has proven to be a suitable approach for studying mechanical cell properties. Whole-cell mechanical behavior has been investigated under various physiological and pathophysiological conditions in numerous studies, a tiny number of which are dealt with in the following.

Hessler *et al.* (2005) used AFM to observe an early-stage apoptosis-induced volume decrease (AVD) of cells undergoing induced apoptosis. The evidence is that dramatic morphological changes, particularly AVD, are an early prerequisite to apoptosis and precede key biochemical time points. Hessler *et al.* (2005) observed that AVD preceded evident key biochemical

hallmarks of apoptosis and suggested that changes in morphological volume occur very early in the induction of apoptosis.

Schneider *et al.* (1997) applied AFM to obtain morphological information about individual cultured endothelial cells of bovine aorta under stationary and strain conditions and to simultaneously measure changes in cell volume in response to aldosterone. This vital mineralocorticoid hormone is known to have acute, nongenomic effects on intracellular pH, intracellular electrolytes, and inositol-1,4,5-triphosphate production. Schneider *et al.* (1997) tested whether under tension endothelial cells change their volume in response to aldosterone. Such changes were already shown in human leukocytes measured by Coulter counter (Grinstein *et al.,* 1986). In contrast to leukocytes that are more or less spherical and live in suspension, endothelial cells exhibit a complex morphology and adhere to a substrate. Thus, measurements of discrete cell volume changes in endothelial cells under physiological conditions were feasible only with more sophisticated techniques. By using AFM Schneider *et al.* (1997) could precisely measure the absolute cell volume of individual living endothelial cells. AFM disclosed a transient swelling of endothelial cells induced by the activation of an aldosterone-sensitive plasma membrane Na^+/H^+ exchanger.

We further investigated the impact of aldosterone and another vital steroid, glucocorticoid, on the structure and function of endothelial cells (blood vessels) (Oberleithner *et al.*, 2006). As endothelial cells are targets for both glucocorticoids and mineralocorticoids, we exposed human umbilical vein endothelial cells to both types of steroids. Our data show that glucocorticoids (dexamethasone) strengthen cell-to-cell contacts (peripheral action), whereas mineralocorticoids enlarge and stiffen cells (central action). This could explain the dexamethasone-mediated retention of fluid in the vascular system, and endothelial dysfunction in states of hyperaldosteronism. More recently, we also applied AFM to investigate cell swelling and to study the effect of another vital hormone, the sex steroid 17β-estradiol (estrogen), on the volume, apical surface, and elasticity in human umbilical vein endothelial cells (HUVEC) (Hillebrand *et al.*, 2006). 17β-Estradiol is known to delay the onset of atherosclerosis in women, but the cellular mechanisms are still unclear. Estrogens bind to specific receptors and initiate a signaling cascade that involves the activation of plasma membrane Na^+/H^+ exchange. We found that 17β-estradiol increases HUVEC water content and HUVEC elasticity mediated by activated estrogen receptors. The estrogen response depended on the activation of plasma membrane Na^+/H^+ exchange. It was therefore concluded that the increase in endothelial cell elasticity could be one of the vasoprotective mechanisms postulated for 17β-estradiol. As yet, and referring to whole-cell investigations, AFM has been dealt with as a high-resolution imaging technique and

a nanosensor for studying whole-cell mechanical behavior under different physiological and pathophysiological conditions.

The application of AFM, however, is not limited to such investigations. More and more studies have been using AFM for further investigations on whole cells. For instance, in our laboratories AFM has been used as a novel approach enabling measurement of proteolytic activity in the microenvironment of tumor cells (Kusick *et al.,* 2005). Proteolytic cleavage of the extracellular matrix (ECM) is a critical feature of tumor cell invasion, and affects cancer cell growth, differentiation, apoptosis, and migration. Malignant cells secrete most proteases as inactive proenzymes that undergo proteolytic cleavage for activation, and proteolytic activity is elevated in close proximity to these cells. Therefore, local activity rather than protease concentration determines ECM proteolysis. Precise quantification of local proteolytic activity, functional investigation, and high-resolution imaging of morphological ECM alterations have proven difficult.

Kusick *et al.* (2005) have demonstrated that AFM can be applied to measure proteolytic activity in the microenvironment of cells. AFM enabled nanoscale volume measurement and 3D reconstruction of single proteins and demonstrated that ECM cleavage is restricted to the proteolytic microenvironment of cancer cells. Hence, AFM has once again proven to be an invaluable approach allowing specific quantification and imaging of local proteolytic processes at a nanometer level, thus providing a unique method for the functional evaluation of the invasiveness and metastatic potential of tumor cells in small scale samples.

In another study AFM was applied to visualize dynamic processes on the plasma membrane surface. Oberleithner *et al.* (1993) applied AFM on migrating cells and investigated *in vivo* plasma membrane turnover to resolve dynamic processes at the nanometer level on the surface of migrating cells. Rapid turnover processes of cytoskeletal elements inside the cell and insertion of new plasma membrane at the leading edge of the cell permit the extension of a cell in a given direction. As an experimental model Oberleithner *et al.* (1993) used migrating kidney cells derived from the Madin–Darby canine kidney (MDCK) cell line that was transformed by alkaline stress. These so-called MDCK-F cells exhibit spontaneous calcium-dependent oscillatory activity of the plasma membrane potential associated with cell locomotion. They imaged cells during migration and observed dynamic invagination processes in the cell surface close to the leading edge, indicating internalization of the plasma membrane. Invaginations were prevented by removal of calcium from the perfusate. During calcium reduction plasma membrane uncoupled from the underlying cytoskeleton and lipidic pores with diameters of about 30 nm could be disclosed and imaged. This study demonstrated that AFM can readily trace dynamic physiological processes *in vivo*, emphasizing the potential role of calcium in maintaining plasma membrane integrity and function.

The AFM whole-cell studies mentioned so far make up a tiny fraction of a large number of similar studies. With regard to all these studies we are led to believe that the variety of AFM applications to whole-cell investigations has not yet reached its limit.

5.2. Membranes

AFM has provided topographs of numerous native and synthetic membranes at unprecedented resolution and in aqueous conditions (Janovjak *et al.,* 2003; Muller *et al.,* 1998; Muller and Engel, 2002; Scheuring *et al.,* 1999, 2004;, Scheuring and Sturgis, 2005). It is therefore not surprising that AFM imaging of various membranes is being broadly applied. Many biological membranes adapt in response to environmental conditions. Scheuring and Sturgis (2005) applied AFM to investigate how the composition and architecture of photosynthetic membranes of a bacterium change in response to light. They showed that structural adaptation ensures efficient photon capture under low-light conditions and prevents photo damage under high-light conditions. Images at unprecedented, submolecular resolution of native membranes have shed light on the architecture of the photosynthetic apparatus in different photosynthetic bacteria, such as *Blastochloris* (*Blc.*) *viridis* (Scheuring *et al.,* 2003), *Rhodospirillum* (*Rsp.*) *photometricum* (Scheuring *et al.,* 2004), *Rhodobacter* (*Rb.*) *sphaeroides* (Bahatyrova *et al.,* 2004), and *Rb. Blasticus* (Scheuring *et al.,* 2005).

AFM has also been used to study the structural response of proteins to physiological stimuli and to analyze trafficking of proteins across membranes. For example, AFM was applied to visualize the ATP-dependent formation of clusters of native proteins protruding from the cytoplasmic membrane surface in cultured transformed kidney cells (Ehrenhoefer *et al.,* 1998), which demonstrated for the first time that functional clusters of proteins are required in native plasma membrane. More recently, we have shown with AFM that the frequency of nuclear pores per nucleus increased from immature to mature stages of oogenesis (Schlune *et al.,* 2006). On the other hand, individual nuclear pores were found to be more active with ribonucleoprotein transport in immature stages. These observations provided, for the first time, an unchallenged structural correlate for evidence emerging from biochemical studies postulating that throughout oogenesis, huge amounts of RNA are produced that are needed for early development; early stages of oocyte development are characterized by high transcriptional activity, whereas translation of maternal RNA dominates late stages (Rosbash, 1974).

Applying the same imaging technique we have described the routes in the nuclear envelope through which mineralocorticoid- and glucocorticoid-triggered macromolecules enter and exit the cell nucleus (Ludwig *et al.,* 2006; Schafer *et al.*, 2002; Shahin *et al.,* 2005a). Mineralocorticoids and

glucocorticoids are vital steroid hormones. While their physiological and therapeutic activities have been dealt with extensively, much remains to be understood about the paths that their triggered macromolecules, essential for steroid hormone action, take through the nuclear envelope (Fig. 6.4) to

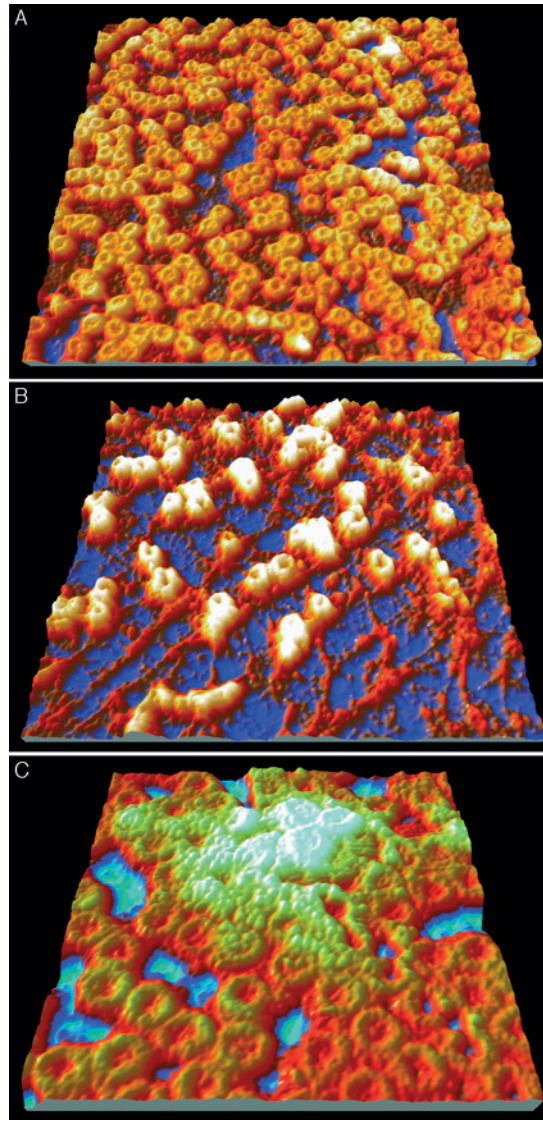

Figure 6.4 (A, B) Atomic force microscopy images (3 μm $\times$ 3 μm) of the cytoplasmic (A) and nucleoplasmic (B) faces of the nuclear envelope of *Xenopus laevis* oocyte. (C) Atomic force microscopy image (1.25 μm $\times$ 1.25 μm) of proteins while being translocated through nuclear pore complexes.

enter and leave the nucleus. Using AFM we could trace the trafficking of steroid-triggered macromolecules across the nuclear envelope at a single molecule level, for the first time structurally describing the paths that these macromolecules take through both sites of the nuclear envelope to enter and leave the nucleus.

Moving on to another cellular membrane, the plasma membrane, we have set out to visualize membrane trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR) (Schillers *et al.,* 2004). Cystic fibrosis is a common hereditary disease that affects the entire body, causing progressive disability and early death. It is caused by a mutation in CFTR. Membrane trafficking of CFTR is supposed to be an important mechanism controlled by the intracellular messenger cAMP. Derived from AFM analysis of the intramolecular domains we concluded that two CFTR molecules line up in parallel, tail by tail, forming a pore in its center. This molecular arrangement was presumed to represent the CFTR chloride channel configuration, operative in native plasma membrane.

So far, we have dealt with the application of AFM on native membranes. The application of AFM, however, is not confined only to native surfaces. Synthetic membranes and reconstituted vesicles have also been imaged with AFM, and at molecular resolution (Zasadzinski *et al.,* 1991). Thin synthetic lipid films supported on solid substrates (e.g., mica or glass) are valuable model systems for mimicking biological surfaces widely used in biophysical research. An advantage of studying synthetic membranes with AFM is that it is possible to change the lipid composition on-line and study lipid–lipid interactions, fluidity, and lipid–protein interactions (El Kirat *et al.,* 2005; Chiantia *et al.,* 2006; Shaw *et al.,* 2003). On synthetic membranes such as supported lipid bilayers, which largely mimic the composition of the lipid leaflet of cellular membranes, it is also possible to observe interactions with a wide range of ligands, among them viruses, bacteria, and toxins, as shown previously (Berquand *et al.,* 2005; Carneiro *et al.,* 2006; Geisse *et al.*, 2004; El Kirat *et al.*, 2005; Hughes *et al.,* 2004; Puntheeranurak *et al.,* 2005; Zuber and Barklis, 2000).

Investigation of such interactions using AFM opened unique perspectives toward understanding particular pharmacological, physiological, and pathophysiological mechanisms. For example, Berquand *et al.* (2004) applied AFM and provided direct evidence that the perturbation of lipid domains by the antibiotic azithromycin strongly depended on the lipid nature; this opened the door for developing new applications in membrane biophysics and pharmacology. El Kirat *et al.* (2006) used AFM to address the crucial question of whether negatively curved lipids influence the interaction of the simian immunodeficiency virus (SIV) fusion peptide with model membranes. Geisse *et al.* (2004) have applied AFM to study the targeting of *Helicobacter pylori* vacuolating toxin to membrane lipids in synthetic phospholipid bilayers. The *Helicobacter pylori* vacuolating toxin VacA

caused several effects on mammalian cells *in vitro*, including intracellular vacuolation, formation of pores in the plasma membrane, and apoptosis. When added to cells, VacA became associated with detergent-resistant membranes, indicating that it bound preferentially to lipid rafts. AFM imaging has allowed an elegant examination of the association of VacA with lipid raft domains in supported lipid bilayers (Geisse *et al.*, 2004). Lipid rafts, in turn, are believed to be of significant biological importance. In the late 1990s, evidence that had accumulated since the 1970s led to the proposal that biological membranes are composed of microdomains of different lipids, which form functional ''rafts'' and are presumably of key physiological importance, for example, for mediating particular signaling pathways (Simons and Ikonen, 1997). The physiological relevance of this concept was initially somewhat controversial but is now much less so (Henderson *et al.,* 2004), not least as a result of intense investigations carried out using AFM (Giocondi *et al.,* 2004; Lawrence *et al.*, 2003; Shaw *et al.,* 2006).

5.3. Single molecules

Because of its piconewton force sensitivity and (sub)nanometer positional accuracy, the atomic force microscope has gradually developed into a powerful tool for exploring the forces and dynamics of the interaction between individual molecules. Numerous studies applied AFM to recognize single molecules while imaging, and to explore the structure, function, and conformational changes of single molecules (Hansma *et al.,* 1992; Hinterdorfer *et al.,* 1996; Muller *et al.*, 1998; Schafer *et al.*, 2002; Hansma *et al.*, 2003; Barrera *et al.,* 2005b; Janovjak *et al.*, 2003; Muller and Engel, 2002; Scheuring *et al.*, 1999, 2003; Raab *et al.,* 1999). As reviewed by Muller *et al.* (2006), more recent studies show that AFM in the context of a ''lab on a tip'' enables the measurement of multiple parameters of membrane proteins. This multifunctional tool can be applied to probe the oligomeric states and conformational changes of membrane protein assemblies in their native environment. The ability to determine diverse properties at high spatial resolution facilitates the mapping of structural flexibilities, electrostatic potentials, and electric currents (Kedrov *et al.,* 2006; Muller and Engel, 2002). By using the AFM tip as a tweezer, it is possible to characterize unfolding and refolding pathways of single proteins and the location of their molecular interactions. These interactions dictate the stability of the protein and might be modulated by ligands that alter the protein's functional state.

Owing to the high signal-to-noise ratio, AFM images on a subnanometer scale allowed the conformational space of membrane protein surfaces to be sampled. This was demonstrated by topographs of porin OmpF, aquaporin-Z, and bacteriorhodopsin, all recorded at a lateral

resolution of <7 Å and a vertical resolution of ~1 Å (Scheuring *et al.,* 2002). More recently, single-molecule force spectroscopy has been used to observe directly the mechanical stepwise folding of numerous single proteins *in vitro*, such as the Na^+/H^+ antiporter NhaA from *Escherichia coli* (Kedrov *et al.*, 2006) and ankyrin repeats (Li *et al.,* 2006). This has been quite beneficial for biological research, as mechanisms of folding and misfolding of membrane proteins are of increasing interest in cell biology.

Another beneficial application of single molecule research with AFM is focused on investigating receptors to better understand their structural organization, stoichiometry, and function among other things. Using the *Xenopus* oocyte expression system, a nicotinic acetylcholine receptor has been imaged at extremely high resolution, which led to a significantly better understanding of the structural organization of this receptor (Lal and Yu, 1993). However, the stoichiometry and arrangements of subunits (architecture) of receptors, which will define the specificity of their actions, remain in many cases elusive.

We recently developed a new method using AFM imaging to determine the architecture of homomeric and heteromeric ionotropic receptors (Barrera *et al.,* 2005a,b; Neish *et al.,* 2002; Ormond *et al.,* 2006). These receptors are ligand-gated ion channels composed of three superfamilies: the ATP-gated P2X channels, Cys-loop channels (for 5-hydroxytryptamine [5-HT], nicotinic acetylcholine, γ-aminobutyric acid [GABA], and glycine), and glutamate-gated channels (Green *et al.,* 1998; Khakh *et al.,* 2005; Lester *et al.,* 2004). They are composed of different subunits and within a specific degree of oligomerization. However, only a fraction of the maximum number of combinations between subunits has been shown to be functionally expressed in cells. Besides, there can be structural interactions between different ionotropic receptors (Khakh *et al.*, 2005), which increase the physiological targets of neurotransmitters.

Purified native receptors, 5-HT-$3_{A/B}$, and antibodies against different epitope tags onto subunits were simultaneously imaged and structurally investigated by AFM. It was found that the subunit stoichiometry of 5-HT-$3_{A/B}$ is 2A:3B and that the subunit arrangement around the receptor rosette is B-B-A-B-A (Barrera *et al.*, 2005a) (Fig. 6.5). The same experimental approach was used to determine the architecture of homomeric $P2X_2$ and $P2X_6$ receptors, and it was concluded that the $P2X_2$ receptor forms a trimeric architecture, whereas the $P2X_6$ receptor had a molecular volume equivalent to a monomeric structure (Barrera *et al.*, 2005b). Once the uncharged region at the N-terminus of the $P2X_6$ was removed, the receptor was assembled as a nonfunctional trimer and exported to the plasma membrane, which suggested that this region contributes to the regulation of $P2X_6$ receptor trafficking (Ormond *et al.*, 2006). The architecture of the ionotropic receptors is critical to the development of signaling

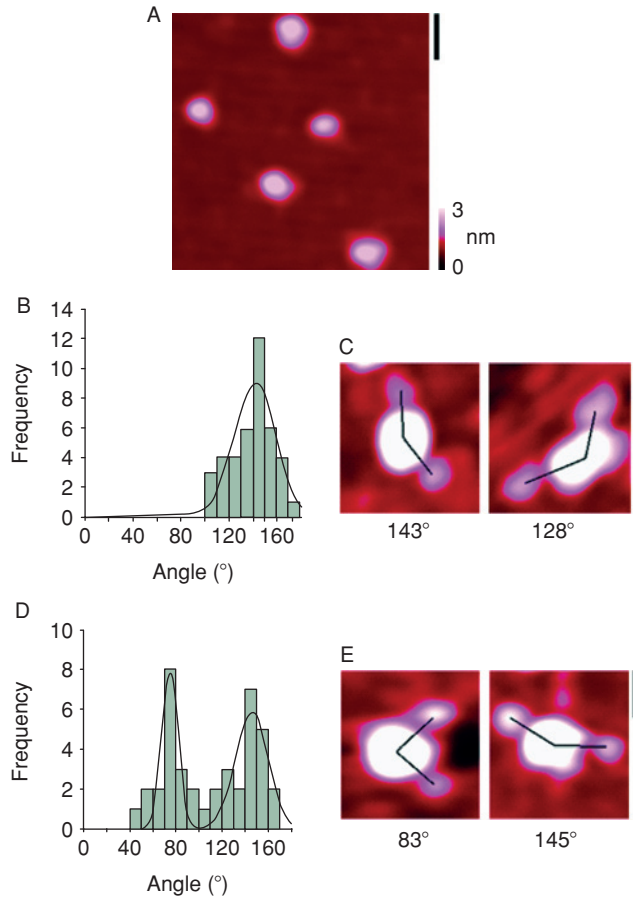

Figure 6.5 Atomic force microscopy images of complexes between 5-hydroxytryptamine$_{3A/B}$ receptors and anti-Myc, and anti-V5 antibodies. (A) Medium-magnification images of 5-hydroxytryptamine$_{3A/B}$ receptors. Scale bar, 50 nm. (B) Frequency distributions of angles between antibodies for receptors doubly bound by anti-Myc antibodies. (C) Zoomed images of receptors that are bound by two anti-Myc antibodies. Scale bar, 20 nm. (D) Frequency distributions of angles between antibodies for receptors doubly bound by anti-V5 antibodies. (E) Zoomed images of receptors that are bound by two anti-V5 antibodies. Scale bar, 20 nm. (Modified from Barrera *et al.*, 2005a.)

transduction pathways. Hence current progress in this area would lead to a finely tuned understanding of the receptor structure–function relationship.

We suggest that the AFM-based methods described in our studies on receptors mentioned previously can be widely applied to other types of multisubunit proteins. Another AFM-based method applicable to particular biological research has recently been introduced by us. We have described

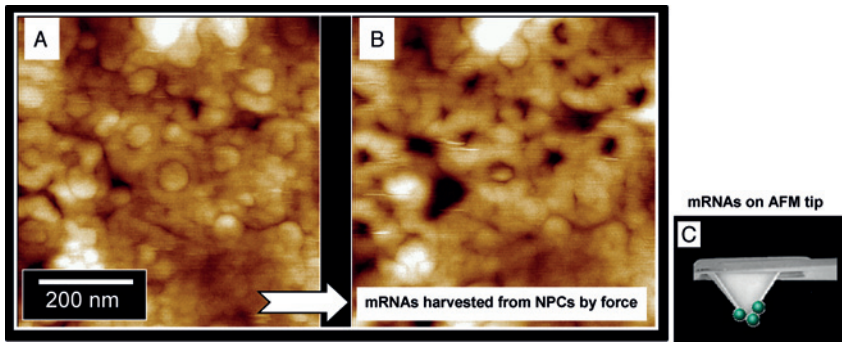

Figure 6.6 (A–C) Nuclear plug harvesting (see text for details). Plugs that are seen being translocated from the nucleus to the cytosol through the central channels of nuclear pore complexes (NPCs) represent mRNA transcripts of steroid hormone (in this case aldosterone)- induced early genes. (Modified from Schafer *et al.*, 2002.)

an approach using AFM that aims to identify transcripts of early genes induced by steroid hormones, taking aldosterone as an example (Schafer *et al.*, 2002). In the first step the hormone is injected into *Xenopus* oocytes. Then the nuclei are mechanically isolated from the respective cells and stripped off their nuclear envelopes 30 min after hormone injection; this time period is needed for the newly synthesized mRNA transcripts, induced by aldosterone, to locate to the nuclear pore central channel for their subsequent export through the nuclear pore channel into the cytosol (Schafer *et al.*, 2002).

AFM is next applied to visualize the cytoplasmic face of nuclear pore complexes (NPCs) whose central channels are occupied by mRNA transcripts, ''plugs,'' that are about to leave the nuclear pores heading for the cytosol (Fig. 6.6). Plugs most likely represent transcripts of aldosterone-induced early genes. For plug removal, the loading force of the AFM tip is gradually increased until plugs come off the NPCs and become attached to the AFM tip. We called this procedure ''plug harvesting'' (Oberleithner *et al.*, 2001). Plugs serve as the starting matter for further mRNA analysis. Plug harvesting can also be applied in the same manner to identify transcripts of early genes induced by steroid hormones other than aldosterone.

6. Conclusions

AFM has resulted in high-resolution imaging of whole cells, membranes, and biomolecules; it has been demonstrated that its application to biological problems can produce fundamental structural and mechanistic information. As the use of this technique is continuously growing, we

expect that new and exciting areas in biology will become frequent targets of AFM imaging.

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CHAPTER SEVEN

Characteristics of Oxysterol Binding Proteins

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Contents

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Abstract

Protein families characterized by a ligand binding domain related to that of oxysterol binding protein (OSBP) have been identified in eukaryotic species from yeast to humans. These proteins, designated OSBP-related (ORP) or OSBP-like (OSBPL) proteins, have been implicated in various cellular functions. However, the detailed mechanisms of their action have remained elusive. Data from our and other laboratories suggest that binding of sterol ligands may be a unifying theme. Work with *Saccharomyces cerevisiae* ORPs suggests a function of these proteins in the nonvesicular intracellular transport of sterols, in secretory vesicle transport from the Golgi complex, and in the establishment of cell polarity. Mammals have more ORP genes, and differential splicing substantially increases the complexity of the encoded protein family. Functional studies on mammalian ORPs point in different directions: integration of sterol and sphingomyelin metabolism, sterol transport, regulation of neutral lipid metabolism, control of the microtubule-dependent motility of endosomes/lysosomes, and regulation of signaling cascades. We envision that during evolution, the functions of ORPs have diverged from an ancestral one in sterol transport, to meet the increasing demand of the regulatory potential in multicellular organisms. Our working hypothesis is that mammalian ORPs mainly act as sterol sensors that relay information to a spectrum of different cellular processes.

Key Words: Cell signaling, Lipid metabolism, Lipid transport, ORP, OSBP, Oxysterol binding protein, Sterol sensor, Vesicle transport.

1. Introduction

1.1. Oxysterols

Oxysterols are 27-carbon derivatives of cholesterol, or by-products of cholesterol biosynthesis, that contain additional oxygen functions as hydroxyl, carbonyl, or epoxide groups (Schroepfer, 2000). Generally, introduction of an oxygen function in cholesterol markedly reduces its half-life and directs it to excretion or to further oxidation to bile acids (Björkhem and Diczfalusy, 2002). Like cholesterol, oxysterols partition into membranes, but due to their greatly increased hydrophilicity, they move much more rapidly between the intracellular membrane organelles and are more easily accessible to receptors in the cytosolic compartment. These compounds are found in healthy mammalian tissues or circulation at very low quantities as compared to cholesterol. However, oxysterol enrichment is associated with certain pathological situations, for example, with the formation of macrophage foam cells and atherosclerotic lesions (Brown and Jessup, 1999; Olkkonen and Lehto, 2004). Oxysterols can be formed through enzymatic cholesterol oxidation, mainly by mitochondrial or microsomal cytochrome P450 family enzymes, or by nonenzymatic

autoxidation processes (Björkhem, 2002; Russell, 2000). The major enzymatically formed oxysterols in human circulation are 27-, 24-, and 7α-hydroxycholesterol (Brown and Jessup, 1999). Enzymatically formed oxysterols are intermediates in the biosynthesis of bile acids and steroid hormones, but they also act as signaling lipids that regulate cholesterol biosynthesis, cellular uptake, and efflux via effects on the major transcription factors responsible for sterol homeostasis (Goldstein *et al.*, 2006; Tontonoz and Mangelsdorf, 2003). The minor enzymatically formed species 25-hydroxycholesterol (25-OHC) is commonly used in cell model studies of cholesterol homeostatic control (see Section 1.3). The major nonenzymatically formed oxysterols, 7-ketocholesterol, 7β-hydroxycholesterol, and 5β,6β-epoxycholesterol, have prominent cytotoxic properties and have been implicated in various pathological states (Lemaire-Ewing *et al.*, 2005; Rimner *et al.*, 2005; Massey, 2006).

1.2. Mediators of oxysterol effects on cellular lipid metabolism

Nuclear receptor proteins designated liver X receptor-α (LXRα) and LXRβ (also known as NR1H3 and NR1H2, respectively) were identified more than a decade ago based on sequence homology with other known receptors (Apfel *et al.*, 1994; Willy *et al.*, 1995). While LXRβ is expressed at relatively even levels in all tissues, LXRα is expressed at high levels in the liver and is less abundant in the adrenal glands, intestine, adipose, macrophages, lung, and kidney (Repa and Mangelsdorf, 2000). The LXRs were initially considered ''orphan'' receptors, because their natural ligands were unknown. However, oxysterols at physiological concentrations were soon found to bind to and activate them (Janowski *et al.*, 1996; Lehmann *et al.*, 1997). The endogenous ligands for the LXRs are likely to be intermediate or end products of sterol metabolic pathways, such as 22(*R*)-hydroxycholesterol, 24(*S*),25-epoxycholesterol, 24(*S*)-hydroxycholesterol, and 27-hydroxycholesterol. LXRs bind to the promoters of specific target genes as heterodimers with the retinoid X receptor (RXR; NR2B1). The sequence elements identified by the LXRs are direct repeat four (DR4)-type motifs, termed LXR responsive elements (LXREs; Repa and Mangelsdorf, 2000; Tontonoz and Mangelsdorf, 2003). Genes regulated by the LXRs are involved in sterol absorption in the intestine, the reverse cholesterol transport process, bile acid synthesis, biliary neutral sterol secretion, hepatic lipogenesis, and synthesis of nascent high-density lipoproteins (Li and Glass, 2004; Tontonoz and Mangelsdorf, 2003). Furthermore, the LXRs modulate macrophage inflammatory functions (Zelcer and Tontonoz, 2006).

The cellular machinery for cholesterol biosynthesis and uptake, as well as for fatty acid biosynthesis, is controlled by transcription factors named sterol regulatory element binding proteins (SREBPs) and their cholesterol-sensing accessory factor, the SREBP cleavage activating protein (SCAP)

(Horton *et al.*, 2002; Eberle *et al.*, 2004; Goldstein *et al.*, 2006). The SREBPs are synthesized as precursors anchored to ER membranes and form complexes with SCAP. When the cellular cholesterol level is low, SREBP–SCAP complexes move to the Golgi complex, where SREBPs undergo a two-step proteolytic processing to release the N-terminal fragment, a basic helix–loop–helix leucine zipper transcription factor. These fragments enter the nucleus and bind to sterol regulatory elements (SRE) in the promoter regions of a number of genes whose products mediate the synthesis of cholesterol and fatty acids. When sterol builds up in cells, SCAP senses cholesterol in the endoplasmic reticulum (ER) membranes and interacts with INSIG proteins, and as a result the SREBP–SCAP complex is retained in the ER (Yabe *et al.*, 2002; Yang *et al.*, 2002). The SREBP machinery of lipid homeostatic regulation is sensitive to both cholesterol and 25-OHC. While SCAP does not bind 25-OHC (Brown *et al.*, 2002; Adams *et al.*, 2004), the INSIG proteins were recently found to directly bind this oxysterol and to mediate the regulatory effect of 25-OHC on SREBP processing (Radhakrishnan *et al.*, 2007; Sun *et al.*, 2007).

1.3. Identification of oxysterol binding protein (OSBP)-related protein (ORP) families

During early studies of feedback inhibition of cholesterol synthesis in cultured cells, it was noted that oxygenated sterols such as 25-OHC were more than 50-fold more potent than cholesterol in reducing the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-controlling enzyme in cholesterol biosynthesis (Brown and Goldstein, 1974; Kandutsch and Chen, 1974; Kandutsch *et al.*, 1978). These studies prompted a search for protein factors that could mediate the effects of oxysterols on cellular lipid metabolism. Protein fractions with oxysterol binding activity were isolated from different sources (Beseme *et al.*, 1986, 1987; Defay *et al.*, 1982; Kandutsch *et al.*, 1977; Kandutsch and Shown, 1981; Kandutsch and Thompson, 1980). Taylor and Kandutsch identified a cytosolic OSBP whose sterol binding specificity correlated with the ability of these compounds to suppress the activity of HMG-CoA reductase, a rate-limiting enzyme in the mevalonate pathway of cholesterol biosynthesis (Taylor *et al.*, 1984; Taylor and Kandutsch, 1985). Therefore, OSBP was regarded as a potential candidate for mediating the effects of oxysterols on the transcriptional regulation of cellular cholesterol homeostasis.

The OSBP protein was purified (Dawson *et al.*, 1989a; Taylor *et al.*, 1989), and cDNAs were cloned from rabbit (Dawson *et al.*, 1989b) and humans (Levanon *et al.*, 1990). OSBP is a homodimeric cytoplasmic protein (Dawson *et al.*, 1989a; Ridgway *et al.*, 1992) that upon treatment of cells with 25-OHC is translocated from a cytosolic or vesicular compartment to membranes of the Golgi apparatus (Ridgway *et al.*, 1992). Discovery of the

SREBP (see Section 1.2), key transcriptional regulators of sterol, triglyceride, and phospholipid homeostasis (Hua *et al.*, 1993; Wang *et al.*, 1993; Yokoyama *et al.*, 1993), turned major interest in the field away from OSBP. Furthermore, LXRs (see Section 1.2) were identified as oxysterol-binding transcription factors with central roles in the control of lipid metabolism and the reverse cholesterol transport pathway (Janowski *et al.*, 1996; Lehmann *et al.*, 1997; Chawla *et al.*, 2001). Despite these discoveries, work aimed at characterizing in detail the function of OSBP continued, and novel interest in the topic has been evoked mainly due to (1) the identification of OSBP-related gene/protein families in eukaryotic organisms from yeast to humans, and (2) recent functional studies revealing clues for important roles of OSBP homologs in cellular lipid metabolism, vesicle transport, and cell signaling.

Proteins with sequence homology to the carboxy-terminal ligand binding domain of oxysterol binding protein (designated the OSBP-related ligand binding domain, ORD) are present in most eukaryotic organisms (Lehto and Olkkonen, 2003; Olkkonen, 2004). These proteins are called either ORP or OSBP-like proteins (OSBPL). In humans (Jaworski *et al.*, 2001; Lehto *et al.*, 2001) and mice (Anniss *et al.*, 2002) the gene family consists of 12 members, and extensive splice variation (see the NCBI database and Collier *et al.*, 2003) substantially increases the number of encoded protein products. The ORPs minimally comprise an ORD, but in mammals a majority of them carry an amino terminal extension that contains a pleckstrin homology (PH) domain (Fig. 7.1). The proteins consisting of an ORD only are here designated ''short ORPs,'' while those carrying a PH domain are called ''long ORPs.'' Each human tissue or cell type expresses a large number of different ORP mRNAs (Lehto *et al.*, 2001). Most ORP messages are expressed ubiquitously. However, there are marked quantitative differences in the tissue- and cell type-specific mRNA expression patterns of the family members, and alterations of ORP expression levels are reported to occur during cell differentiation processes (Gregorio-King *et al.*, 2001; Johansson *et al.*, 2003; Lehto *et al.*, 2004). The function of ORPs has mainly been investigated in mammalian cells and in the yeast *Saccharomyces cerevisiae* (Schulz and Prinz, 2007; Yan and Olkkonen, 2007). However, individual reports have also been published on ORP family members in *Drosophila melanogaster* (Alphey *et al.*, 1998), *Caenorhabditis elegans* (Sugawara *et al.*, 2001), *Dictyostelium discoideum* (Fukuzawa and Williams, 2002), the parasitic protist *Cryptosporidium parvum* (Zeng and Zhu, 2006), as well as in several plants (Avrova *et al.*, 2004; Skirpan *et al.*, 2006). The widespread presence of the gene family throughout the phylogenetic tree of eukaryotes, and the fact that even the unicellular *S. cerevisiae* has seven *OSBP homolog* (*OSH*) genes (Schmalix and Bandlow, 1994; Jiang *et al.*, 1994; Beh *et al.*, 2001), provides evidence for a fundamental function that originated very early in eukaryotic evolution.

Figure 7.1 The human ORP protein family. Domain structures of the major ORP variants are shown. The black brackets on the right delineate subfamilies formed by closely related proteins. PH (green), pleckstrin homology domain; D (light blue), dimerization motif defined for oxysterol binding protein; FFAT (black vertical line), two phenylalanines in a acidic tract motif; ORD (red), oxysterol binding protein-related ligand binding domain; ANK (pink), region containing ankyrin repeats; TM (orange), putative transmembrane segment. The yellow vertical line within the ORD represents the highly conserved "oxysterol binding protein fingerprint," EQVSHHPP, used to align the proteins. Variants containing both a PH domain and an ORD are denoted as long (L) and those truncated at the amino terminus, thus lacking the PH domain, as short (S). In the case of ORP3 (1) variants are full length, while (2) variants contain a short C-terminal sequence unrelated to the ORD (dark blue). Splice variation leading to minor changes in mRNA structure is found for several ORPs. Therefore, the amino acid scale given is not precise but rather is indicative.

2. Subcellular Distribution of the ORPs

2.1. The PH domain region and ankyrin repeats of long ORPs

To gain insight into ORP function, one of the main avenues of research has been the analysis of their subcellular localization and of the determinants that interact with specific components on organelle-limiting membranes. The ORPs are in principle cytosolic proteins, but several of them have been

shown to associate peripherally with specific subcellular membrane compartments. The Golgi targeting of OSBP (Ridgway *et al.*, 1992) was found to be specified by a PH domain in the N-terminal part of the protein (Lagace *et al.*, 1997; Levine and Munro, 1998). PH domains are also present in the N-terminal region of 10 ''long'' mammalian OSBP homologs (Fig. 7.1). The amino terminal extensions present in these ORPs contain predominant targeting information: ORP9, the Golgi complex (Wyles and Ridgway, 2004); ORP1L, late endosomes (Johansson *et al.*, 2003, 2005); and ORP3, 6, and 7, plasma membrane (Lehto *et al.*, 2004). It has been suggested that 25-OHC binding to the C-terminal domain of OSBP induces a conformational change that unmasks the PH domain, thus inducing a shift of the protein to Golgi membranes. An analogous 25-OHC-induced shift from cytosolic and ER distribution to the plasma membrane was reported for a chimeric ORP3:OSBP fusion protein carrying the ORD of OSBP (Lehto *et al.*, 2005), suggesting that a regulatory mechanism similar to that of OSBP may also be operational in other family members. The interaction of the OSBP PH domain with phosphatidylinositol-4-phosphate (PI-4-P) is crucial for targeting of this protein to the Golgi complex and essential for its function (Lagace *et al.*, 1997; Levine and Munro, 1998, 2002). Similarly, the PH domain of *S. cerevisiae* Osh1p displays Golgi targeting specificity and interacts with PI-4-P (Roy and Levine, 2004). In addition, the Golgi localization of the OSBP and Osh1p PH domains depends on ARF (Levine and Munro, 2002; Roy and Levine, 2004), small GTPases with key roles in transport vesicle formation (Godi *et al.*, 2004). When expressed as fragments detached from their protein context, the PH domains of several ORPs, unlike that of OSBP, can be targeted to a localization different from that of the full-length protein (Johansson *et al.*, 2003; Lehto *et al.*, 2005; Wyles and Ridgway, 2004). Thus, additional targeting information in determinants flanking the PH domain seems to play a role in the specific membrane association of several ''long'' ORP proteins.

The mammalian ORP1 long, ORP1L (Lehto *et al.*, 2001; Johansson *et al.*, 2003), and two of the long ORPs in *S. cerevisiae*, Osh1p and Osh2p (Schmalix and Bandlow, 1994; Beh *et al.*, 2001), have at their very N-terminus a region containing ankyrin repeats (ANK), motifs typically involved in protein–protein interactions (Sedgwick and Smerdon, 1999). In the case of ORP1L, the ANK region interacts with the GTP-bound active form of the late endosomal (LE) small GTPase Rab7, and plays an important role in targeting of ORP1L to these compartments (Johansson *et al.*, 2005). The ANK repeat region of yeast Osh1p was reported to target the protein to the nucleus–vacuole junction (NVJ; see Section 2.3) (Levine and Munro, 2001). Since Osh1p was shown to interact physically with the NVJ protein component Nvj1p (Kvam and Goldfarb, 2004), this protein is most likely recognized by the Osh1p ANK region. The data of Johansson *et al.* (2003)

suggest that the ORP1L ANK region and PH domain synergize in targeting the protein to LE. Apparently, the ANK repeat region is used to achieve specific membrane targeting of select ORP family members via protein–protein interactions.

2.2. Roles of the C-Terminal ORD and the FFAT motif

In the absence of bound ligand, the carboxy terminal ORDs appear to have negative regulatory impact on the targeting function of the amino terminal PH domain regions (Ridgway *et al.*, 1992; Johansson *et al.*, 2003; Lehto *et al.*, 2005). The short ORPs tend to show a more cytosolic distribution than their long counterparts (Johansson *et al.*, 2003; Laitinen *et al.*, 2002). However, the short ORPs also show affinity for membranes. This is most likely mediated by surface regions enriched in charged amino acid residues, which interact with acidic phospholipids in biological membranes (Hynynen *et al.*, 2005; Im *et al.*, 2005; Raychaudhuri *et al.*, 2006). In addition, eight of the mammalian ORPs (OSBP, ORP1, 2, 3, 4, 6, 7, and 9) carry, between the PH domain and the ORD, a sequence motif denoted FFAT (two phenylalanines in an acidic tract) with the consensus sequence EFFDAxE (Loewen *et al.*, 2003) (Fig. 7.1). The FFAT motif binds to VAMP-associated proteins (VAP), transmembrane proteins of the ER. Even though eight ORPs have an FFAT motif, ER targeting has been reported only for OSBP, ORP9, ORP3, ORP6, and ORP7. In OSBP, this localization is largely cryptic, and becomes readily detectable only in a mutant form of the protein (Wyles *et al.*, 2002). For ORP1, ORP2, and ORP4, no ER localization has been reported. ORP5 and ORP8 have a putative C-terminal transmembrane segment that most likely specifies ER targeting (D. Yan, M. Lehto, and V. M. Olkkonen, unpublished observations).

2.3. Models on ORP function based on the localization data

What could be the role of the ER targeting of ORPs through the FFAT motif? The ER receptors for the FFAT motifs, the VAP proteins, are suggested to act as ER docking sites for several proteins with functions in lipid metabolism, including the ORPs, Goodpasture antigen binding protein (GBPB)/ceramide transporter (CERT; Hanada *et al.*, 2003), the retinal degeneration B (rdgB)/Nir proteins (Amarilio *et al.*, 2005; Lev, 2004), and *S. cerevisiae* Opi1p, a transcriptional repressor of inositol synthesis. The association of Opi1p with a yeast VAP homolog, Scs2p, is regulated by the phospholipid composition of ER membranes (Loewen *et al.*, 2004). Phosphatidic acid (PA) retains Opi1p bound to Scs2p in the ER membranes, while the addition of inositol consumes the ER PA, resulting in the detachment and nuclear translocation of Opi1p. It is a tempting possibility

that the ORPs could also bind to VAP dependent on a lipid signal, such as the ER phospholipid composition or occupancy of the ORP ORD by a ligand, possibly oxysterol or cholesterol. These signals could induce the detachment of ORPs from the ER and to their movement to another compartment, specified by the targeting determinants in the amino terminal PH domain region (Fig. 7.3A). After executing a function at the non-ER target organelle, the ORP could undergo a conformational change and return to its docking site at the ER. This functional cycle could involve transfer of the bound ORD ligand between the two membranes, but the ligand could also have a mere signal function.

Junctions between ER membranes and those of several other organelles (termed ER junctions, ERJ) have been implicated in several vital cellular processes such as store-operated Ca^{2+} entry, excitation–contraction coupling in striated muscle, coupling of Ca^{2+} transport between the ER and mitochondria, and intercompartmental lipid transport (Levine, 2004).

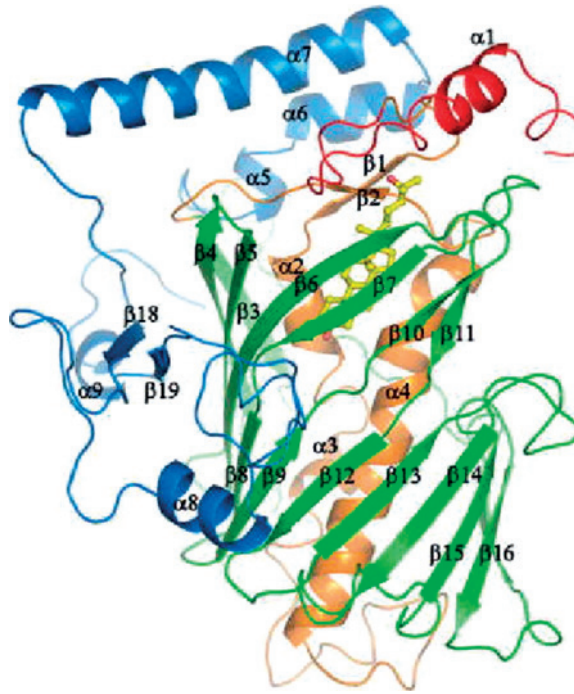

Figure 7.2 High-resolution structure of an ORP—*S. cerevisiae* Osh4p/Kes1p. The lid formed by the N-terminal part of the protein (residues 1 to 29) is shown in red, the central helices (30 to 116) in orange, the β-barrel (117 to 307) in green, and the C-terminal subdomain (308 to 434) in cyan. An oxysterol (25-OHC), with the 3β-hydroxyl group oriented toward the bottom of the binding pocket, is shown in yellow. (Reprinted by permission from Macmillan Publishers Ltd: Im *et al.*, Nature 437, 154–158, copyright 2005.)

Interestingly, *S. cerevisiae* Osh1p was reported to localize to the Golgi apparatus and an ERJ, the NVJ, a structure characteristic of this organism, while Osh2p and Osh3p were suggested to target ER–plasma membrane junctions (Levine and Munro, 2001; Loewen *et al.*, 2003). Prompted by these findings, a model was proposed in which ORPs interact simultaneously with ER and non-ER membranes via the FFAT motif and the amino terminal PH domain region, respectively (Fig. 7.3B). This model would predict a function of long ORPs in the generation of ERJ or in their activities (Levine, 2004; Olkkonen and Levine, 2004). However, Osh1p was shown not to be involved in the formation or stability of the NVJ, and its targeting to the NVJ was demonstrated to occur via a direct interaction with an NVJ protein component, Nvj1p (Kvam and Goldfarb, 2004). Moreover, the yeast Osh proteins are not required for the nonvesicular transport of phosphatidylserine to the sites at which the nonmitochondrial decarboxylase converts it into phosphatidylethanolamine (Routt *et al.*, 2005). Despite these findings undermining the ORP–ERJ hypothesis, the study of Kvam and Goldfarb (2004) demonstrates that the yeast Osh proteins are required for normal piecemeal microautophagy of the nucleus, an NVJ-mediated autophagic process that involves the blebbing of nonessential portions of the nucleus into invaginations of the vacuolar membrane.

Since the short mammalian ORP variants have a more cytosolic distribution than their long counterparts, they can be expected to be more mobile and to diffuse more freely through the cytosolic compartment than the long variants. Therefore, if ORPs serve as intercompartmental lipid carriers, the short ORPs are in our opinion the best candidates for this function (see Section 4.1; Fig. 7.3C).

3. Role of the Mammalian ORPs in Lipid Metabolism

3.1. Ligands of the mammalian ORP proteins

Identification of ORD ligands for the ORPs is crucial for the elucidation of their functions. Of the mammalian ORP proteins, OSBP and its closest homolog, ORP4 (also designated OSBP2 or HLM; Fournier *et al.*, 1999; Moreira *et al.*, 2001), were known to bind oxysterols (Moreira *et al.*, 2001; Taylor *et al.*, 1984; Wang *et al.*, 2002), and the ligands binding to the ORDs of the remaining family members have so far remained unknown. The ORDs of ORP1, ORP2, ORP9, and ORP10 were shown to bind phosphoinositides (PIPs) (Xu *et al.*, 2001; Fairn and McMaster, 2005a,b; Hynynen *et al.*, 2005), but it is unclear whether these interactions involve a pocket such as that found in yeast Osh4p/Kes1p (Im *et al.*, 2005), or if the findings rather reflect interactions of the negatively charged PIPs with

clusters of positively charged amino acid residues on the surface of the ORPs. A recent study employing purified recombinant proteins demonstrates that ORP1 and ORP2 bind 25-OHC and are thus true oxysterol binding proteins (Suchanek *et al.*, 2007). Moreover, we showed by molecular homology modeling and site-directed mutagenesis that ORP2 has a fold, with a distinct sterol binding pocket, similar to that described for *S. cerevisiae* Osh4p (Im *et al.*, 2005) (Fig. 7.3). Furthermore, live cell photo-cross-linking with [^{3}H]photo-25-OHC and [^{3}H]photo-cholesterol supported the *in vitro* data for ORP1 and ORP2 and suggested that another eight ORPs are also capable of sterol binding (Suchanek *et al.*, 2007). There is thus evidence suggesting that at least 11 members of the ORP family are able to bind sterols. However, the photo-cross-linking data have to be confirmed using purified proteins before a firm conclusion can be drawn. Moreover, it is possible that the physiological ligands of some ORP family members could represent lipid classes other than sterols. Interestingly, our recent experiments suggest that in addition to 25-OHC, ORP1L also binds 22(*R*)-OHC, while OSBP did not bind this oxysterol detectably (Yan *et al.*, 2007b). This indicates that there are differences in the oxysterol ligand specificity of the ORPs, a finding with crucial functional implications. We envision that differences in the ligand specificity and cell type-specific expression patterns of the ORPs and other proteins liganded by oxysterols (LXRs, INSIGs), as well as in the oxysterol content of distinct cell types, create a functional matrix resulting in a complex and fine-tuned reading-out of oxysterol signals.

3.2. The roles of OSBP in cholesterol and sphingomyelin metabolism

Of the mammalian ORP proteins, OSBP has been characterized most extensively. The protein is translocated from a cytosolic or vesicular/ER localization to the Golgi complex upon addition of its high-affinity ligand 25-OHC (Ridgway *et al.*, 1992). OSBP overexpression in Chinese hamster ovary (CHO) cells results in increased cholesterol biosynthesis and a reduction of cholesterol esterification, with consistent changes observed in the mRNAs for the key enzymes involved in these processes (Lagace *et al.*, 1997). Manipulations of the cellular sterol status were reported to affect the Golgi localization of OSBP, suggesting that the Golgi sterol content or the dynamic flux of cholesterol through the Golgi complex is sensed by OSBP (Ridgway *et al.*, 1998; Storey *et al.*, 1998; Mohammadi *et al.*, 2001). Furthermore, OSBP undergoes cholesterol-sensitive phosphorylation of specific serine residues, dephosphorylation accompanying the Golgi association of the protein. However, 25-OHC does not affect the phosphorylation status of OSBP, nor does phosphorylation affect the binding of 25-OHC by OSBP, indicating that the subcellular localization of the

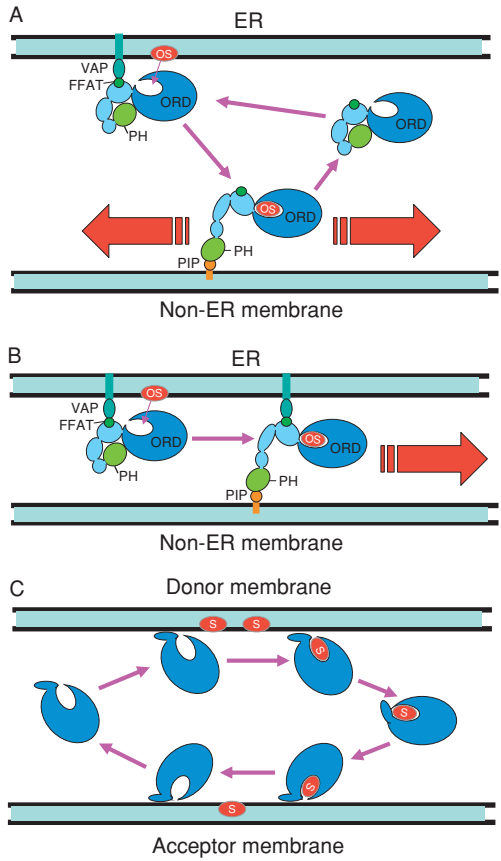

Figure 7.3 Models for ORP function. (A, B) Most of the long ORPs interact with VAMP-associated proteins (VAP) at the endoplasmic reticulum (ER) via a two phenylalanines in an acidic tract (FFAT) motif. Oxysterol (OS) binding to the oxysterol binding protein-related ligand binding domain (ORD) results in a conformational change that exposes the pleckstrin homology domain (PH). The PH domain interacts with phosphoinositides (PIP) in non-endoplasmic reticulum membranes. Oxysterol binding to the ORD and following phosphoinositide and protein–protein interactions induce signals (striped red arrows) that impact various cellular processes. The ORP may (A) shuttle between the endoplasmic reticulum and a non-endoplasmic reticulum compartment or (B) interact simultaneously between the two membranes at contact sites between the endoplasmic reticulum and other organelles. (C) The short ORPs may act as sterol carriers. The proteins interact with acidic membrane phospholipids, which facilitates sterol (S) loading. The bound sterol induces a change of lid conformation, leading to detachment of the ORP from the donor membrane. Unloading occurs at the acceptor membrane, followed by recycling of the protein to the donor compartment for another functional cycle. Adapted from Future Lipidology 2(1), 85–94 (2007) with permission of Future Medicine Ltd.

protein may be regulated by phosphorylation only in the absence of a "master" 25-OHC signal (Ridgway *et al.*, 1998). The mechanisms by which OSBP affects cholesterol metabolism, and whether these impacts are oxysterol dependent, has remained unclear. When added to cells as such, 25-OHC has effects on cholesterol homeostasis opposite to those of OSBP overexpression. Thus, OSBP and 25-OHC do not appear to act in a synergistic fashion. This is explained by the recent finding that 25-OHC regulates cellular cholesterol homeostasis through a direct interaction with the INSIGs, proteins that retain SREBP–SCAP complexes in the ER when sterols are abundant (Radhakrishnan *et al.*, 2007; Sun *et al.*, 2007) (see Section 1.2). The newly discovered role of INSIGs as 25-OHC receptors is in agreement with a study employing siRNA-mediated knock-down of OSBP to demonstrate that the endogenous OSBP in HeLa cells plays no role in the suppression of cholesterol biosynthesis by 25-OHC (Nishimura *et al.*, 2005).

Overexpression of OSBP in CHO cells in the presence of 25-OHC was reported to enhance the synthesis of sphingomyelin (Lagace *et al.*, 1999). A mutant OSBP with an amino acid substitution in the PH domain displayed enhanced association with ER membranes and was found to arrest a fluorescent ceramide analogue at sites in the ER. This suggested that the function of OSBP may involve transport of ceramide from the ER to Golgi sites where sphingomyelin synthase is located (Wyles *et al.*, 2002). Convincing evidence for this hypothesis was provided by a recent study showing that the Golgi translocation and activation of ceramide transport protein, CERT (Hanada *et al.*, 2003), are abolished when OSBP expression in silenced through RNA interference (Perry and Ridgway, 2006). Prompted by these findings, it was proposed that OSBP acts as a sterol sensor whose function is to integrate, via regulation of CERT function, the cellular sterol status with sphingomyelin biosynthesis. It is well established that ceramide transport from the ER to the Golgi occurs largely via a nonvesicular mechanism (Funato and Riezman, 2001; Hanada *et al.*, 2003; Perry and Ridgway, 2005). The domain structure of CERT resembles that of OSBP: it has a C-terminal ligand binding domain (a START domain that binds ceramide), an FFAT motif, and a PH domain that interacts with PI-4-P. It would therefore be tempting to speculate that OSBP acts to promote the formation of ER–Golgi junctions and recruitment of CERT to these sites via dual interactions with both Golgi PI-4-P and ER VAP proteins. Another putative mechanism might be enhancement of PI-4-P synthesis on Golgi membranes by OSBP, leading to an increased affinity of CERT for this compartment. The study of Perry and Ridgway (2006) is not the sole evidence for a role of ORPs in integrating sterol and sphingolipid metabolism: in yeast *S. cerevisiae*, disruption of OSBP homolog genes has been shown to increase the cellular levels of certain sphingolipids

or to grant resistance to an inhibitor of sphingolipid biosynthesis (Daum *et al.*, 1999; Yano *et al.*, 2004).

3.3. OSBP and hepatic lipogenesis

In a recent study (Yan *et al.*, 2007a), we showed that adenoviral overexpression of rabbit OSBP in mouse liver leads to an increase of plasma very-low-density lipoprotein (VLDL) and liver tissue triglycerides (TG). The increase of plasma TG was attributed to an increase of hepatic TG secretion. Investigation of the underlying mechanism revealed upregulation of SREBP-1c expression and an increase of the active nuclear form of the lipogenic transcription factor in the OSBP-transduced liver. Importantly, we also showed that silencing of OSBP in cultured hepatocytes attenuated the insulin induction of SREBP-1c and fatty acid synthetase (FAS), as well as TG synthesis, in cultured mouse hepatocytes. Furthermore, OSBP overexpression was shown to inhibit phosphorylation of the extracellular signal-regulated kinases (ERK) in both cultured hepatocytes and in live animals. Changes in ERK activity were reported to have an impact on the stability of nuclear SREBP-1c (Botolin *et al.*, 2006), providing one putative mechanistic explanation of the OSBP overexpression phenotype. The findings of this study demonstrate a new role of OSBP as a regulator of TG metabolism and suggest its involvement in the insulin signaling cascades that control hepatic lipogenesis.

3.4. The involvement of mammalian OSBP homologs in cellular lipid metabolism

Our understanding of the functions of the OSBP homologs in mammals is still in its infancy. The closest relative of OSBP, ORP4/OSBP2, exists as two major variants, ORP4L (long) and ORP4S (short) (Wang *et al.*, 2002). Like OSBP, ORP4 was shown to bind the oxysterols 25-OHC and 7-ketocholesterol (Moreira *et al.*, 2001; Wang *et al.*, 2002). Both ORP4S and ORP4L were reported to localize on vimentin intermediate filaments in CHO cells (Wyles *et al.*, 2007). Unlike OSBP, the subcellular localization of the ORP4 variants was not affected by treatment of cells with 25-OHC. Interestingly, ORP4S overexpression induced abnormal bundling/aggregation of the vimentin filaments and significant inhibition of the esterification of low-density lipprotein (LDL)-derived cholesterol, indicative of a functional role of this protein in cholesterol transport to the ER (Wang *et al.*, 2002). Accordingly, it was suggested that ORP4 uses vimentin filaments as a scaffold or tracks for transport of cholesterol or regulatory oxysterols between the endocytic compartments and the ER (Wyles *et al.*, 2007). Furthermore, evidence was provided that ORP4L heterodimerizes with OSBP, an interesting finding that may have important functional

implications, considering that each cell type expresses a large number of different ORPs simultaneously.

There is increasing evidence for roles of the closely related ORP1 and ORP2 in cellular lipid metabolism. The long variant of ORP1, ORP1L, localizes to late endocytic compartments (LE), while ORP1S is cytosolic (Johansson *et al.*, 2003). The ankyrin repeat region at the N-terminus of ORP1L mediates interaction with the late endosomal GTPase Rab7 (Johansson *et al.*, 2005). Overexpression of ORP1L or the ANK region leads to enhanced recruitment of microtubule-dependent dynein/dynactin motor complexes on LE and clustering of the endosomes in the juxtanuclear region. Furthermore, ORP1L overexpression was reported to induce autophagy (Johansson *et al.*, 2005). Since the ORD of ORP1L binds both PIPs (Fairn and McMaster, 2005a) and sterols (Suchanek *et al.*, 2007), and the PH domain interacts with PIPs (Johansson *et al.*, 2005), ORP1L can be envisioned to act as a lipid sensor that in complex with Rab7 and its other effector protein RILP (Johansson *et al.*, 2007) modulates the motility and/or distribution of LE according to lipid cues. Our latest work on the role of macrophage ORP1L in the development of atherosclerosis (see Section 3.5) provides compelling evidence for a functional role of ORP1L in macrophage sterol metabolism (Yan *et al.*, 2007b).

Overexpression of ORP2, a short human ORP, in CHO or HeLa cells results in an upregulation of cellular cholesterol efflux to all acceptors (Laitinen *et al.*, 2002; Hynynen *et al.*, 2005). Furthermore, the transport of newly synthesized cholesterol from the ER to the cell surface was enhanced by an excess of ORP2 (Hynynen *et al.*, 2005). Cholesterol esterification and ACAT activity were significantly reduced in the CHO cell model, an effect not detected in the HeLa cells with inducible ORP2 overexpression. The HeLa cells expressing ORP2 also showed, obviously as a homeostatic response to cholesterol loss, upregulation of LDL receptor expression and LDL uptake, as well as increased HMG-CoA reductase activity (Hynynen *et al.*, 2005). These results were consistent with enhancement of intracellular cholesterol transport by ORP2. The mechanism underlying this function is so far unclear, and we do not know whether ORP2 binds only oxysterols or also cholesterol. However, it is possible that ORP2 could, in analogy with yeast Osh4p (Raychaudhuri *et al.*, 2006) (see Section 4.1), transport sterols between subcellular membrane compartments. Interestingly, we also observed in the ORP2 expressing CHO cells a defect in neutral lipid (both triglyceride and cholesterol ester) storage, associated with altered phospholipid fatty acid composition especially under conditions of lipoprotein starvation (Käkelä *et al.*, 2005). This suggests that ORP2 may also act to regulate neutral lipid metabolism via a yet unidentified mechanism. In addition to the effects on cholesterol and neutral lipid metabolism, ORP2 overexpression has also been reported to result in disturbances of vesicle transport from the Golgi complex (Xu *et al.*, 2001;

Laitinen *et al.*, 2002). The underlying mechanism is unclear, but it may involve alterations in the lipid composition or organization of Golgi membranes.

We find it likely that most of the ORPs are able to bind sterols (Suchanek *et al.*, 2007). Therefore, an excess of any of these proteins in cells could cause disturbances of sterol homeostatic control, even if their true function were to act as lipid sensors impacting on cellular processes other than sterol metabolism itself. Furthermore, a number of ORPs associate with the ER via interaction with VAPs or through carboxy terminal membrane-spanning segments, and their overexpression causes distortions of the structure and function of the ER (Wyles and Ridgway, 2004; Lehto *et al.*, 2005), which harbors major enzymatic and regulatory machineries responsible for maintaining cellular lipid homeostasis. Therefore, to obtain reliable information on the physiological role of mammalian ORPs, RNA interference studies in cell models and gene-deficient animal models are absolutely necessary.

3.5. Putative connections between the ORP, the LXR, and the SREBP

It is an intriguing possibility that ORPs could modulate the access of oxysterols to the LXRs or the INSIGs. However, the evidence for this type of connection is at the moment scarce. Overexpression of ORP1L but not ORP1S was found to enhance the LXR-mediated transactivation of a reporter gene, dependent on the presence of LXR agonist, either 22(*R*)-OHC or a synthetic nonsterol agonist (Johansson *et al.*, 2003). However, the mechanism underlying this effect remained unclear. In a recent study (Yan *et al.*, 2007b), we showed that macrophage ORP1L overexpression in LDL receptor-deficient mice increased the size of atherosclerotic lesions. The transgenic macrophages were shown to display a defect in cholesterol efflux to spherical high-density lipoproteins (HDL) and reduced expression of ATP-binding cassette transporter G1 (ABCG1) and apolipoprotein E, as well as increased expression of phospholipid transfer protein (PLTP). All these genes are subject to transcriptional regulation by the LXR. Furthermore, ORP1L overexpression in cultured mouse macrophages was shown to attenuate the response of the ABCG1 mRNA to the LXR agonist 22(*R*)-OHC, which was also shown to be a ligand of ORP1L. One possible interpretation of the results is that ORP1L modulates LXR–ligand interactions, thereby affecting the expression of LXR target genes and the development of atherosclerosis. However, we find it equally probable that other, more indirect mechanisms may account for the observed phenotypic effect. Regarding the regulation of SREBP maturation, it is a tempting possibility that a member(s) of the ORP family could modulate the access of oxysterol ligands to the INSIGs. The potential functional connections

between the ORP and the LXR and SREBP systems of lipid homeostatic control are an attractive topic for future investigations, relevant for the mechanisms underlying the development of dyslipidemias and atherosclerosis.

4. The Yeast *S. cerevisiae* ORPs

4.1. Role of yeast osh proteins in sterol metabolism

Sterol homeostasis in *S. cerevisiae* shares a number of similarities with that in mammalian cells (Henneberry and Sturley, 2005). In yeast, the predominant sterol is ergosterol, the structure of which differs only slightly from cholesterol: it has two additional double bonds and a methyl group. Most of the ergosterol synthetic enzymes are localized in the ER, but ergosterol concentrates at the plasma membrane (Zinser *et al.*, 1991), similar to cholesterol in mammalian cells (Maxfield and Wustner, 2002; Soccio and Breslow, 2004). Under anaerobic conditions yeast sterol biosynthesis is inhibited and the cells are able to take up sterol from the growth medium. Sterol uptake can also be achieved in specific genetic setups (Schulz and Prinz, 2007). The sterol taken up at the plasma membrane can be transported to the ER for esterification, via a mechanism that involves ATP-binding cassette transporters, relatives of the transporters that mediate lipid efflux from mammalian cells (Alimardani *et al.*, 2004; Wilcox *et al.*, 2002). Sterol transport between the yeast ER and plasma membrane occurs via nonvesicular mechanisms (Baumann *et al.*, 2005; Li and Prinz, 2004), even though it is possible that a yet unknown Sec18p-independent vesicle transport mechanism may account for part of the transport (Schulz and Prinz, 2007). Importantly, *S. cerevisiae* lacks homologs of the putative sterol carriers found in mammalian cells, START domain-containing proteins (Alpy and Tomasetto, 2005), sterol carrier protein 2 (SCP2; Atshaves *et al.*, 2003; Puglielli *et al.*, 1995; Vila *et al.*, 2004), and caveolins (Smart *et al.*, 2004; Uittenbogaard *et al.*, 1998, 2002). Yeast has, however, seven ORPs, Osh1p–Osh7p, three of which (Osh1–3p) belong to the category of long ORPs and four of which (Osh4–7p) are of the short subtype (Beh *et al.*, 2001). The first ORP high-resolution structure, that of a short yeast ORP, Osh4p/Kes1p, revealed that Osh4p is a sterol-binding protein (Im *et al.*, 2005). It was crystallized in complex with five different sterols, and has a sterol binding pocket formed by 19 β-strands in an antiparallel arrangement (Fig. 7.2). The sheet bends to an almost complete roll that is, in the presence of bound ligand, closed by a lid containing an amphipathic α-helix connected by a flexible linker. Sterols bind within the pocket oriented with the 3β-hydroxyl group at the bottom of the hydrophobic binding tunnel. The sterol side chain interacts with the lid, stabilizing its closed

conformation. Importantly, many of the interactions of the bound sterol are mediated via water molecules within the pocket, giving the ligand interaction substantial flexibility. This explains the ability of the pocket to accommodate structurally different sterols, and possibly also other types of lipid ligands in a lid-open conformation. The structure of Osh4p suggested that this protein and its homologs might act as sterol transporters and possibly also as mediators of sterol signals (Im *et al.*, 2005).

Even before solution of the Osh4p structure, several genetic and biochemical studies provided convincing evidence that the yeast Osh proteins are involved in sterol metabolism. Jiang *et al.* (1994) investigated strains mutant for *OSH1, OSH4/KES1*, and *OSH5/HES1*. They found in double or triple mutants pleiotropic sterol-related phenotypes, including tryptophan transport defects and nystatin resistance, as well as mild reductions of membrane ergosterol levels. Beh *et al.* (2001) carried out an exhaustive study in which the phenotypic effects of all 127 permutations of *OSH* deletion alleles were determined. The results demonstrated that the individual *OSH* genes were not essential, but deletion of all seven was lethal, suggesting that the genes together share a function essential for viability. The viable combinations of *OSH* deletions displayed distinct sterol-related defects, and depletion of all seven proteins resulted in cellular sterol accumulation, evidence for a disturbance of sterol homeostatic control. Beh and Rine (2004) reported that elimination of *OSH* function resulted in a redistribution of ergosterol from the plasma membrane to intracellular locations, vacuolar fragmentation, and cellular accumulation of lipid droplets. Moreover, *OSH* defects caused disturbances of endocytosis, cell budding, and cell wall deposition. These findings suggest that function of the yeast Osh proteins involves the subcellular sterol distribution, the other phenotypic features possibly being secondary to this. In accordance with this notion, Raychaudhuri *et al.* (2006) presented evidence for function of the Osh proteins (Osh4p, Osh5p, and Osh3p) in sterol transport from the yeast plasma membrane to the esterification compartment. Consistent with the *in vivo* findings, it was shown that Osh4p is capable of transferring cholesterol and ergosterol from donor to acceptor vesicles *in vitro*. The sterol transfer was shown to take place more rapidly between membranes that contain PIPs, suggesting that interactions of ORPs with the negatively charged PIP headgroups on membrane surfaces significantly facilitate the sterol transport function. Therefore, in addition, the interactions of several short mammalian ORPs with PIPs (Fairn and McMaster, 2005a,b; Hynynen *et al.*, 2005) may play an important role in a putative function as intercompartmental lipid carriers.

Studies by the group of H. Yang show that the function of *S. cerevisiae* Osh6p and Osh7p involves sterol metabolism, and that the association of

these proteins with cellular membranes is regulated by the AAA family ATPase Vps4p (Wang *et al.*, 2005a,b). The C-terminal coiled-coil domain of Osh7p was shown to determine the interaction with Vps4p. It was also shown that deletion of *VPS4* or *OSH6–OSH7* double deletion resulted in a defect of sterol ester synthesis in the presence of normal triglyceride synthesis, and that Osh7p overexpression partially replenished sterol esterification in the *vps4*Δ strain. These findings indicate that the deletions did not impair fatty acid uptake by the yeast cells, but caused a specific defect in sterol esterification, probably in sterol transport to the ER. Thus, Osh6p and Osh7p could act as sterol transfer proteins, and Vps4p catalyzes their dissociation from membranes as an essential part of their functional cycle. Alternatively, Os6p and Osh7p could regulate the activity of Vps4p. Since Vps4p also acts to dissociate the ESCRT III complex responsible for sorting of cargo proteins to the multivesicular body (Babst *et al.*, 2002a,b), Osh6p and Osh7p could via Vps4p relay information from lipid cues to the control of endosomal sorting/membrane trafficking. Furthermore, the finding that Vps4p is required for the correct sorting of Ncr1p (Zhang *et al.*, 2004), a yeast homolog of the mammalian Niemann–Pick C-1 (NPC1) protein, suggests that there may be a functional connection between the ORPs and the NPC proteins in the transport of sterols out of the endosomal compartments (Yang, 2006).

As a conclusion, the current evidence suggests that in yeast, the short ORPs (Osh4–Osh7p) have a function in the nonvesicular transport of sterols. They are likely to act as sterol carriers, even though other modes of action cannot be excluded. However, trafficking of sterols may not be the only function of yeast ORPs. A recent study demonstrated that the Osh proteins also play important roles in yeast cell polarization by maintaining the proper subcellular localization of septins, the Rho GTPases Cdc42p and Rho1p, and the Rab GTPase Sec4p (Kozminski *et al.*, 2006). Furthermore, Osh3p was suggested to regulate nuclear fusion during yeast mating (Park *et al.*, 2002), and to play a role in the control of pseudohyphal growth of *S. cerevisiae* and *Candida albicans* under nitrogen starvation (Hur *et al.*, 2006). Moreover, a function of Osh4p as a regulator of post-Golgi secretory vesicle transport is well established (see Section 4.2).

4.2. Osh4p regulates post-golgi secretory vesicle transport

Osh4p acts as a negative regulator of Golgi secretory function. Fang *et al.* (1996) demonstrated that deletion of *OSH4* leads to by-pass of the temperature sensitivity of mutants in *SEC14*, a gene encoding a phosphatidylinositol transfer protein (PITP; Sec14p) essential for secretory vesicle biogenesis (Bankaitis *et al.*, 2005). Disruption of the other yeast *OSH* genes fails to produce this by-pass phenotype. Sec14p is thought to maintain a membrane

composition permissive to Gcs1p, a GTPase-activating protein (GAP) for Arf1, a small GTPase with a central role in transport vesicle formation (Yanagisawa *et al.*, 2002). This function most likely involves promotion of diacylglycerol (DAG) formation at the expense of phosphatidylcholine. Even though the precise function of Osh4p in this context is poorly understood, it was suggested that it exerts its effect via regulation of Arf1 activity (Li *et al.*, 2002). A recent study sheds further light on the dilemma: Osh4p was shown to carry, in the lid of its sterol binding pocket, a specific type of amphipathic helix, which inserts into membrane domains with high curvature (Drin *et al.*, 2007). Gcs1p inactivates Arf1 bound to curved membranes or ones with conical lipids such as DAG (Antonny *et al.*, 1997; Bigay *et al.*, 2005). Therefore, Osh4p could counterbalance Sec14p activity by locally modifying the membrane sterol content and/or lipid organization, to modulate Gcs1p recruitment (Drin *et al.*, 2007). Analysis of site-specific *osh4* mutants revealed no clear correlation between sterol binding capacity and the ability to inhibit Golgi-derived vesicular transport (Im *et al.*, 2005). Therefore, the precise relationship between the functions of Osh4p in post-Golgi membrane trafficking and in sterol transport is as yet unclear. Of the mammalian ORPs, ORP1S and ORP9S, but not ORP2 or the ORP10 ORD, were shown to be capable of functionally replacing Osh4p/Kes1p (Xu *et al.*, 2001; Fairn and McMaster, 2005b). The difference between ORP9S and ORP10 was suggested to be due to different PIP binding specificity (Fairn and McMaster, 2005b).

5. Role of ORPs in Cell Signaling

Alphey *et al.* (1998) identified one of the *Drosophila* ORPs, designated OSBP-Dm, by its ability to overcome the cell cycle arrest induced by Wee1p overexpression in fission yeast. *Drosophila* and *C. elegans* are unable to synthesize the four-ring sterol structure and are dependent on dietary sterol supply. Therefore, it was reasoned that the function of OSBP-Dm is not in the regulation of sterol biosynthesis, but the protein has another type of function that involves cell signaling and/or cell cycle control. The impact of OSBP-Dm on the cell cycle may, however, be very indirect, and it cannot be excluded that, for example, modulation of intracellular sterol transport could lead to the observed phenotypic effects. In *Dictyostelium*, an ORP designated OSBPa was reported to be involved in the regulation of the slug-fruiting body switch (Fukuzawa and Williams, 2002). The mechanism through which the ORP impacts this process, however, remained unclear.

More convincing evidence for a signaling function of an ORP was provided by the study of Sugawara *et al.* (2001), who identified a

C. elegans ORP, designated BRAM-interacting protein, BIP, as a modulator of transforming growth factor (TGF)-β signaling. They carried out a two-hybrid screen using BMP receptor-associated protein (BRAM) as a bait, and identified a *Xenopus* ORP (BIP) as a BRAM binding partner. They then isolated the *C. elegans* homolog of this cDNA, showed that it interacts with the *C. elegans* BRAM homologs BRA-1 and BRA-2, and demonstrated that inhibition of BIP expression by RNA interference produces an Sma phenotype characteristic of disturbance of the *C. elegans* TGF-β pathway that regulates body length. In this case, the documented interaction of the ORP (BIP) with the BRA proteins is evidence of a direct role as a modulator of the signaling pathway. It will also be of interest to assess whether one or several of the mammalian ORPs could play a role in the TGF-β signaling cascades.

Recently, Skirpan *et al.* (2006) identified and characterized a *Petunia inflata* ORP called PiORP1, which interacts with the kinase domain of a receptor-like kinase PRK1. Moreover, PiORP1 was shown to be phosphorylated by PRK1. Since PRK1 activity is essential for pollen development and plays a role in pollen tube growth, PiORP1 may be involved in PRK-1-mediated signaling in pollen.

In mammalian cells, there is increasing evidence for roles of ORPs in cell signaling. Wang *et al.* (2005c) identified OSBP as a sterol-sensing scaffolding factor that regulates the dephosphorylation and hence the activity of ERK, key components of the mitogen-activated protein kinase (MAPK) signaling pathways (Zebisch *et al.*, 2007). This work suggests that OSBP binds both cholesterol and 25-OHC, and that the cholesterol-bound state scaffolds a protein phosphatase complex (PP2A serine/threonine phosphatase and PTPPBS tyrosine phosphatase) that dephosphorylates and thereby inactivates ERK. Reduction of the cellular cholesterol content or addition of 25-OHC dissociated the phosphatase complex acting on ERK, leading to hyperphosphorylation of the kinases. An important implication of these findings is that other members of the ORP family could have lipid-specific scaffolding functions that control signaling pathways. In support of this idea, Lessmann *et al.* (2007) demonstrated that ORP9 contains a phosphoinositide-dependent kinase-2 (PDK-2) phosphorylation site, the phosphorylation of which was dependent on PKC-β and mTOR in bone marrow-derived mast cells. They also provided RNA interference and immunoprecipitation evidence that ORP9 interacts with these kinases to negatively regulate phosphorylation of the PKD-2 site at Akt/protein kinase B, a major controller of cell survival, cell cycle progression, and glucose metabolism (Hanada *et al.*, 2004). Interestingly, ORP3 and ORP7 were recently found to interact physically with R-Ras, a small GTPase that regulates cell adhesion and migration (Goldfinger *et al.*, 2007; Kinbara *et al.*, 2003), implying a functional role of these ORPs in Ras signaling.

As a number of ORPs have been shown to bind lipids, several of them both PIPs and sterols, one can envision that these proteins may act as lipid sensors that integrate information from the cellular lipid status with the function of signaling cascades. In addition to the type of regulatory mechanism suggested for OSBP (Wang *et al.*, 2005c), such a function could be executed by regulating the distribution of signaling complexes in lipid microdomains, for example, ones enriched in PIPs or sterols. Furthermore, ORPs may impact signaling through effects on the vesicle transport and subcellular localization of signaling proteins. Elucidation of the connections between the ORPs and the pathways of cell signaling is a central future task that may lead to important medical applications, especially since there are a number of studies reporting an association of altered ORP expression levels with malignant cell phenotypes (Fournier *et al.*, 1999; Difilippantonio *et al.*, 2003; Henshall *et al.*, 2003; Jelinek *et al.*, 2003; Almstrup *et al.*, 2004; Pizzatti *et al.*, 2006).

6. Future Perspectives

The current information on ORP function in different organisms is summarized in Table 7.1. This information is still fragmentary, and our understanding of the mechanisms through which the ORPs impact the various cellular processes is in its infancy. Mammals have a large number of ORPs, most of which belong to the long subtype. In these proteins the ORD acts as one domain in a complex structure. We envision that during evolution, the functions of the ORP ligand binding domain (ORD) have diverged from an ancestral one in mediating sterol transport to meet the increasing demand of regulatory potential in multicellular organisms. Interest in the protein family is constantly expanding, and the groundbreaking studies published in the past few years have paved the way for creating new functional hypotheses. Testing these hypotheses in cultured cell set-ups and in animal models, as well as in nonvertebrate systems readily amenable for genetic manipulation, will in the near future increase our understanding of the physiological role of ORPs. ORP gene silencing in cultured cells and live animals, as well as gene-deficient animal models, will be instrumental in reaching this goal. It is likely that many central functions of the ORPs will turn out to involve regulation of cellular and body lipid metabolism. However, lipid homeostasis must be integrated with a number of other regimes. It is therefore likely that numerous new functional connections of the ORPs with the control of intracellular vesicle transport, cell differentiation, proliferation, polarity, adhesion, migration, and survival/death will be discovered.

Table 7.1 Suggested ORP Functions

Organism	Protein	Suggested function	References
Mammals	OSBP	Sterol-dependent regulation of ERK dephosphorylation and sphingomyelin synthesis; modulation of insulin signaling and hepatic lipogenesis	Wang *et al.*, 2005c; Perry and Ridgway, 2006; Yan *et al.*, 2007a
	ORP1L	Motility and distribution of late endosomes; autophagy; macrophage lipid metabolism	Johansson *et al.*, 2003, 2005, 2007; Yan *et al.*, 2007b
	ORP1S	Vesicle transport from Golgi	Xu *et al.*, 2001
	ORP2	Vesicle transport from Golgi; sterol transport; neutral lipid metabolism	Xu *et al.*, 2001; Laitinen *et al.*, 2002; Hynynen *et al.*, 2005; Käkelä *et al.*, 2005
	ORP4	Vimentin-dependent sterol transport and/or signaling	Wang *et al.*, 2002; Wyles *et al.*, 2007
	ORP9	Regulation of Akt phosphorylation	Lessmann *et al.*, 2007
Drosophila melanogaster	OSBP-Dm (CG6708)	Cell cycle control	Alphey *et al.*, 1998
Caenorhabditis elegans	BIP (obr-3)	Modulation of TGF-β signaling	Sugawara *et al.*, 2001
Dictyostelium discoideum	OSBPa	Regulation of slug-fruiting body switch	Fukuzawa and Williams, 2002
Cryptosporidium parvum	CpORP1	Lipid transport across the parasitophorous vacuole membrane	Zeng and Zhu, 2006
Solanum tuberosum	StOBP1	Function in a nonspecific defense pathway	Avrova *et al.*, 2004
Petunia inflata	PiORP1	Pollen development	Skirpan *et al.*, 2006

(*continued*)

Table 7.1 (*continued*)

Organism	Protein	Suggested function	References
Saccharomyces cerevisiae	Osh1p	Postsynthetic sterol regulation; piecemeal microautophagy of the nucleus; cell polarity establishment	Jiang *et al.*, 1994; Beh *et al.*, 2001; Kvam and Goldfarb, 2004; Kozminski *et al.*, 2006
	Osh2p	Sterol metabolism; cell polarity establishment	Daum *et al.*, 1999; Beh *et al.*, 2001; Kozminski *et al.*, 2006
	Osh3p	Sterol transport, regulation of nuclear fusion during mating and of pseudohyphal growth; sphingolipid metabolism	Raychaudhuri *et al.*, 2006;Park *et al.*, 2002; Hur *et al.*, 2006; Yano *et al.*, 2004
	Osh4p/ Kes1p	Sterol transport and metabolism; post-Golgi vesicle transport; cell polarity establishment	Jiang *et al.*, 1994; Raychaudhuri *et al.*, 2006; Fang *et al.*, 1996; Li *et al.*, 2002; Kozminski *et al.*, 2006
	Osh5p	Sterol transport and metabolism	Jiang *et al.*, 1994; Raychaudhuri *et al.*, 2006
	Osh6p	Sterol transport; regulation of Vps4p function; cell polarity establishment	Wang *et al.*, 2005a,b; Kozminski *et al.*, 2006
	Osh7p	Sterol transport; regulation of Vps4p function	Wang *et al.*, 2005b
Candida albicans	Osh3	Regulation of pseudohyphal growth	Hur *et al.*, 2006

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