

# Chapter 4

## The Role of the Cytoskeleton in Cell Migration, Its Influence on Stem Cells and the Special Role of GFAP in Glial Functions

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## Abbreviations

AD	Alzheimer's disease
Arp2/3	Actin-related proteins 2/3
bFGF	Basic fibroblast growth factor
CCL	C-C motif chemokine
CCR7	C-C chemokine receptor type 7
CDC42	Cell division control protein 42 homolog
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSF	Cerebrospinal fluid
CX3CR1	CX3C chemokine receptor 1
CXCR	C-X-C chemokine receptor
DOCK180	Dedicator of cytokinesis protein
ECM	Extracellular matrix
ELMO1	Engulfment and cell-motility protein 1
ERK	Extracellular signal-regulated protein kinase
ERM	Ezrin/Radixin/Moesin
FAK	Focal adhesion kinase
G proteins	guanine nucleotide-binding proteins
GABA	Gamma amino butyric acid
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GPCRs	G-protein-coupled receptors

HD1	<i>Hemidesmosomal 1 protein</i>
hMSC	Human mesenchymal stem cells
HSPC	Hematopoietic stem/progenitor cell
IF	Intermediate filament
JAK	Janus kinase
MAPKs	Mitogen-activated protein kinases
mDial1	Diaphanous homolog 1 of <i>Drosophila</i>
MFs	Microfilaments
MLC	Myosin light chain
MSCs	Mesenchymal stem cells
MTs	Microtubules
NSCs	Neural stem cells
PBSF	Pre-B cell growth-stimulating factor = SDF1, stromal cell-derived factor 1
PKB	Protein kinase B
PNS	Peripheral nervous system
RAC1	Ras-related C3 botulinum toxin substrate 1
RDC-1	G protein-coupled receptor = CXCL12
RNAi	RNA interference
ROCK	Rho-associated coiled-coil forming protein kinases
SDF-1	Stromal cell-derived factor-1= CXCL12
STAT3	Signal transducer and activator of transcription 3
TGFβ1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
VASP	Vasodilator-stimulated phosphoprotein
XCR1	C sub-family of chemokine receptors 1

## Introduction

The cytoskeleton is a dynamic network of proteins organized in fibrillar or globular filaments in the cell cytoplasm [1]. Three types of filaments are common to many eukaryotic cells: (1) intermediate filaments provide mechanical strength and resistance to shear stress; (2) microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport; and (3) actin filaments determine the shape of the cell's surface and are necessary for cell migration. Actin filaments also interact with accessory proteins that link them to other cellular components, as well as to each other [1].

This chapter initially addresses the role of the cytoskeleton in one of its main functions, cell migration. The dynamics and the mechanical aspects of the actin filaments are essential to this process, as are the signaling pathways induced by the chemokines and their receptors. We also give special attention to the cytoskeletal proteins of the stem cells, in the origin of cell functions. Finally, the chapter discusses the participation of the cytoskeleton in functions performed by different types of glial cells, focusing on the role of a particular intermediate filament, the glial fibrillary acidic protein (GFAP), in the health and disease.

## An Overview of Cell Migration

Cell migration has fascinated cell biologists, biochemists, and recently also physicists and mathematicians. This is not surprising, since it is an essential process that occurs during different stages and at different times, ranging from organism development to normal adult life and also during disease states [2–4].

Cells in multicellular organisms can move in different directions, through the extracellular matrix, over each other, or even between each other. Cells move in three basic steps: (1) extending the plasma membrane forward at the front, or leading edge, of the cell in a protrusion; (2) moving the cell body; and (3) retracting the rear part of the cell [5]. These steps involve two main cytoskeleton filaments, microtubules and actin filaments; the microtubules are required for polarization [6, 7] while the actin filaments are the main players during migration and protrusion formation [8].

Cells are able to extend four different types of protrusions at the leading edge, lamellipodia, filopodia, blebs, and invadopodia. All these structures have their own functions and contribute to cell migration in specific ways. Lamellipodia are able to extend long distances through the extracellular matrix, pulling cells through the tissues [2]. Filopodia explore the cell's surroundings [9, 10]. Membrane blebs help in cell migration during development [11], and invadopodia are protrusions that allow degradation of the extracellular matrix, and help cells to pass through tissues [12].

In the following parts of this section we discuss the mechanical aspects of the formation of a protrusive migratory structure called the lamellipodium, and how the plasma membrane regulates the behavior of this structure, as well as its influence during cell migration. We also discuss some chemokines that induce migratory processes followed by cytoskeletal changes.

### *Lamellipodium*

The thin protrusive region at the leading edge of migrating fibroblasts in culture was termed the “lamellipodium” by Abercrombie et al. [13]. Abercrombie et al. [14] showed that these structures contain actin filaments arranged in a branched structure, but not microtubules. First described in fibroblasts, lamellipodia have also been observed in many other cell types such as precursor cells, epithelial cells, and neural crest cells [2, 15].

For many years, a group of proteins called the Actin-Related Proteins 2/3 (Arp2/3) complex was thought to be the primary mediator of actin polymerization in lamellipodia. First described as a nucleator of actin polymerization [16], the Arp2/3 complex binds to actin filaments and induces the formation of branched actin networks [17]. Branched actin networks were also observed in electron-microscopy images of lamellipodia [18]. However, it is now known that the extent of actin filament branching can vary depending on the cell type and conditions, as a recent report found only a few branches in the leading edge of cells [19]. Not only

branching but also the balance of other known actin-binding proteins can contribute to the extension of the lamellipodium. For example, more capping protein activity reduces actin length and increases nucleation by Arp2/3 [20]. On the other hand, an increase in the expression of vasodilator-stimulated phosphoprotein (VASP) (a protein known to promote filament elongation) was reported to generate longer filaments [21, 22]. More recently, other actin nucleators were found to contribute to lamellipodial protrusion, including several members of the Formin family of proteins. Formins were described as protecting actin filaments from capping and also as promoting filament elongation without branching. One of these proteins, diaphanous homolog 1 of *Drosophila* (mDia1), was first reported to localize at the lamellipodia of migrating cells [23].

The final essential factor in cell movement is the plasma membrane. The lamellipodial protrusion will encounter the physical barrier imposed by the membrane, and this barrier will also restrict cell migration [24].

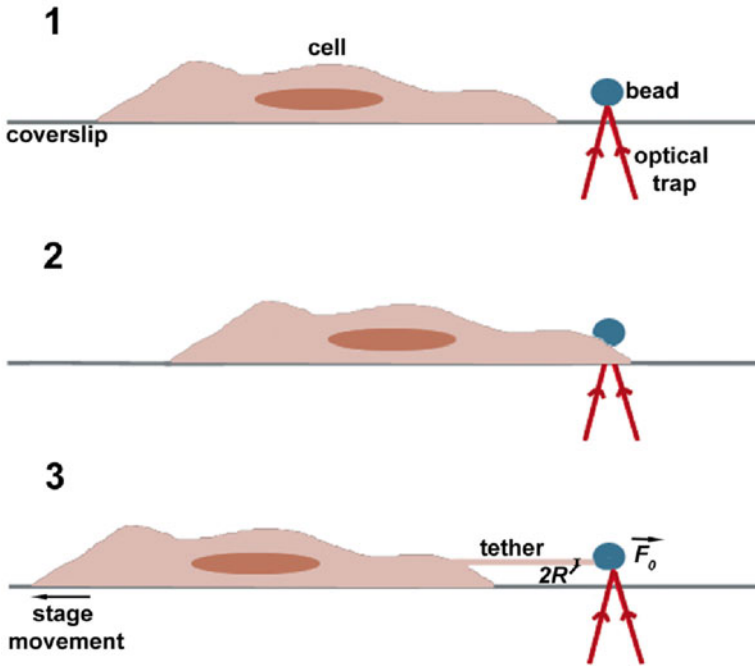
### ***Membrane Mechanical Properties Orchestrates Cell Migration***

The mechanical characteristics of the plasma membrane, particularly its plasma membrane tension and bending modulus, play central roles in cell motility and cytoskeleton remodeling [25–28].

A direct way to assess these responses to forces is by measuring two elastic parameters of the cell membrane: its bending modulus [28] and its membrane tension [29], using a technique based on extracting the membrane tether from the cell by pulling on it with an attached microsphere trapped in an optical tweezers [30]. The experimental procedure is illustrated in Fig. 4.1. Analysis of the force-extension curve, together with measurement of the tether radius, yield these two elastic parameters and also information regarding the membrane–cytoskeleton interaction [31]. Tether pulling with optically trapped beads is the only known direct method for these measurements [26].

The mechanical load exerted by the membrane at the leading edge of cells can locally influence the dynamic growth and organization of the actin network [25, 32–34]. The high membrane tension in the lamellipodia of motile cells directly influences the protrusion [35–37]. Simultaneously at the rear of the cell, the same membrane load can exert a pulling force that induces retraction [38, 39]. However, this mechanical load imposed by the membrane was also reported to be influenced by forces generated from the actin cytoskeletal protrusion itself [27, 31, 32, 40, 41].

One possibility is that the membrane mechanical parameters are determined primarily through a balance of forces between the cytoskeleton and the hydrostatic pressure acting on the membrane [29, 42]. Another possibility is that these parameters are controlled mainly by the interaction between the membrane and the cytoskeleton [31, 43]. Regardless of the exact mechanism, membrane mechanical properties have emerged as important regulators that coordinate local dynamics over cellular scales.



**Fig. 4.1** Schematic representation of a tether extraction experiment. Situation (1): a bead is trapped in an optical trap. Situation (2): a bead is placed against the cell surface. Situation (3): by moving the microscope stage, a membrane tether, with radius  $R$  and force  $F_0$ , is formed

Apart from these dynamic aspects, recent data also suggest that cell specialization and/or differentiation can account for the differences in the mechanical properties of the membrane, and that these differences are reflected in their specialized functions [44]. A question that remains unanswered is how the threshold value of these membrane parameters are set. The answer is still not clear, and may vary with different cell types. Pontes et al. [43] began to test this hypothesis by measuring the membrane tension and bending modulus for a variety of cells. These authors observed that the elastic parameters for neurons are close to those obtained for an isolated cell membrane (a membrane disconnected from the cytoskeleton), suggesting a weaker interaction between the membrane and the adjacent F-actin cortex in this cell type. They also observed that the parameters did not change within the different neuronal cell regions, i.e., the cell body, neurite and growth cone. They found very similar membrane mechanical parameters for astrocytes and glioblastoma cells, supporting the idea that these two cell types have the same origin and also share similar functions, for example giving support to neurons in the brain [45]. Macrophages and microglial cells have substantially higher values for the membrane mechanical parameters. When activated, these two phagocytic cells decrease their bending modulus by a factor of 3. This reduction can be interpreted as an easier way to bend the cell surface, which is advantageous during phagocytosis.

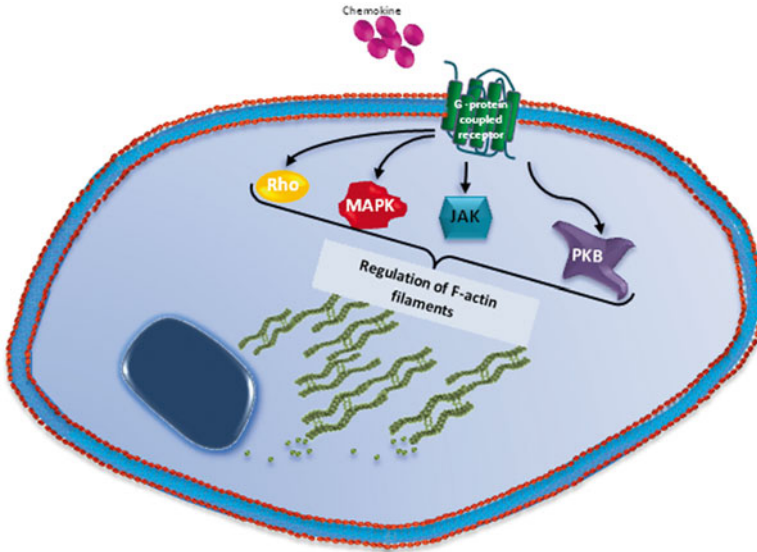
Taken together, these observations are striking examples which demonstrate that different cells performing different functions show different mechanical parameters. These new findings suggest the possibility of characterizing cells based not only on morphological and biochemical analyses, but now on their mechanical properties as well.

### ***Chemokines Induce Migratory Processes***

Chemokines are *chemotactic cytokines*, comprising a large superfamily of small peptides (approximately 8–17 kDa) that currently number 47 in humans [46, 47]. They can be classified according to their amino-acid structure into four groups, based on the variations of a conserved cysteine motif in the mature sequence of the proteins [48, 49]. Chemokines bind to the chemokine receptor subfamily of class A G-protein-coupled receptors (GPCRs), which comprises ten CCR family members, seven C-X-C chemokine receptors (CXCR) family members, the “C” sub-family of chemokine receptors 1 (XCR1) and CX3C chemokine receptor 1 (CX3CR1) [46, 50]. These GPCRs signal through heterotrimeric G-proteins, and regulate a diversity of signal transduction pathways involved in chemotaxis and cell survival.

Chemokines were first described for their role in chemotaxis and migration of leukocytes to lymphoid tissues and sites of injury, and the signaling pathways activated by their receptors lead to changes and reorganization within the cytoskeleton proteins. They also proved to be important in the development and homeostasis of the immune system and various other organs, and in pathophysiological processes associated with osteoporosis [51], obesity and insulin resistance [52], viral infections [53, 54], immune responses [55, 56], mobilization of progenitors to the bone marrow [57] and autoimmune encephalomyelitis [58]. More recently, chemokines emerged as key mediators of cancer progression, by interfering with the homing of cancer cells to metastatic sites and the recruitment of a number of different cell types to the tumor microenvironment, such as tumor-associated macrophages, tumor-associated neutrophils, lymphocytes, cancer-associated fibroblasts, myeloid-derived suppressor cells and endothelial cells [48, 49, 59, 60, 61].

Processing the chemokine gradients into migratory or adhesive responses occurs in multiple dynamic steps that regulate changes in the cytoskeleton and cellular adhesion [46, 62–65]. Binding of the chemokines to their G-coupled receptors can lead to downstream activation of different signaling pathways (Fig. 4.2), such as protein kinase B (PKB/Akt) and mitogen-activated protein kinases (MAPKs) [66, 67]. Another activated pathway may be the Janus kinase family (JAK), activated in a G $\alpha$ -independent fashion [68, 69]. The Rho family of GTPases and their downstream effectors were also implicated in chemokine-elicited migration. One of the important groups of Rho effectors is the Rho-associated coiled-coil forming protein kinases (ROCK) I and II, which enhance myosin light chain (MLC) phosphorylation by both inhibiting MLC phosphatase and phosphorylating MLC, thereby regulating actin–myosin contraction [66, 70]. ROCK isoforms also regulate lymphocyte polarity



**Fig. 4.2** Signaling pathways involved in cytoskeleton regulation. Chemokines bind to G-protein-coupled receptors, and consequently can activate different signaling pathways, such as protein kinase B (PKB), mitogen-activated protein kinases (MAPKs), the Janus kinases family (JAK) and the Rho family GTPases. All these signaling pathways are involved in modulating the cytoskeleton proteins, leading to their reorganization, more specifically of the F-actin filaments

and migration through members of the Ezrin/Radixin/Moesin (ERM) family of proteins [66, 71]. Rho GTPases also control cytoskeletal remodeling through effector proteins from the mDia family of formins, which, as mentioned above, are actin-nucleating proteins favoring the formation of long straight actin filaments. Lack of mDia1 expression significantly reduces T cell homing to secondary lymphoid organs [72].

Little is known about the correlation between chemokines and the cytoskeleton. At least C-C motif chemokine 19 (CCL19)/CCL21-CCR7 and CXCL12-CXCR4 constitute an exception.

### **The Influence of the Chemokines CCL21/CCL19 and Their Receptor CCR7 on the Cytoskeleton**

From a physiological perspective, all these intracellular events that occur in the lymphocyte homing process depend on a combination of interactions between different chemokines and their receptors, according to the cell type involved: T-cell homing and traffic of lymphocytes into and within secondary lymphoid tissues rely largely on CCR7 and its ligands CCL21/CCL19 [73, 74], as well as a minor contribution from CXCL12-CXCR4 interactions [75, 76]. Bardi et al. [77] reported that



both the CCR7-mediated polarization and chemotaxis are dependent on the Rho kinases, but not on MAPK extracellular signal-regulated protein kinase (ERK)-2, as previously described [78–80]. The C-C chemokine receptor type 7 (CCR7) and other chemokine receptors such as CXCR4 also activate leukocyte integrins, which are important for the endothelial adhesion and arrest of rolling lymphocytes [81–83], possibly through downstream activation of RhoA [84–86].

Although B cell integrin activation is also primarily induced by CCR7 and CXCR4, their homing also requires the activation of CXCR5, whose expression is restricted to B cells and a subset of CD4+ T cells [76].

The expression of CCR7 and CCL21 has been described in many cancers (especially melanoma, breast cancer, and head and neck cancers), and was correlated with actin polymerization and lamellipodium formation, which contribute to increased tumor-cell migration, invasion and metastatic potential [87–91].

### **The Influence of the Chemokine CXCL12 and Its Receptor CXCR4 on the Cytoskeleton**

CXCL12, better known as stromal cell-derived factor-1 (SDF-1), was first described as pre-B cell growth-stimulating factor (PBSF) [92], and activates integrins in B-cells as mentioned above. The chemokine CXCL12 and its receptor CXCR4 are well known for their role in the metastasis of breast cancer [93, 94]. However, CXCL12 is constitutively expressed in a broad range of tissues, e.g. in bone marrow, spleen, liver, lung and brain, as well as in most types of tumors [92, 95]. This chemokine is the only known ligand for CXCR4, also known as Fusin/LESTR/CD184 [96–98]. CXCR4 is a G-protein-coupled seven transmembrane receptor and is widely expressed by many different cell types including hematopoietic cells, leukocytes, endothelial cells, central nervous system (CNS) cells, and cells of the gastrointestinal tract.

Physiologically, CXCL12 is important for the homing of CXCR4-expressing hematopoietic cells to the bone marrow [99] and for guiding CXCR4-positive cells from different tissues to their niche [100]. CXCL12/CXCR4 knockout is lethal and leads to several impairments in CNS development and hematopoiesis in mice [101]. Furthermore, the CXCL12-CXCR4 axis plays a role in angiogenesis and inflammation (e.g. recruitment of lymphocytes). The chemokine CXCL12 can also bind to another chemokine receptor, CXCR7/G Protein-Coupled Receptor (RDC-1) [102, 103]. However, the connection between this pathway and the cytoskeleton is poorly understood.

Li et al. [104] showed that after CXCR4 stimulation, a signaling pathway that leads to the reorganization of the actin-cytoskeleton becomes activated. After binding of CXCL12 to CXCR4, the heterodimeric G-protein dissociates into the G $\alpha$ i and G $\beta$  $\gamma$ -subunits. The G $\alpha$ i2-subunit interacts with the N-terminus of the engulfment and cell-motility protein 1 (ELMO1) which forms a complex with the DOCK180 (Dedicator of cytokinesis) protein. The ELMO1/DOCK180 complex serves as a guanine nucleotide exchange factor (GEF), activating the small GTP-binding protein (G protein) Rac1 [104].

It is well known that small GTPases such as RhoA, Rac and CDC42 control the dynamics of the cytoskeleton [105]. Rac, which is activated by ELMO1/DOCK180, can remove the capping proteins and activate the Arp2/3 complex, which induces the growth of actin filaments and the formation of new actin branches from existing ones [106, 107].

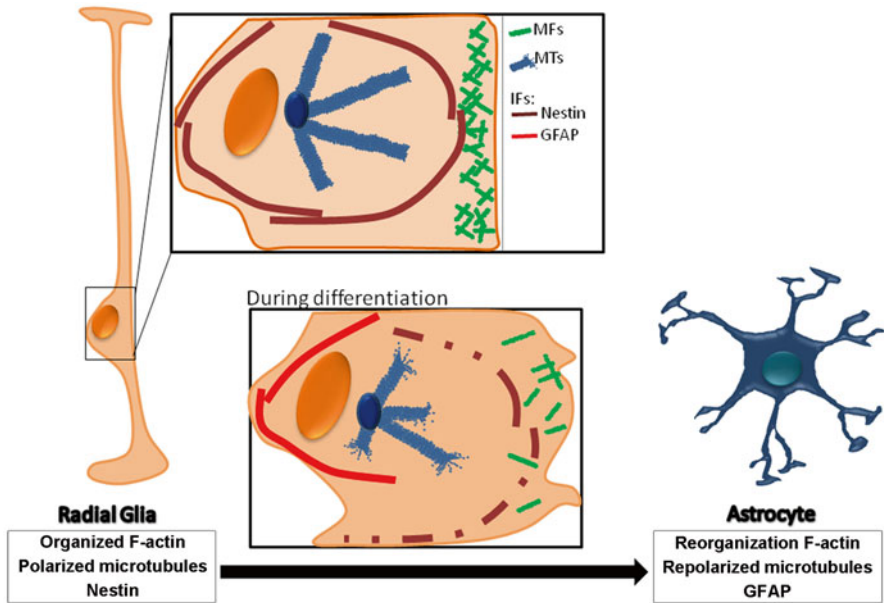
Another cytoskeleton modulation by CXCL12 is the activation of Focal Adhesion Kinase (FAK) and Paxillin. After CXCL12 binds to CXCR4, the Janus kinase 2 (JAK2) and the MAP-kinase ERK1/2 pathways become activated by phosphorylation. Activated JAK2 phosphorylates Signal transducer and activator of transcription 3 (STAT3), and pSTAT3 and pERK are able to phosphorylate FAK and Paxillin, activating these proteins, which leads to actin cytoskeleton reorganization [108]. In conclusion, CXCL12 is known to influence the cytoskeleton reorganization in two different ways, through the G $\alpha$ i2-ELMO1/Dock180-Rac1 activation and the JAK2-pSTAT3/pERK-pFAK/paxillin activation.

Apart from the chemokines previously described in detail, the CXCL9/Mig chemokine is also known to activate the small GTPases Rac1 and RhoA via its receptor CXCR3 on human melanoma cells, also leading to cytoskeletal changes [109].

### *Role of Cytoskeleton in Maintenance of Stem-Cell Properties*

Nowadays, the application of stem cells in regenerative medicine is one of the major fields in biomedical research. Because of their ability to self-renew and differentiate into specific lineages, stem cells play an important role in the development of cell-based therapies [110–112]. The implementation of these new therapies made it necessary to investigate the cellular and molecular mechanisms involved in the regulation of stem-cell differentiation, growth, and phenotypic expression. The most recent studies have indicated that the regulation of stem-cell growth and fate is also dependent on the crosstalk between the extracellular matrix (ECM) ligands and the stem-cell surface receptors [113, 114]. Therefore, during their differentiation into specific lineages, stem cells are subjected to extracellular stimuli that determine a number of morphological alterations associated with the expression of cytoskeletal proteins, actin filaments, microtubules, intermediate filaments, and their downstream effectors [115–117]. These alterations are also crucial in establishing the migration phenotype observed in different types of stem cells, as was previously documented in mesenchymal stem cells (MSCs) and in the embryonic neural stem cells (NSCs) [113, 115–118]. A brief summary of the most important conclusions that explain the role of the cytoskeleton in stemness maintenance and its contribution to stem-cell migration and differentiation is given below.

Increasing evidence shows that a diverse array of environmental factors contributes to the control of stem-cell activity, differentiation and migration. According to the tissue microenvironment factors, stem cells are believed to modulate their cytoskeleton, to migrate and move away from their niche, and then to differentiate [119]. Studies on myocardial development and on capillary endothelial cells have



**Fig. 4.3** Changes of the cytoskeleton in a neural stem cell during differentiation. During the differentiation process, the neural stem cell (represented by radial glia) undergoes cytoskeletal changes, in the microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs). Alterations in the stem-cell cytoskeleton may involve the disorganization of MFs, which become more dispersed; destabilization of the MTs; and also modulation of the expression of IF proteins such as Nestin and GFAP, leading to the differentiation of stem cells into astrocytes, for example

demonstrated that alterations in cell shape might regulate cellular differentiation [113, 115, 116, 118, 120]. These results show that the cytoskeleton is a key player in the differentiation and migration of stem cells [121] (Fig. 4.3).

Recent studies showed that during osteogenic differentiation, the actin cytoskeleton of MSCs becomes more dispersed, similarly to that of osteoblasts, and the disruption of the actin cytoskeleton decreases osteogenesis in favor of adipogenesis [121]. These results have been crucial in the area of tissue engineering of bone and cartilage, which attempts to develop new therapeutic strategies for the treatment of musculoskeletal trauma and diseases [43, 122, 123]. A number of studies have shown that the stem-cell fate and the adhesive interactions between the stem cells and the substrate can be influenced through the control of their shape by artificial extracellular matrices.

Alterations in the cytoskeleton are also dependent on microtubules that contribute to migration and to stem-cell polarization. The regulation of microtubules is usually dependent on the Rho GTPases, in particular RhoA, Rac1 and Cdc42. Previous studies have reported that migrating hematopoietic stem and progenitor cells growing on MSCs display a polarized morphology, with the formation of an uropod at the rear pole and a leading edge at the front, which is involved in microtubule

destabilization. The uropod formation seems to be dependent on the activity of RhoA and its downstream effector Rho-associated coiled-coil containing protein kinase (ROCK I). When RhoA is inhibited using the Rho kinase inhibitor (Y-27652) or RNA interference (RNAi), the polarization of the hematopoietic stem/progenitor cells (HSPCs) and their migration capability are considerably decreased, indicating the crucial role of microtubules in stem-cell migration [124, 125]. Vertelov et al. [126] showed that in hypoxic conditions, the human mesenchymal stem cells (hMSCs) showed increased RhoA activity, and consequently it may contribute not only to increasing migration, but also to preserving MSCs in an undifferentiated state, as compared to normoxic conditions. Moreover, the microtubules seem to be important to maintain the migration capacity as well as the polarity of NSCs [127].

Two of the most thoroughly studied IF proteins are nestin and GFAP [128] (Fig. 4.3). In the 1990s, nestin was first identified as a marker of neuroepithelial stem/progenitor cells in the CNS by Lendahl and collaborators [129]. Nowadays, it is considered to be a marker for distinguishing precursor from differentiated cells [130–132]. A study performed by Mellodew et al. [133] also showed that loss of nestin expression could be a predictive signal for differentiation of NSCs. GFAP is classically known as a marker of mature astrocytes. However, several studies have been conducted in order to evaluate its contribution to the maintenance of stem-cell features. Previous studies suggested that primary astrocyte cultures from the post-natal and adult mouse brain could contain GFAP-expressing cells that may act as multipotent NSCs when transferred to neurogenic conditions [134, 135]. GFAP functions are addressed in detail in section “GFAP Expression and Its Functions in Astrocytes”.

### **Cytoskeleton Alterations During Disease Progression: The Role of Stem Cells in Cancer**

The cancer stem-cell theory predicts that not all cancer cells in a tumor exhibit the same tumor-growing ability, and that only a small population of cells with stem-cell properties drives tumor growth. The proliferation, survival and migration of tumor stem cells seem to be dependent on the local microenvironment. Although highly controlled during embryonic development, the ECM is commonly deregulated in cancer [136, 137] and seems to contribute to the development of chemo- and radio-resistance of tumor cells. Under normal conditions, the ECM receptors allow stem cells to anchor to the local microenvironment where their properties can be maintained [136, 137]. This anchorage physically constrains stem cells to make direct contacts with the microenvironment cells, which produce paracrine-signaling molecules that are essential for maintaining stem-cell properties [136, 137].

Considering that tumor cells possess an increased proliferation and migration ability, we could hypothesize that due to the occurrence of genetic mutations and microenvironment alterations, the characteristics of the stem-cell cytoskeleton become distorted. The most recent studies have shown that cancer cells express the same cytoskeleton markers as benign stem cells [130]. Therefore, the problem

seems to be associated with the degree of expression of the cytoskeleton markers and with the signaling pathways that become activated.

One of the first lines of evidence that the cytoskeleton is involved in the tumor phenotype was the experiment conducted by Vasioukhin et al. [138], who set up conditional gene targeting to knockout genes in the stem cells and basal epidermal layer of mouse skin. They started to knock out the  $\alpha$ -catenin, and observed that mouse skin rapidly took on the appearance of squamous cell carcinoma in situ. More recently, Rampazzo et al. [139] demonstrated that the treatment of tumor stem cells isolated from glioblastoma samples, with Wnt ligands, or the induction of  $\beta$ -catenin overexpression mediates neuronal differentiation and halts proliferation in primary glioblastoma cells.

In prostate cancer, deregulation of the non-canonical Wnt/Ca<sup>2+</sup> pathway leads to F-actin filament rearrangements and consequently to the reduction of cancer progression [140].

Understanding the complexities of the stem cell cytoskeleton in cell homeostasis and in tumor development is a challenging exercise, not only to understand the physiology of many diseases but also to implement new therapeutic strategies.

## **Role of Intermediate Filaments in Glial Cells: Example**

### ***Glial Fibrillary Acidic Protein (GFAP) in Health and Disease***

The intermediate filaments are components of the cytoskeleton that are specific to each cell type. These filaments confer mechanical force and resistance on the cells, and are regulated developmentally and tissue-specifically. GFAP is the main intermediate filament (IF) protein in astrocytes, although other intermediate filament proteins such as nestin, vimentin and synemin can also be found in these cells [141]. A combination of vimentin and nestin is observed in immature astrocytes, while vimentin and GFAP are found in mature astrocytes [37, 142, 143] (Fig. 4.3). Only GFAP seems to be capable of forming homodimers [143].

GFAP was discovered in the brains of patients with multiple sclerosis. It was initially termed ‘plaque protein’ and was first isolated, purified and the amino-acid content determined over 40 years ago. Immunostaining for GFAP has been performed since 1975 [144, 145].

Although GFAP was originally thought to be an astrocyte-specific IF [145], curiously, several investigators have shown that it is present in different amounts in various types of tissues, such as enteric glia [146], Schwann cells [147], and even outside the CNS, in chondrocytes, fibroblasts and myoepithelial cells [147, 148], lymphocytes [149] and stellate cells from the liver, kidney, pancreas, lungs, and testes [150, 151].

Astrocytes are heterogeneous, and their biology varies according to the particular physiological state and time frame, and also to their location in the CNS [151, 152].

It is a challenging task to determine the specific role of GFAP in these multiple environments and in different physiological and pathological conditions.

The human GFAP polypeptide consists of 432 amino acids and has a molecular mass of 55 kDa. The gene contains 9 exons and spans over 10 Kb in chromosome 17p21 [153]. GFAP belongs to the family of type 3 intermediate filaments.

The constitutional transcription of the human GFAP gene is controlled by a TATA-like sequence CATAAA, located 29 base pairs downstream of the RNA start site [154]. Multiple sites seem to be involved in the regulation of GFAP expression, with important roles for phosphorylation and DNA methylation in GFAP transcription [155]. The demethylation of the GFAP promoter activates GFAP transcription [156]. GFAP expression during development is also controlled by acetylation in neural stem cells, and has been shown to be significantly reduced by acetylation in mature astrocytes [157, 158].

The expression of GFAP has multiple regulatory factors, including various hormones, cytokines and growth factors, including interleukins 1 and 2, tumor necrosis factor (TNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), transforming growth factor beta 1 (TGF $\beta$ 1) and glutamate. Interestingly, this regulation seems to be partially controlled by the interaction between astrocytes and cortical neurons, mainly through TGF $\beta$ 1; and varies in different regions of the brain [159–163].

### GFAP Alternative Splicing-Isoforms

At least nine isoforms of GFAP mRNA exist, and are generated by alternative mRNA splicing and polyadenylation signal selection [155, 164–169]. Please refer to Middeldorp and Hol for an excellent review of GFAP biology and GFAP isoforms [155]. Seven of these isoforms are present in humans.

GFAP $\alpha$  was the first to be identified, and is also the most abundant and the most studied.

GFAP $\beta$  has a transcription site located 169 nucleotides above the site for GFAP $\alpha$ , which corresponds to a 5' region that is not transcribed in the main isoform [170]. GFAP $\beta$  is the main isoform in Schwann cells from the rat peripheral nervous system (PNS), but its mRNA comprises only 5–10 % of the total GFAP mRNA in the CNS. It has been found in normal hamster brain and in a case of human glioma [171].

GFAP $\gamma$  was first described in spleen and bone marrow from mice. GFAP $\gamma$  lacks exon 1 and includes the last 126 bp of intron 1–2, comprises around 5 % of all GFAP mRNA in the CNS in mice, and is also present in a small proportion in humans [167].

The splice variants GFAP $\Delta$ Ex6, GFAP $\Delta$ 164, and GFAP $\Delta$ 135 skip sequences in exon 6/7 and have been observed in tissue from patients with Alzheimer's disease, focal lesions in chronic epilepsy, and a specific astrocyte subtype. These out-of-frame splice forms completely lack the tail domain [166].

GFAP $\kappa$  is the result of an alternative splicing at the 3' end of the GFAP pre-mRNA, and the consequent inclusion of an alternative exon termed 7B [164].

The C-terminal domain of GFAP $\kappa$  is therefore different from those present in other isoforms.

GFAP $\zeta$  was described in mice, originating from the initial report of a transcript including the last 284 bp of intron 8–9 [167, 172].

GFAP $\delta$  is the most often studied isoform, after the canonical form GFAP $\alpha$ . It was initially described in rats in 1999, from an alternative splicing, resulting in substitution of the last two exons by an alternative exon called 7+ [165]. Nielsen and coworkers [168] described the corresponding RNA in humans, terming it 7a, and naming the isoform GFAP epsilon; and more recently have come to a consensus on the name GFAP $\delta$  [173]. The exon 7a has its own polyadenylation signal inhibiting the expression of exons 8 and 9. It has been isolated only in mammals, and seems to be subject to a different evolutionary pressure than the other exons [174]. Among the higher primates, the exons 7+ are 100 % identical, with the exception of an alanine on codon 426, conserved in only 9 % of human alleles, and replaced by a valine in 21 % and by a threonine in 70 %. The potential phosphorylation of this threonine residue could explain a positive selection for this change [175].

Incorporation of exon 7a results in a substitution of the 42 C-terminal amino acids by a new c-terminal domain of 41 amino acids. Among the different isoforms, only GFAP $\delta$  and GFAP $\kappa$  show a modification in the C-terminal portion. As a consequence, GFAP $\delta$  and GFAP $\kappa$  are incapable of forming homodimers, but are able to form heterodimers with GFAP $\alpha$  and vimentin [164, 175]. GFAP $\kappa$  mRNA comprises around 5–10 % of the total GFAP mRNA in humans [164, 175, 176].

GFAP $\delta$  is expressed in proliferating neurogenic astrocytes during development, in the adult human brain as well as in radial glia cells [169, 177–179]. It can be detected by immunohistochemistry, particularly in astrocytes of the *glia limitans* and in different forms of gliosis [172, 180], in contrast to earlier studies on post-mortem material [169]. Moreover, its expression seems to parallel that of vimentin in normal and reactive astrocytes, but not in glial tumors [180]. Interestingly, differential GFAP isoform expression in mice does not seem to be linked with aging or reactive gliosis [172]. The specific functions of the different GFAP isoforms have not been well established. These isoforms often differ from GFAP $\alpha$  in the C-tail domain, the region responsible for interaction with other cell components. The interaction of GFAP $\alpha$  was specifically shown with presenilins 1 and 2 [168] and  $\alpha\beta$ cristallin [176]. Therefore an assembly-compromised role of GFAP $\delta$  as a modulator of the GFAP filament surface has been postulated [155].

## GFAP Expression and Its Functions in Astrocytes

GFAP is phylogenetically ancient. The human GFAP polypeptide shows a 90 % homology with its murine and porcine counterparts, and about 85 % homology with goldfish GFAP [174].

During development, GFAP is expressed in radial glia, bipolar cells which express vimentin and nestin and which have been shown to be neural precursors [181, 182] (Fig. 4.3). Studies differ with respect to the exact moment when GFAP expression can first be detected in these cells, varying from gestational week 6–12;



these differences are probably due to the location in the brain or to the detection techniques used [155, 183, 184]. Nervous-system neural precursor cells show a progressive shift in intermediate filament expression, from vimentin to GFAP. In the normal adult brain, only certain subpopulations of astrocytes seem to co-express vimentin and GFAP such as Bergmann Glia, subpopulations of corpus callosum, hippocampus, subpial, and rare white-matter astrocytes [185, 186].

The functions of GFAP are not yet completely elucidated, and include a role in the long-term maintenance of the brain parenchyma structure, the proper functioning of the blood–brain barrier [187], myelination [188], astrocyte proliferation [189, 190], and astrocytic modulation of some neuronal functions, such as the formation and protection of synapses [191, 192]. GFAP is involved in other important and fundamental cellular processes, and is probably implicated in astrocyte motility [193, 194] and exocytosis of astrocytic gliotransmitters [195, 196]. GFAP is also important in the regulation and maintenance of glutamate transporters in the astrocyte plasma membrane, a key mechanism for glutamate uptake and its metabolism and for the formation of GABA [197].

## **GFAP, Pathological States and Disease**

### **GFAP Knockouts**

Knockout mice for intermediate filaments (GFAP<sup>-/-</sup>, Vimentin<sup>-/-</sup>, GFAP<sup>-/-</sup>, and vimentin<sup>-/-</sup>) do not show major changes in their development, adult life, and reproduction [198–200]. These authors found no major differences in brain architecture and cellularity in comparison to wild-type animals. However, another group of researchers working on GFAP-null rodents reported contrasting results, showing myelination defects in the spinal cord, optic nerve and corpus callosum, and hydrocephalus in half GFAP-null mice after 18 months [201]. Astrocytes lacking intermediate filaments exhibit normal morphology, but lack the ability to form normal glial scars [202], have restricted motility in vitro [203], and are highly sensitive to ischemia and trauma [204]. GFAP<sup>-/-</sup> mice are also more sensitive to neurodegeneration induced by kainic acid or mechanical trauma, which is not observed in wild-type animals [205].

### **GFAP and Gliosis**

GFAP expression rises as a consequence of inflammation and various CNS diseases such as trauma, ischemia, genetic diseases, toxic lesions, and degenerative diseases [206]. In all these situations, astrocytes react to injury, in a process usually called astrogliosis or simply gliosis. Despite having general morphological features in common, astrocytes may vary morphologically and chronologically in their responses according to the nature, intensity and localization of the lesion. The kinetics of this



response is usually rapid, and can be detected 1 h after the insult, with a maximum intensity at between 3 and 7 days [207].

Astrocytic gliosis has classically been described morphologically by the hypertrophy of the cell soma and processes, which is roughly proportional to the severity of the insult and the proximity of the astrocyte to it. More recently it has been well established that there is an increase in the GFAP cellular content, and, depending on the severity of the reaction, also an increase in the number of astrocytes [208–210]. Constitutional GFAP expression is heterogeneous among different astrocyte populations, and in the normal state not all astrocytes express detectable levels of GFAP. With increasing intensity of gliosis, most astrocytes will express GFAP, and in severe gliosis one also observes astrocyte proliferation, with subsequent overlap and disruption of individual astrocyte domains [151]. Therefore, GFAP has been generally used as a marker of gliosis [206], even though astrocyte reactivity and GFAP upregulation due to different stimuli may be associated with different changes in transcriptome profiles and cell function [211]. In other words, GFAP levels can be generally increased in various CNS pathological states such as trauma, ischemia, infections, and neurodegenerative diseases.

Interestingly, low levels of GFAP can be detected in the cerebrospinal fluid (CSF) in healthy individuals. The reason has not been well established, but it has been postulated that the presence of GFAP might be related to some degree of astrocyte death and release of the protein into the extracellular space, as normal astrocytes do not secrete GFAP [212]. GFAP levels in the CSF can be elevated in association with several conditions, including traumatic, vascular, developmental, inflammatory, neoplastic, and degenerative diseases. We briefly describe certain diseases in which GFAP seems to have a particular role.

### Alzheimer's Disease

Alzheimer's disease (AD) is characterized mainly by two neuropathological alterations, the formation of neurofibrillary tangles, and amyloid deposits in the brain [213]. Reactive astrogliosis has been well described in AD, although its role in this disease is not yet completely understood. Reactive astrogliosis in AD is usually focal, and reactive astrocytes are intimately associated with amyloid plaques or diffuse amyloid deposits, surrounding them and forming miniature scars all around [151]. The intensity of reactive astrogliosis, as determined by GFAP levels, has been reported to increase in parallel with increasing disease morphological burden [214, 215], and in some studies can be correlated with cognitive impairment [216].

### Alexander's Disease

A gradual increase in astrocyte GFAP content is usually observed during adult life in mice, primates, and humans [217, 218]. Mice induced to overexpress GFAP die for reasons that are currently unknown, and their astrocytes exhibit accumulation

of the protein in numerous cytoplasmic Rosenthal fibers [219]. Likewise, Alexander's disease, a rare human disease, is caused in 95 % of cases by mutations of GFAP, leading to its accumulation in the cytoplasm, associated with proteins such as small heat shock, ubiquitin and  $\beta$  cristallin in the form of Rosenthal fibers [220, 221]. There are two clinical forms, one with early onset, which progresses with childhood leukodystrophy with striking clinical signs of megalencephaly, seizures and psychomotor delay; and a later-onset form, often revealed by difficulty swallowing and speaking, autonomic dysfunction and ataxia. Several different mutations have been reported in the human GFAP gene in Alexander's disease, mostly heterozygous missense changes predicting the production of full-length mutant and wild-type proteins, and subsequently alterations such as small in-frame insertions and deletions, and in-frame skipping of an entire exon or frameshifts at the extreme C-terminal end [222]. The pathophysiology is explained by the increase in the toxic function of abnormal tissue deposits [223]. For the list of GFAP mutations in this disease please refer to the Waisman Center of the University of Wisconsin-Madison (<http://www.waisman.wisc.edu/alexander/mutations.html>).

Interestingly, Rosenthal fibers are also present in certain pathological conditions, namely tumors such as pilocytic astrocytomas and gangliogliomas, and even some types of chronic gliosis, for example, those observed in the periphery of craniopharyngiomas or hemangioblastomas.

## GFAP and Gliomas

The development of immunohistochemistry for GFAP was an important advance in surgical neuropathology, including the diagnosis of brain tumors. The expression of GFAP in primary glial tumors has been extensively studied since the beginning of the 1980s, and is now widely used in diagnostic neuropathology [224–226]. Independently of the histological grade, every tumor with astrocytic differentiation is expected to show at least some positivity for GFAP [227]. GFAP-positive cells can also be observed in some other glioma types, such as oligodendroglioma, where often small cells named “minigemistocytes” express GFAP [227]; and in ependymomas [226, 228]. A significant proportion of choroid-plexus tumors can also express GFAP, even focally [226].

Some studies have shown *in vitro* a negative correlation between GFAP expression levels and the malignant transformation of astrocytes [229–231]. However in experimental astrocytoma murine models, GFAP expression does not seem to affect tumor progression [232].

GFAP serum levels seem to be significantly elevated in patients with glioblastoma multiforme [233]. This implies damage to the blood–brain barrier, since this protein is not usually detectable in the serum or is present in very low levels. Some other conditions that can lead to its rise in the serum are stroke, hemorrhage, trauma and multiple sclerosis [212].

### ***Enteric Glia Cytoskeleton***

GFAP is also the main marker of enteric glia, a peripheral glial-cell type derived from the neural crest cells that has close morphological, molecular and functional similarities to astrocytes (reviewed by Coelho-Aguiar et al. [234]). These glial cells are crucial for the proper functioning of the gut. Their disruption in mice by the targeting of GFAP-positive cells leads to increased permeability of the mucosal epithelium, followed by an inflammation process and disruption of the ileum and jejunum structure [235–237].

Interestingly, as in the CNS, GFAP is also expressed in glia-like progenitors in the gut. Some of these GFAP-positive cells can generate multilineage colonies in vitro and also give rise to glial and neuronal cells in vivo in graft studies, in injury conditions or in experiments of myenteric plexus ablation with benzalkonium chloride detergent [238–245].

Similarly to reactive gliosis in the CNS, there is an increase of GFAP levels in enteric glia of inflamed gut regions, notably in inflammatory bowel diseases [246]. An increase in GFAP expression was also observed in colon biopsies of patients with *Clostridium difficile* infections [246]. Patients with Parkinson's disease show  $\alpha$  synuclein aggregates (the pathological trait of Parkinson's disease) also in the ENS, and also an increased expression and reduced phosphorylation of GFAP, which is also observed in neurodegenerative processes in the CNS [247, 248]. These informations suggest an association between enteric inflammation and glial dysregulation.

Furthermore, specific roles are known for other components of the cytoskeleton in enteric glia, the F-actin filaments and microtubules. Little is known about this, but  $\text{Ca}^{2+}$  dependent responses have been identified in enteric glia, related to their functions in neurotransmission. Calcium enters enteric glial cells through a mechanism termed capacitative calcium entry, which is responsible for the maintenance of calcium storage in different cell types. It has been shown that in cultured myenteric glia, the disruption of the actin filaments or microtubules can decrease and even completely inhibit calcium entry [249]. These experiments confirmed the importance of the cytoskeleton for the physical interactions between the calcium storage organelle and the plasma membrane as a capacitative calcium-entry mechanism, which replenishes the cell after depletion of the intracellular calcium store. It is probable that the same events occur in astrocytes and other cell types to maintain their intracellular calcium store, but this has not yet been investigated.

### ***Schwann Cell Cytoskeleton***

Another neural crest-derived GFAP-expressing type of glia is the Schwann cells, which are responsible for the myelination of the entire peripheral nervous system (PNS). Schwann cells can be found in myelinating or non-myelinating forms. This cell type is capable of great migratory and differentiation capacities during

development and even in adults, to repair and replace the myelin of injured axons. The physical interaction of these cells with the surrounding microenvironment involves changes in the cytoskeleton.

These cells constitute the other example of peripheral glia that express GFAP. However, it is clear that they show a different GFAP isoform expression from that of astrocytes and enteric glia. Moreover, they express this IF at lower levels than astrocytes, and even more reduced levels in relation to enteric glia [250].

GFAP expression is a marker of non-myelinating Schwann cells. GFAP appears after differentiation of Schwann-cell precursors into immature Schwann cells [251]. Its expression depends on contact with non-myelinated axons, and is downregulated in myelinating Schwann cells. On the other hand, vimentin is expressed in myelin-forming Schwann cells. In experiments with sciatic-nerve transection, a reduction of GFAP mRNA levels was observed. Moreover, the immunodetection of GFAP also decreased, while vimentin expression increased [252].

Interactions among Schwann cells, the extracellular matrix and axons are mediated by surface receptors and are transduced by the cytoskeleton proteins. These interactions are essential to the recovery of neuronal transmission after axonal nerve injury. The axonal regeneration process follows well-established steps. After disruption of myelin sheets, Schwann cells dedifferentiate and proliferate. There is an increase in the expression of adhesion molecules and cytoskeleton proteins such as GFAP and vimentin. Then, these glial cells form bands of Büngner, which serve as a substrate for axonal regrowth. After that, they enwrap the axons and form myelin [253]. All these stages require continuous reorganization of the Schwann cell cytoskeleton.

In GFAP-null mice, the development of peripheral axons and their myelin is normal, as is their functioning. However, lack of GFAP leads to retardation in nerve regeneration after injury, probably because of a problem in Schwann cell proliferation. This study, developed by Triolo and coworkers [253], revealed that GFAP interacts with integrin  $\alpha v \beta 8$ , which interacts with fibrin, thus acting in the early steps of Schwann-cell proliferation. These investigators also showed that vimentin interacts with integrin  $\alpha 5 \beta 1$ , which connects to fibronectin, acting in the subsequent steps of Schwann-cell proliferation and nerve regeneration [253].

Another cytoskeleton protein associated with integrins in Schwann cells is merlin, also known as schwannomin. Merlin is a perinuclear protein that translocates to cytoplasm during differentiation and becomes associated with integrin  $\beta 1$  in myelinating Schwann cells. This study suggests that merlin links the integrin to the microfilaments, supporting myelination [254]. Other proteins associated with intermediate filaments interact with integrins, such as hemidesmosomal 1 protein (HD1), plectin and dystonin [255]. Targeted disruption of dystonin, for example, results in problems in the interaction with the axon basement membrane and in PNS dysmyelination [256].

## Conclusion

As set out in this chapter, the first step in cell locomotion is the formation of cytoskeleton protrusions such as lamellipodia at the periphery of a cell. This protrusion is composed of F-actin filaments. Study of the mechanical characteristics of

cytoskeleton proteins and plasma membrane tension in lamellipodia was advanced by the use of optical tweezers, which enable measurement of the bending modulus and membrane tension. Important inductors of the migratory phenotype are the chemokines, small peptides that elicit cytoskeleton responses through signaling by their G protein-coupled receptors. CCL19/CCL21 and their receptor CCR7, and CXCL12 and its receptor CXCR4 have physiological roles in guiding different cell types to their niches, as well as acting in tumor-cell migration by eliciting reorganization of the actin cytoskeleton.

We next discussed the role of the cytoskeleton in the performance of stem cells. Actin microfilaments and microtubules are reorganized during differentiation, and are important for stem-cell fate and polarization. Intermediate filaments, specific to each cell type, may also be involved. Neural stem and precursor cells, for example, express nestin, while GFAP is typical for mature astrocytes but is also found in neural precursors. In the last section we explored the specific characteristics of the intermediate filament GFAP in its multiple isoforms. GFAP is essential for astrocyte functions, and its disruption is implicated in several disorders and diseases. Peripheral glial cells that also express GFAP are the enteric glial cells, which are the counterparts of astrocytes in the gut; and the Schwann cells, which are responsible for the myelination of the peripheral nerves.

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