Cell-Extracellular Matrix Interactions in Cancer

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Editors

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Springer

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ISBN 978-1-4419-0813-1 e-ISBN 978-1-4419-0814-8
DOI 10.1007/978-1-4419-0814-8
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2009932115

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Cancer is a term used for diseases that affect any organ in the body, in which abnormal cells divide in an uncontrolled manner and in some instances are able to invade other tissues. Cancer is not just one disease, but many diseases with origins in multiple different organ systems. The defining abnormality in cancer is an imbalance between cell proliferation and death and is caused by mutations in DNA that code for proteins that regulate these cellular processes. Genes that promote cell growth in tumors are called proto-oncogenes, while tumor suppressor genes code for proteins that mediate antiproliferation signals and suppress mitosis and cell growth. Upregulation of proto-oncogenes and/or downregulation of tumor suppressor genes are a common event in cancer.

Cells do not exist in isolation and require interactions with extracellular matrix (ECM) components in order to undergo normal morphogenesis with respect to organogenesis. ECM, which is composed of large macromolecules (e.g., collagens, fibronectin, laminins) and polysaccharides (e.g., glycosaminoglycans such as hyaluronan), plays a significant role in regulating numerous cellular functions including cell shape, adhesion, migration, proliferation, polarity, differentiation, and apoptosis. In physiological conditions, ECM levels are tightly regulated by a fine balance between synthesis and degradation. However, in pathological conditions, such as cancer, both increased synthesis of certain ECM components (i.e., collagens, fibronectin, and laminins) and/or increased breakdown with consequent generation of ECM cleavage products (i.e., laminin- or collagen-cleavage products) can contribute to cancer growth and progression. Finally, the observation that many growth factors (i.e., FGF, VEGF) are stored in the ECM milieu and released upon protease-dependent cleavage further confirms the importance of ECM in regulating cell functions.

Cells interact with ECM via a family of transmembrane receptors, known as integrins. These receptors are not only important for cells to adhere on ECM, but are also important regulators of cell signaling that controls cell processes such as proliferation, apoptosis, and migration. These signals are either transduced via the integrins upon binding to ECM components or by a crosstalk between integrins and

growth factor receptors. Both integrins and growth factor receptors have been shown to bind either directly and/or indirectly with numerous signaling and scaffolding molecules that have been linked to oncogenesis.

The overall goal of this book is to describe how ECM creates a niche for tumor formation and the contribution of ECM components and their respective receptors in the development and spread of cancer.

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Chapter 1
The Extracellular Matrix: An Overview

Jeffrey H. Miner

Abstract The extracellular matrix encompasses the very large number of constituent macromolecules that are synthesized and secreted by cells into the space surrounding them, followed in most cases by further assembly, cross-linking, and/or polymerization of the secreted proteins to form an organized structure. The extracellular matrix has a number of critical roles in tissue and organ development, function, and repair after injury. In addition, there are numerous serious and debilitating genetic diseases whose bases lie in mutations in genes encoding extracellular matrix proteins. There are also acquired diseases, such as scurvy, chronic obstructive pulmonary disease, and cancer, that can be caused by damage to or are influenced by changes in the organization or integrity of the extracellular matrix. The goal of this chapter is to provide an overview of the extracellular matrix by discussing the different classes of extracellular matrix molecules and presenting a subset of individual extracellular matrix proteins from each class in greater detail in order to demonstrate their importance.

Keywords Basement membrane • collagen • laminin • proteoglycan • elastic fiber • bone • fibrosis

Introduction

The extracellular matrix encompasses the very large number of constituent macromolecules that are synthesized and secreted by cells into the space surrounding them, followed in most cases by further assembly, cross-linking, and/or polymerization

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_1,

of the secreted proteins to form an organized structure. The extracellular matrix has a number of critical roles in tissue and organ development, function, and repair after injury. There are numerous serious and debilitating genetic diseases whose bases lie in mutations in genes encoding extracellular matrix proteins. In addition, there are acquired diseases, such as scurvy, chronic obstructive pulmonary disease, and cancer, that can be caused by damage to or are influenced by changes in the organization or integrity of the extracellular matrix. The goal of this chapter is to provide an overview of the extracellular matrix by discussing the different classes of extracellular matrix molecules and presenting a subset of individual extracellular matrix proteins from each class in greater detail in order to demonstrate their importance.

Classes of Extracellular Matrix Molecules

Overall, extracellular matrix components are an extremely diverse group of proteins and nonprotein constituents. Given this diversity, it is difficult, if not impossible, to easily divide them into different classes in a consistent manner (e.g., molecular classes, such as collagens and proteoglycans, versus functional classes, such as basement membranes and elastic fibers). For the sake of meaningful discussion, I have chosen to describe these proteins from a functional standpoint and so will primarily use the latter classification scheme. However, important matrix components that do not fit well into functional classes will also be discussed.

Generalizations

A common (though not universal) theme regarding extracellular matrix proteins is that they are modular; certain domains appear repeatedly in different types of extracellular matrix components. This is most likely the result of partial gene duplication and exon shuffling during the course of evolution (Hutter et al. 2000). Though they will not be discussed here, some of these domains are also present within cell surface proteins or even within intracellular proteins.

Basement Membranes

Basement membranes are thin sheets of specialized extracellular matrix found at the basal side of epithelial and endothelial cells and surrounding all muscle fibers, fat cells, the entire central nervous system, and Schwann cell/axon units in peripheral nerves. They are important for compartmentalizing tissues, for serving as cellular and muscle fiber adhesion sites, for promoting cell proliferation, differentiation,

and migration, and for establishing the filtration barriers in the kidney and in the choroid plexus. All basement membranes have at a minimum four major components: laminin, collagen IV, nidogen, and heparan sulfate proteoglycans such as perlecan and agrin (Timpl 1989).

Laminin

Laminin describes a family of 12 large evolutionarily and structurally related glycoproteins. Of the four major basement membrane components, laminin appears to be the only one that is absolutely necessary for basement membrane formation (Smyth et al. 1999). Laminins are secreted as heterotrimers composed of one α, one β, and one γ chain; there are five α, four β, and three γ chains that assemble nonrandomly to form at least 15 different heterotrimers (Miner and Yurchenco 2004). This complexity forced the development of a nomenclature to identify the trimers using three Arabic numerals that define the chain composition (Aumailley et al. 2005). For example, the laminin α1β1γ1 trimer is known as laminin-111 or LM-111. The α3β3γ2 trimer, frequently discussed in the literature as laminin-5 as per the previous nomenclature (Burgeson et al. 1994), is now known as LM-332.

Laminins share several structural features. All the chains have an α-helical long arm that forms a coiled-coil; intertwining of α, β, and γ chain long arms leads to the formation of laminin trimers, which are further stabilized by limited interchain disulfide bonding. In addition, most of the chains have a short arm composed of alternating globular and laminin type EGF-like repeats. The most NH2-terminal domain, called the LN (laminin N-terminal) domain, is crucial for polymerization of laminin trimers once they are secreted into the extracellular space. Finally, laminin α chains have a large COOH-terminal domain called the LG (laminin globular) domain. The LG domain interacts with receptors, such as integrins, dystroglycan, and Lutheran/Basal cell adhesion molecule, on the surface of adjacent cells. These interactions can facilitate basement membrane formation, mediate cell/matrix adhesion, and initiate signaling events that influence cell behavior.

The importance of individual laminin chains to basement membrane structure and function is demonstrated by the severe effects of mutations in humans and genetically altered mice. Mutations that affect laminin α2 cause a congenital form of muscular dystrophy (Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim); OMIM# 607855). Mutations that affect the components of LM-332 (laminin α3, β3, or γ2) cause a severe skin-blistering disease, junctional epidermolysis bullosa (OMIM# 226700). The absence of laminin β2 causes Pierson syndrome (OMIM# 609049), which is characterized by kidney disease and neurological defects. In mice, removal of laminin α5 causes developmental defects in neural tube closure, placentation, digit and lung lobe septation, kidney development and function, and tooth development (Miner and Yurchenco 2004; Fukumoto et al. 2006). Laminin α5 also plays an important role in the intestine (Bolcato-Bellemin et al. 2003; Mahoney et al. 2008). Finally, peri-implantation mouse embryos lacking either laminin β1 or γ1, which are present in all laminins

expressed early in development, lack basement membranes (Miner and Yurchenco 2004). These studies demonstrate the importance of laminin for basement membrane formation.

Collagen IV

Collagen IV is unique among the collagen superfamily because it is found primarily in basement membranes. Like all collagens, collagen IV exists in the extracellular matrix as trimers whose individual chains contains Gly-X-Y amino acid triplet repeats. However, unlike most collagens, collagen IV chains contain multiple interruptions of these repeats. This is thought to be related to the fact that basement membranes need to be flexible, and the interruptions dampen the rigidity that is imparted by the trimerized Gly-X-Y repeats (Hudson et al. 2003).

There are six genetically distinct collagen IV chains, α1 through α6. These form three different heterotrimers. The α1α1α2 heterotrimer is essentially ubiquitous in basement membranes. The α3α4α5 heterotrimer is found primarily in basement membranes associated with the kidney glomerulus, lung alveoli, seminiferous tubules, choroid plexus, and neuromuscular junctions. The α5α5α6 heterotrimer is associated primarily with smooth muscle basement membranes but is also prominent in Bowman's capsule in the kidney (Ninomiya et al. 1995). That there are only three different heterotrimers is consistent with the fact that the COOH-terminal noncollagenous (NC1) domain present in all six chains provides a "code" for an assembly into heterotrimers (Khoshnoodi et al. 2008).

As with laminin, the importance of collagen IV to the basement membrane's structure and function, is well demonstrated by the fact that mutations in the genes encoding the collagen IV chains cause human disease. Mutations in either *COL4A3*, *COL4A4*, or *COL4A5* cause Alport syndrome, a glomerulonephritis associated with deafness and ocular abnormalities (OMIM# 301050 and 203780). The Alport kidney glomerular basement membrane shows abnormal thickening and splitting, as also observed in the *Col4a3*$^{-/-}$ mouse model of this disease (Fig. 1.1) (Miner and Sanes 1996). Recently, a number of mutations in *COL4A1* have been discovered to be associated with porencephaly, stroke, kidney disease, and muscle cramps (OMIM# 607595, 175780, and 611773) (Gould et al. 2006; Plaisier et al. 2007).

Nidogen

Nidogen 1, also known previously as entactin, was originally isolated as the "fourth laminin chain" because it binds tightly to the laminin γ1 chain short arm (Timpl 1989). Nidogen 1 also has affinity for collagen IV and had been thought to be absolutely essential for basement membrane formation as a linker between the laminin and collagen IV networks, which are selfpolymerizing. Mice lacking nidogen 1, however, are viable and fertile, a finding attributed to the compensation by an increased expression and/or deposition, into basement membranes of nidogen 2 (Murshed et al. 2000).

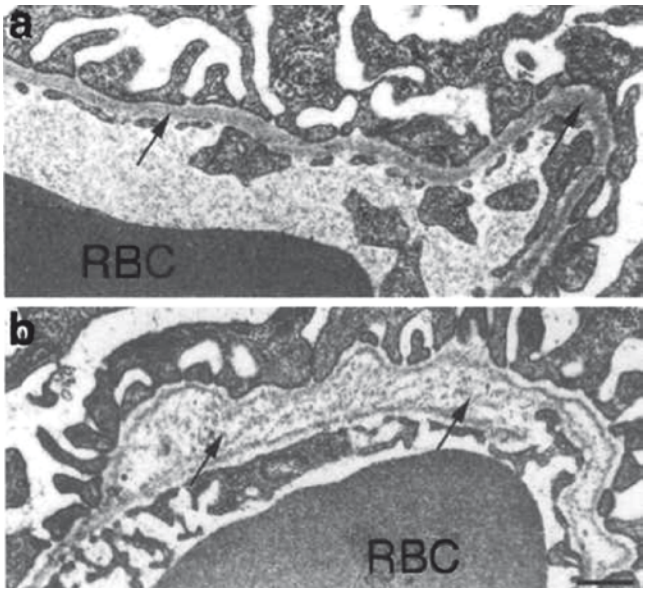

Fig. 1.1 Electron micrographs of glomerular basement membranes in approximately 2-month-old control **a** and *Col4a3*$^{-/-}$ (Alport) bmice. Arrows point to the glomerular basement membranes. RBC, red blood cell (Reproduced from Miner and Sanes (1996) with permission from Rockefeller University Press.)

Mice lacking nidogen 2 are also normal, but mice lacking both nidogens die perinatally with lung and heart defects. But surprisingly, these mice manifest only focal basement membrane defects, suggesting that nidogen is not required for basement membrane formation at all (Bader et al. 2005). Mutations in the genes encoding nidogens 1 or 2 have not been found in humans.

Heparan Sulfate Proteoglycans

All basement membranes contain one or more heparan sulfate proteoglycans (HSPGs), which consist of a core protein with O-linked glycosaminoglycan side chains that are variably sulfated. Perlecan and agrin are the most widely distributed HSPGs in basement membranes. Perlecan has affinity for both laminin and collagen IV (Timpl 1989), whereas agrin binds tightly to a specific site on the laminin γ1 long arm (Kammerer et al. 1999); agrin also interacts with receptors on cells via laminin-like globular domains (Sanes et al. 1998). In addition to its deposition in basement membranes, perlecan is also present in cartilaginous matrices (Costell et al. 1999), and mutations in perlecan (*HSPG2*) cause severe skeletal deformations in humans (OMIM# 224410 and 255800) (Arikawa-Hirasawa et al. 2001).

A splice form of agrin expressed by motor neurons is absolutely required for normal neuromuscular junction structure and function; its mutation results in an

absence of neuromuscular junctions and total fetal paralysis (Gautam et al. 1996; Burgess et al. 1999). Mutations in the human agrin gene have not been found, likely because the effects of significant mutations would be so severe as to cause prenatal lethality. Collagen XVIII is a HSPG that is also distributed widely in basement membranes and is best known as the precursor of endostatin, an antiangiogenic peptide formed by the cleavage and release of the COOH-terminal domain of collagen XVIII (Marneros and Olsen 2005). Perhaps unrelated to this, mutations in *COL18A1* cause Knobloch syndrome type I (retinal detachment and occipital encephalocele; OMIM# 267750) (Marneros and Olsen 2005).

Elastic Fibers

Elastic fibers provide the required elasticity to tissues for which reversible extensibility or deformability is important for function, including the walls of large blood vessels, the lung air sacs, and the skin. Elastic fibers are laid down during organogenesis, usually by smooth muscle cells or fibroblasts, via a complex series of secretory and extracellular enzymatic cross-linking events. This makes their repair in adults difficult, if not impossible.

Elastic fiber biogenesis begins with the laying down of microfibrils, which are composed of fibrillin, fibulin, emilin 1, and microfibril-associated glycoproteins (MAGPs); more than 30 different proteins have been shown to be associated in some way with microfibrils (Shifren and Mecham 2006). Following the assembly of mature microfibrils, secreted tropoelastin monomers aggregate and associate with the microfibril. Secreted lysyl oxidase extensively cross-links tropoelastin monomers with each other to form elastin polymers that are reversibly deformable and highly resilient (Shifren and Mecham 2006), and sufficient to last throughout the lifetime of the organism. (It is important to note here that not all microfibrils are associated with elastic fibers; parallel microfibrils in the eye attach the suspended lens to the ciliary body, which controls the shape of the lens for focusing.) The major components of elastic fibers that will be reviewed here are fibrillin, elastin, and fibulin.

Fibrillin

There are three genetically distinct fibrillins, designated fibrillin-1, -2, and -3. Fibrillins-1 and -2 are the best studied of the three proteins; interestingly, there is not a complete fibrillin-3 gene in mouse or rat (Corson et al. 2004). All are large, ~350-kDa glycoproteins whose primary structures consist mostly of calcium-binding EGF domains that adopt a rod-like configuration in the presence of calcium ions. Fibrillins polymerize into stereotypical “beads-on-a-string” structures that associate with each other laterally to form the microfibrillar lattice (Ramirez et al. 2007) that forms the basis for elastic fiber biogenesis. Fibrillins bind a number of other extracellular

proteins, including tropoelastin, various MAGPs, HSPGs, and members of the transforming growth factor (TGF) β superfamily (Shifren and Mecham 2006). Indeed, many of the defects associated with mutations in fibrillins that are discussed in the next paragraph may relate to abnormally robust signaling by TGFβ and related molecules (Ramirez and Dietz 2007).

The importance of fibrillins to elastic fiber biogenesis is underscored by the effects of mutations in the genes encoding them. Mutations in *FBN1* cause Marfan syndrome (OMIM# 154700), an autosomal dominant disease of fibrous connective tissue characterized by increased height, long limbs and digits, scoliosis, crowded teeth, myopia, and variable life-threatening cardiovascular defects, including aortic aneurysm and aortic dissection (Ramirez and Dietz 2007). In contrast, mutations in FBN2 cause a more limited autosomal dominant disease, congenital contractural arachnodactyly (OMIM# 121050), which lacks the potentially lethal cardiovascular defects (Park et al. 1998).

Elastin

Elastin is synthesized and secreted as the ~72-kDa soluble tropoelastin monomer. Tropoelastin is a highly hydrophobic protein, with 90% of all the amino acid residues being nonpolar; one-third of elastin's amino acids are glycine, with a preponderance of Ala, Val, Leu, and Ile. For tropoelastin to generate the elastic fiber, it must be cross-linked into polymers, which is initiated by the activity of the enzyme lysyl oxidase (Shifren and Mecham 2006). Of tropoelastin's 40 lysine residues, approximately 35 are involved in cross-linking (Mecham 1999). It is thought that elastin's elasticity is imparted by its high hydrophobicity, with the possibility that nonpolar side chains are exposed to water when elastic fibers are stretched; recoil then occurs when the force is removed and the hydrophobic groups reaggregate and expel water (Mecham 1999).

Heterozygous mutations, either null or missense, in *ELN* cause supervalvular aortic stenosis (SVAS; OMIM# 185500), a congenital narrowing of the ascending aorta. Much of the evidence obtained from identifying pathogenic mutations in patients suggests that the mechanism for this condition involves primarily haploinsufficiency (Metcalfe et al. 2000). Evidence from mice carrying one null mutation in *Eln* is in agreement; interestingly, in mice and humans with haploinsufficiency, there is an increase in the number of elastic laminae in the aorta, apparently in compensation for the approximately 50% reduction in the amount of elastin mRNA and protein (Li et al. 1998).

Fibulin

Fibulins are a family of seven extracellular matrix proteins (de Vega et al. 2007; Kobayashi et al. 2007) that are associated with a diverse set of extracellular superstructures, including elastic fibers, basement membranes, and fibrillar fibronectin

(Chu and Tsuda 2004). The characteristic domain structure of the fibulins includes tandem arrays of calcium-binding epidermal growth factor-like motifs and a C-terminal fibulin type module. Of the seven family members, fibulins 3, 4, and 5 have been shown to be involved in elastic fiber assembly by binding directly to tropoelastin and perhaps also to the enzyme lysyl oxidase like 1 (Rahn et al. 2009). The relevant fibulin knockout mice have defects in elastic fibers of various tissues, including those in the aorta, the lungs, the skin, the abdominal wall, and the pelvis, with disruption of the latter two causing hernias and vaginal prolapse (Rahn et al. 2009). In humans, mutations in *FBLN5* and *FBLN4* cause autosomal recessive cutis laxa (OMIM# 219100), which is characterized by loose skin and a range of other elastic fiber defects. *FBLN5* mutations have also been found in patients with age-related macular degeneration (OMIM# 608895). It is therefore highly relevant that fibulin 5 protein has been localized to Bruch's membrane at the back of the eye and to the extracellular deposits (drusen) that characterize this disease (Mullins et al. 2007).

Acquired Diseases of Elastic Fibers

Besides the genetic defects mentioned above that impact elastic fiber structure and function, there are also acquired diseases. The most prevalent and best known of these is emphysema, most cases of which are caused by cigarette smoking. Emphysema is a progressive lung disease in which the walls of the lung air sacs are destroyed. The deposition of smoke particles in the lung induces the infiltration of macrophages that ingest the particles and secrete proteases such as macrophage elastase (matrix metalloproteinase 12) that degrade the elastic fibers (Hautamaki et al. 1997). This leads to a loss of lung elasticity, making exhalation difficult and reducing the surface area for gas exchange. Given that elastic fibers cannot be easily repaired or replaced, emphysema cannot be reversed. There is also an acquired form of cutis laxa that is caused by the action of infiltrating immune cells that degrade elastic fibers; this can be initiated by an infection or other inflammatory insult. However, new data suggests that mutations in elastic fiber proteins (elastin and fibulin 5) that are normally innocuous may play a causative role in this disease (Hu et al. 2006).

Bone, Cartilage, and Other Connective Tissues

The major extracellular matrix protein found in bone, cartilage, and other connective tissues, such as tendons and ligaments, is collagen. These tissues, which consist of cells embedded in extracellular matrix, are also rich in proteoglycans (for example, perlecan and aggrecan) and hyaluronic acid, a very large nonproteinaceous glycosaminoglycan. These glycosaminoglycans provide a high negative charge density. This attracts water, forming the hydrated gel-like substance that is especially

evident in and important for the cushioning function of cartilage. But due to its prominence in these connective tissues, collagen will be the primary focus of this section of the chapter.

Collagen

The form of collagen present in most connective tissues is fibrillar collagen, which is also the most abundant type of protein in animals. As mentioned above, all collagens are triple helical trimers (homotrimers or heterotrimers) whose individual chains are characterized by Gly-X-Y amino acid triplet repeats, with the Y amino acid frequently Pro. The presence of Gly at every third residue is crucial because it is the only amino acid with a small enough side chain to fit at the center of the triple helix (Olsen and Ninomiya 1999a). As previously discussed, however, the chains of type IV collagen possess numerous collagenous domain interruptions, which are thought to be important for their ability to form flexible networks rather than fibrils. In contrast, fibrillar collagens do not have interruptions, which allow them to form the stiff superstructures that are required for imparting tensile strength, for example, as found in bones and tendons (Kadler et al. 2008).

There are 28 different types of collagen (Khoshnoodi et al. 2006), and each has been assigned a distinct Roman numeral, for the most part based upon the order of discovery. These diverse collagen types have been divided into several different classes: fibrillar (types I, II, III, V, and XI), fibril-associated collagens with interrupted triple helix (FACIT; types IX, XII, XIV, XVI, and XIX), basement membrane-associated (types IV, VIII, XV, and XVIII), (trans)membrane (types XIII, XVII, XXIII, and XXV), and "other collagens" that form specialized structures in a variety of tissues (types VI, VII, and X) (Olsen and Ninomiya 1999a; Ricard-Blum and Ruggiero 2005). As part of this overview, one member of each class will be discussed; a member of the basement membrane-associated class (type IV) has already been discussed above.

Collagens are secreted as trimeric "protomers" that have been enzymatically modified to varying degrees, depending on the collagen type (Fig. 1.2). These modifications are important for imparting stability to the triple helix and to the fibril and usually include hydroxylation of prolines to form hydroxyproline and hydroxylation of lysines to form hydroxylysine (Lamande and Bateman 1999). Once outside the cell, in some cases there is cleavage of COOH and/or NH2-terminal noncollagenous domains; these cleavages are carried out by specific enzymes, such as the procollagen C proteinase bone morphogenetic protein 1 (BMP1) (Kessler et al. 1996). Protomers then self-associate with each other to form higher order structures, such as fibrils. The various enzymatic modifications and cleavages are crucial for regulating protomer–protomer interactions and for subsequent cross-linking of collagens into stable higher order structures (Kadler et al. 2008).

Protomers (i.e., homotrimers or heterotrimers) are formed by the assembly in the endoplasmic reticulum of newly synthesized α chains belonging to a particular type of collagen. Given that a particular cell may be synthesizing a number of different

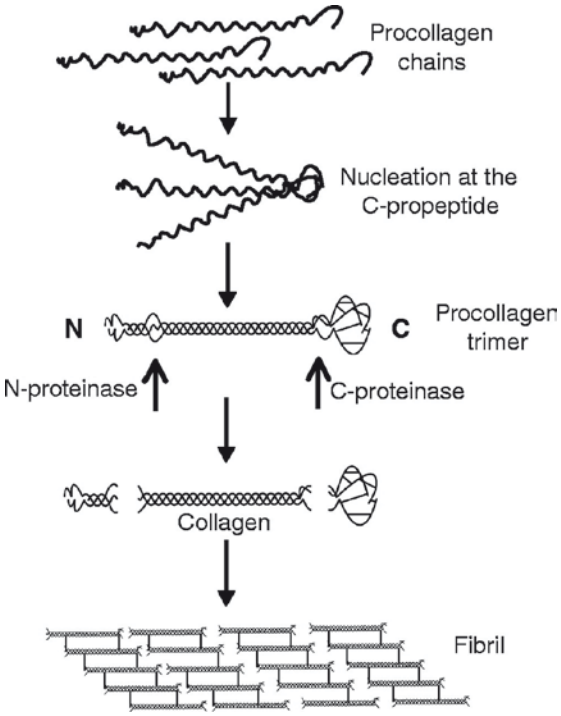

Fig. 1.2 Overview of the steps involved in the production of collagen fibrils. See text for details (Reproduced from Canty and Kadler (2005) with permission from The Company of Biologists.)

types or subtypes of collagen, a recognition system for ensuring that only the appropriate protomers are assembled has evolved. This system appears to rely almost exclusively on the COOH-terminal noncollagenous domains (Khoshnoodi et al. 2006). Once the noncollagenous domain mediates the appropriate associations amongst the three constituent chains of the protomer, the collagenous domains are able, with the help of molecular chaperones such as HSP47, to mediate self-winding into a triple helix from the COOH- to the NH2-terminus (Figs. 1.1 and 1.2), thus completing protomer assembly (Lamande and Bateman 1999). It is important to note here that in some cases, one α chain type can heterotrimerize with α chains of a different, usually closely related type (Olsen and Ninomiya 1999b).

Fibrillar Collagen

Collagen I is the prototypical fibrillar collagen. It is present in bone, tendon, ligament, skin, tooth, blood vessels, and in some interstitial matrices. There are two collagen α chains, α1 and α2, that contain ~338 Gly-X-Y repeats. Once secreted the C- and N-propeptides are cleaved (respectively) by BMP1 and members of the "a disintegrin-like and metalloprotease domain, with thrombospondin type 1 motifs"

(ADAMTS) family, such as ADAMTS2 and ADMATS3 (Le Goff et al. 2006). Collagen protomers then become capable of associating with each other to form fibrils (Fig. 1.2). For some collagens, such as type V, fibrillogenesis is regulated in part by the extent to which the N-propeptides are removed, as their continued presence influences fibril diameter (Linsenmayer et al. 1993).

Once formed, collagen fibrils are greatly strengthened by covalent cross-links that form between protomers (Robins 2007). The enzyme lysyl oxidase deaminates specific lysine and hydroxylysine side chains to form reactive aldehyde groups; these form covalent bonds with each other or with other lysine/hydroxylysine residues. In the bone, the collagen fibrils become mineralized primarily with hydroxyapatite, which is composed of calcium phosphate (Clarke 2008).

Due to the great abundance of fibrillar collagens in the body, either acquired or genetic defects in collagen assembly can have widespread effects on multiple connective tissues. One of the best known (but now infrequent) acquired diseases of collagen is vitamin C deficiency, also known as scurvy, the symptoms of which include spongy gums, bleeding from mucous membranes, skin spots, and loss of teeth (Peterkofsky 1991). Vitamin C/ascorbic acid is required as a cofactor for the activities of prolyl and lysyl hydroxylases, both of which are crucial for stable collagen fibril assembly, as discussed above. The known genetic diseases of collagen affect the chain genes themselves as well as those encoding enzymes required for collagen modification and cross-linking. Various forms of osteogenesis imperfecta (OMIM# 166200 et al.), for example, are caused by mutation of either *COL1A1* or *COL1A2*, which encode the two chains of type I collagen (Byers 2000). Affected patients have brittle, easily broken bones due to the abnormal collagen fibrils. Mutations in either *COL1* gene that removes the exon containing the N-propeptide cleavage site causes Ehlers Danlos syndrome type VII (OMIM# 130060), which is characterized by very loose and unstable joints. Others forms of this disease are caused by *ADAMTS2* and lysyl hydroxylase mutations (OMIM# 225410 and 225400, respectively).

FACIT Collagens

The FACIT collagens contain multiple collagenous domains that are separated by noncollagenous interruptions (Canty and Kadler 2005). All also contain a large NH2-terminal domain that includes a thrombospondin 1 NH2-terminal like domain immediately before the first collagenous domain, and several contain fibronectin type III repeats and von Willebrand factor A-like domains (Ricard-Blum and Ruggiero 2005). Not very much is known about the functions of the FACIT collagens, but they may serve as molecular bridges between fibrillar collagens and other components of the extracellular matrix (Shaw and Olsen 1991). The first identified member of this class is collagen IX (Ricard-Blum and Ruggiero 2005), which is comprised of $\alpha 1\alpha 2\alpha 3$(IX) heterotrimers. Mutations in *COL9A2* and *COL9A3* cause multiple epiphyseal dysplasia (OMIM# 600204 and 600969, respectively), which is characterized by short stature, stubby hands, waddling gait, and stiffness and/or pain in the knees.

Transmembrane Collagens

Whether or not transmembrane molecules are formally considered to be part of the extracellular matrix, it is clear that trimeric transmembrane proteins with collagenous ectodomains are well-suited to mediate cell–matrix interactions by associating with bona fide extracellular matrix components. These unique collagens, all of which are homotrimeric, have an NH2-terminal noncollagenous cytoplasmic tail, a transmembrane domain, and a variable number of collagenous ectodomains. The largest of these is collagen XVII, which is also known as bullous pemphigoid antigen 180 (BP180) (Ricard-Blum and Ruggiero 2005). Collagen XVII is expressed on keratinocytes and is a structural component of the hemidesmosomes that anchor the epidermis to the basement membrane. Mutations in *COL17A1* cause a relatively mild skin-blistering disease (OMIM# 226650), demonstrating its functional importance.

Other Collagens

Not all collagens can be easily grouped into structural or functional classes such as those discussed above. For example, type VII collagen is the major component of the anchoring fibrils that link the epidermal basement membrane to the underlying dermis. Collagen VII is a large homotrimer (each α chain is 2,944 amino acids) bearing large NH2-terminal and small COOH-terminal noncollagenous domains, called NC-1 and NC-2, respectively. The NC-2 domain mediates the organization of trimeric protomers into antiparallel dimers that become stabilized by disulfide bonds. A portion of NC-2 is cleaved by BMP1 outside the cell, leading to formation of mature anchoring fibrils (Rattenholl et al. 2002). The NC1 domains (one at each end of the antiparallel dimer) interacts with laminin-332 in the epidermal basement membrane, while the remainder of the anchoring fibril loops into the dermis where it interacts with components of the dermal extracellular matrix (McMillan et al. 2003). Mutations in COL7A1 cause dystrophic epidermolysis bullosa (OMIM# 131750), a disfiguring skin-blistering disease in which the epidermal basement membrane tears away from the dermis; in some cases, the esophagus is similarly affected. This disease is also frequently accompanied by epidermal cancers, and tumorigenic activity has been linked to the collagen VII NC-1 domain in these patients (Ortiz-Urda et al. 2005).

Interstitial Matrices

For the purposes of this overview, the interstitium is meant to include those tissue compartments that lie between the main functional components and serve as the “glue” that holds the tissues together. (In this sense, the interstitium can also be considered a subset of connective tissue.) For example, the interstitium of the kidney separates

the basement membranes of neighboring tubules, the interstitium of skeletal and cardiac muscles separate the individual muscle fibers, and the interstitium of the lung separates adjacent air spaces and capillaries. In addition, during embryogenesis cells lay down temporary amorphous extracellular matrices for mediating cell adhesion and migration. Although collagens and elastic fiber components are commonly found in most of these extracellular matrices, there are a sizeable number of other matrix proteins that are deposited. Many of these proteins are made by interstitial fibroblasts in mature organs or by migrating mesenchymal cells during organogenesis. They include fibronectin, tenascin, vitronectin, and secreted protein acidic and rich in cysteine (SPARC or osteonectin). Fibronectin will be discussed in some detail.

Fibronectin

Fibronectin in the extracellular matrix is primarily a fibroblast or mesenchymal cell product and is one of the most widely studied extracellular matrix proteins. Fibronectin is secreted as a disulfide-bonded dimer, but upon interacting with the appropriate cell surface integrin receptors, fibronectin undergoes conformational changes that expose hidden sites that promote its polymerization into fibrils (Kadler et al. 2008). Like other matrix proteins, fibronectin is a large modular glycoprotein (Hynes 1999; Mao and Schwarzbauer 2005). It contains the eponymous fibronectin type I, type II, and type III repeats that are present in many other secreted and transmembrane proteins. Importantly, fibronectin harbors the Arg-Gly-Asp (RGD) triplet that is a specific ligand for several integrin receptors, including α5β1, α8β1, αvβ3, and αvβ5 (Hynes 2002), which also direct fibronectin polymerization. Fibronectin therefore serves as an important mediator of diverse cell–matrix interactions.

Fibronectin is not only important for cell–matrix interactions, but it also plays a major role in organizing the components of the extracellular matrix. This is due to the fact that fibronectin is a very "sticky" protein, as it has binding sites for other extracellular proteins, including fibrin and collagen (Mao and Schwarzbauer 2005). Recent data suggests that fibronectin, together with integrins, contribute to specifying the sites of collagen fibril assembly (Kadler, Hill and Canty-Laird 2008).

The importance of fibronectin to multiple developmental processes is demonstrated by the fact that fibronectin knockout ($Fn^{-/-}$) mice die during gestation, with abnormalities in morphology evident at embryonic day 8.0 (George et al. 1993). Fibronectin is deposited widely in the developing embryo, and its absence from specific sites is associated with defects in the neural tube, in the head folds, in the heart, in the vasculature, in mesoderm and blood cell formation, and in somitogenesis (George et al. 1993). Besides these important roles in developing tissues, fibronectin is moderately abundant in soluble form in plasma, but the activity of plasma fibronectin, which is synthesized primarily by hepatocytes (Sakai et al. 2001), has remained obscure. There is evidence that it plays a role in platelet aggregation and thrombogenesis under certain circumstances (Cho and Mosher 2006).

Fibrosis

A discussion of the extracellular matrix would not be complete without at least a brief mention of fibrosis, which describes the deposition of excess extracellular matrix (usually including fibrillar collagen) within the functional compartments of tissues. This can eventually lead to scarring and tissue dysfunction and/or failure. Fibrosis can occur as a response to tissue injury, whether acquired or genetic, or, in the case of scleroderma (systemic sclerosis), can be initiated by an autoimmune response. One of the major promoters of matrix deposition in all pathological scenarios is TGF-β. Given the morbidity and mortality associated with fibrosis, TGF-β and its receptors are targets of potential antifibrotic therapies in multiple tissues, including kidney, lung, heart, muscle, skin, and liver (Varga and Pasche 2008). Successful attenuation of excess extracellular matrix deposition could therefore have an enormous impact on the treatment of diverse diseases with significant fibrotic components.

Acknowledgments This work was supported by R01DK078314 and R01GM060432 (both to JHM).

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Chapter 2
Integrin Structure and Function

Manakan Betsy Srichai and Roy Zent

Abstract Integrins are a large family of heterodimeric glycoprotein receptors first discovered over twenty years ago. They exist as two noncovalently bound a- and b- subunits that function as adhesion molecules and play key roles in many biological processes including actin cytoskeleton organization and transduction of intracellular signals regulating cellular functions. Integrins bind a variety of extracellular matrices including collagens and laminins. The phenotypes observed from the generation of integrin knockout mice have provided a wealth of information on the unique biological functions of specific integrin heterodimers. Structural data obtained from X-ray crystallography and nuclear magnetic resonance (NMR) have provided insight into the structural basis for integrin activation and subsequent transduction of bidirectional signals bidirectionally, important for controlling biological cellular functions.

Keywords Cell-Extracellular matrix interactions • Cell signaling • Cell adhesion • Cell migration

General Introduction

Integrins are a large family of type I transmembrane heterodimeric glycoprotein receptors that function as the major metazoan receptors for cell adhesion and connect the intracellular and extracellular environments. Integrins are found in organisms ranging from sponges, corals, nematodes, and echinoderms to mammals (Burke 1999). Integrins exist as two noncovalently bound α and β subunits, which pair to form heterodimers. There are 18α and 8β known subunits which combine to form at least 24 distinct integrin heterodimers (Hynes 2002). Each heterodimer consists of a large extracellular domain which binds proteins in the extracellular environment, a single-membrane-spanning transmembrane domain, and a generally

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_2,

short intracellular cytoplasmic tail domain, which forms links with the cytoskeletal elements via cytoplasmic adaptor proteins (Hynes 2002). Integrins can bind to extracellular matrix (ECM) glycoproteins including collagens, fibronectins, laminins, and cellular receptors such as vascular cell adhesion molecule-1 (VCAM-1) and the intercellular cell adhesion molecule (ICAM) family (Hynes 2002; Plow et al. 2000). In addition, integrins also play key roles in the assembly of the actin cytoskeleton as well as in modulating signal transduction pathways that control biological and cellular functions including cell adhesion, migration, proliferation, cell differentiation, and apoptosis (Schwartz et al. 1995). Since their discovery approximately 20 years ago, significant progress has been made in the integrin biology field that has resulted in a greatly improved understanding of their structure and function. In this chapter, we discuss the in vivo work with integrin knockout mice that has led to an improved understanding of the biological significance of integrins. We also discuss how the unique structure of integrins enables these cell receptors to signal bidirectionally, a critical function of integrins. Finally we will describe the fundamental mechanisms by which integrins assemble and link to the actin cytoskeleton as well how they transduce intracellular signals to modulate cellular functions.

Classification of Integrins

The specificity of integrin binding to ECM components including laminins, collagens, and fibronectin depends on the extracellular domains of the α and β integrin subunits (Fig. 2.1). Integrins α1β1, α2β1, α10β1, and α11β1 represent the primary collagen receptors (Hynes 2002; Ruggiero et al. 1996; Camper et al. 1998; Velling et al. 1999); integrins α3β1, α6β1, α6β4 and α7β1 are the major laminin receptors (Tashiro et al. 1999); and integrins α5β1, α8β1, αIIbβ3 and the αvβ integrins are the major fibronectin receptors that bind in an RGD-dependent manner (van der Flier and Sonnenberg 2001). Redundancy exists with respect to certain integrin–ECM interactions as some integrins bind the same extracellular ligands albeit at different affinities and conversely, some ligands are recognized by different integrins. For example integrins α1β1, α2β1, α10β1, and α11β1 all bind collagen, yet integrin α1β1 also binds laminins. Most integrin heterodimers are widely expressed in many tissues; however, some are more restricted in their expression. For example, αIIbβ3 is only found on platelets, α6β4 on keratinocytes, and αEβ7, α4β7, α4β1, and the β2 integrin families are restricted to leukocytes (Takada et al. 2007).

Phenotypes of Integrin Knockout Mice

Integrins play diverse and important roles in most biological processes (Hynes 2002). Mice that lack integrin expression either constitutively or in specific cell types exhibit a wide range of phenotypes. These knockout mice have provided

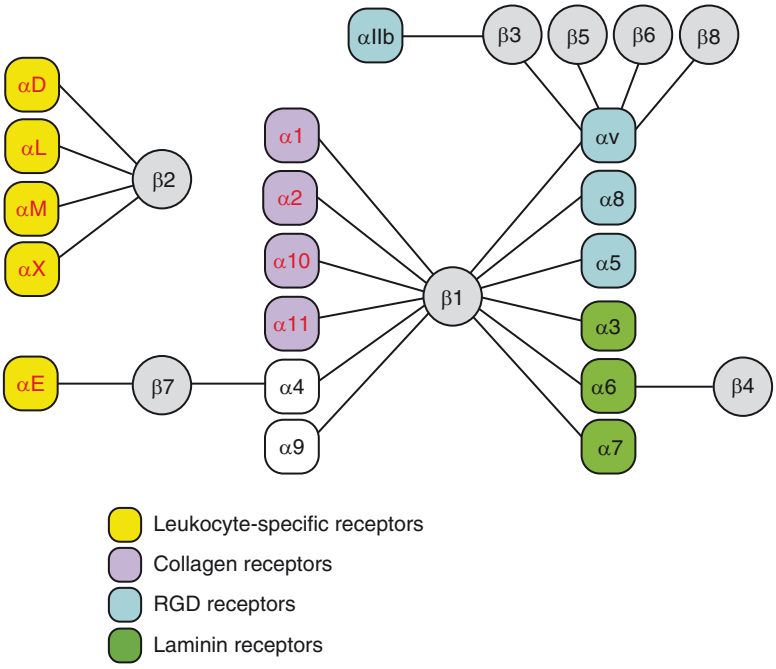

Fig. 2.1 Classification of integrin family of heterodimers. The nine α domains with inserted (I) domains (1, 2, 10, 11, D, L, M, X, E) are indicated in red

much insight into the functions of specific integrin heterodimers, reflecting the unique roles of the various integrins. In general, each of the 24 integrins has a specific, nonredundant function. Genes for each of the β subunits and all but two of the α subunits have been deleted in mice (Table 2.1). Integrin β1 is ubiquitously expressed and can bind multiple α partners, and thus it is not surprising that knockout of β1 results in embryonic lethality due to a complete block in preimplantation development. In contrast, knockouts of α1, α2, α10, and α11 integrin subunits, which each exclusively heterodimerize with β1 to function as primary collagen receptors, are all viable and fertile but possess distinct characteristic abnormalities. For example, α1 integrin knockout mice develop increased collagen synthesis and display reduced tumor vascularization (Gardner et al. 1999; Pozzi and Zent 2003), while the α2 integrin knockout mouse has a more subtle phenotype with mild platelet function abnormalities and increased vascularization following wounding (Chen et al. 2002). These findings support the idea that there is significant redundancy and compensation amongst the collagen receptor integrins. In contrast when the primary laminin-binding (α3β1, α6β1/α6β4, α4β7) and the primary RGD-binding integrins (α5β1, α8β1, αv and αIIbβ3) are deleted, the phenotypes are more severe, suggesting less redundancy and compensation.

Table 2.1 Classification of the phenotypes in integrin knockout mice

Gene		Phenotype
α_1	V,F	Increased collagen synthesis, reduced tumor vascularization
α_2	V,F	Few developmental defects. Delayed platelet aggregation
α_3	L,birth	Defects in kidneys, lungs, and cerebral cortex; skin blistering
α_4	L, E11–E14	Defects in chorioallantois fusion and cardiac development
α_5	L,E10	Defects in extraembryonic and embryonic vascular development
α_6	L,birth	Defects in cerebral cortex and retina; skin blistering
α_7	V,F	Muscular dystrophy
α_8	L+V/F	Small or absent kidneys; inner ear defects
α_9	L, perinatal	Bilateral chylothorax
α_{10}	V,F	Dysfunction of growth plate chondrocytes
α_{11}	V,F	Dwarfism most likely resulting from severely defective incisors
α_v	L,E12-birth	Defects in placenta and in CNS and GI blood vessels; cleft palate
α_D	…	No reported knockout available
α_L	V,F	Impaired leukocyte recruitment and tumor rejection
α_M	V,F	Impaired phagocytosis and PMN apoptosis; obesity; mast cell development
α_X	…	No reported knockout available
α_E	V,F	Inflammatory skin lesions
α_{IIb}	V,F	Defective platelet aggregation
β_1	L,E5.5	Inner cell mass deterioration
β_2	V,F	Impaired leukocyte recruitment; skin infections
β_3	V,F	Defective platelet aggregation; osteosclerosis
β_4	L,perinatal	Skin blistering
β_5	V,F	No apparent phenotype
β_6	V,F	Skin and lung inflammation and impaired lung fibrosis
β_7	V,F	Abnormal Peyer's patches; decreased no. of intraepithelial lymphocytes
β_8	L,E12-birth	Defects in placenta and in CNS and GI blood vessels; cleft palate

Table adapted from Bouvard/Fassler et al. (2001 Circ Res)
V, viable; F, fertile; L, lethal; L+V/F, disrupted development in some but survival in a fraction of others

Mutations in integrin subunits have been found to cause clinical disorders in man and these correlate well with mice in which the same integrins are deleted. There are three well-described inherited autosomal recessive diseases in humans linked to germline mutations in integrin subunits. Mutations in αIIb and β3 integrin subunits are associated with Glanzmann's thrombasthenia, a clinical entity associated with platelet dysfunction and bleeding disorders (Hogg and Bates 2000). Both the β3 and αIIb knockout mice exhibit features of defective platelet aggregation similar to that observed in Glanzmann's thrombasthenia (Hogg and Bates 2000; Tronik-Le Roux et al. 2000; Hodivala-Dilke et al. 1999). Point mutations and gene deletion in β2 integrin in humans and mice, respectively, have been associated with Leukocyte Adhesion Deficiency (LAD) (Hogg and Bates 2000; Scharffetter-Kochanek et al. 1998). Finally mutations in α6 and β4 integrin result in junctional epidermolysis bullosa with skin blistering (Pulkkinen et al., 1997, 1998; Ruzzi et al. 1997; Takizawa et al. 1997; Vidal et al. 1995) and a similar phenotype is seen in mice lacking these integrins (Georges-Labouesse et al. 1996; van der Neut et al. 1996).

Structure

Each subunit of the integrin αβ heterodimers contains a large extracellular domain, a single-spanning transmembrane domain, and a short cytoplasmic tail (with the exception of β4). Over the past decade, we have begun to recognize and understand better the importance of integrins in mediating adhesion and signaling. This recognition has yielded the emergence of important structural information of integrin conformations. Solving high-resolution structures of integrins has proved challenging, particularly because integrins are large membrane proteins, and thus difficult to purify. Many years of effort have been devoted to understanding and characterizing integrin structure, and knowledge of the structural basis for integrins has helped gain insight to further characterize the mechanisms underlying integrin activation.

Extracellular Domain

The extracellular domain of integrins are generally large, ~80–150 kDa structures. Most of the structural data of the extracellular domains comes from high-resolution X-ray crystallography. The first extracellular domain crystal structure solved was the inserted (I)-domain from αMβ2 in 1995 (Lee et al. 1995). It took six additional years to solve the first complete extracellular domain of an integrin (αvβ3) (Xiong et al. 2001). The extracellular portion of the α and β subunits are comprised of several subdomains organized into a globular ligand-binding N-terminal head domain standing on two long and extended C-terminal legs that connect to the transmembrane and cytoplasmic domains of each respective subunit (Fig. 2.2) (Nermut et al. 1988).

The α subunit head consists of a folded seven-bladed β propeller head domain, a thigh domain and two calf domains (Xiong et al. 2001; Springer 1997). Half of the α subunits contain an additional inserted (I)-domain of ~200 amino acids that is inserted within the β propeller domain. When present, the α I-domain represents the exclusive extracellular-binding site for ligands. The α I-domain contains a conserved "metal-ion-dependent adhesive site" (MIDAS) that binds divalent metal cations (Mg^{2+}) and plays important roles in protein ligand binding. Ligand binding alters the coordination of the metal ion and shifts the I-domain from a closed, resting state to an open, active conformation which results in increased ligand affinity and promotes subsequent integrin activation (Liddington and Ginsberg 2002). This mode of activation is analogous to small G proteins, whereby GTP hydrolysis leads to altered coordination of a Mg^{2+} ion and subsequent conformation changes.

The β subunit is composed of an I-like domain, which is structurally similar to the I-domain in α subunits, a PSI (plexin/semaphorin/integrin) domain, a hybrid domain, four EGF repeats, and a membrane proximal β tail (βTD) domain. The β subunit plays important roles in ligand binding in α subunits which lack the I-domain. In these integrin heterodimers, ligands bind to a crevice in the head domain between the αβ subunit interfaces. The ligand interacts with a metal-ion-occupied MIDAS located within the β subunit and the propeller domain of the α subunit.

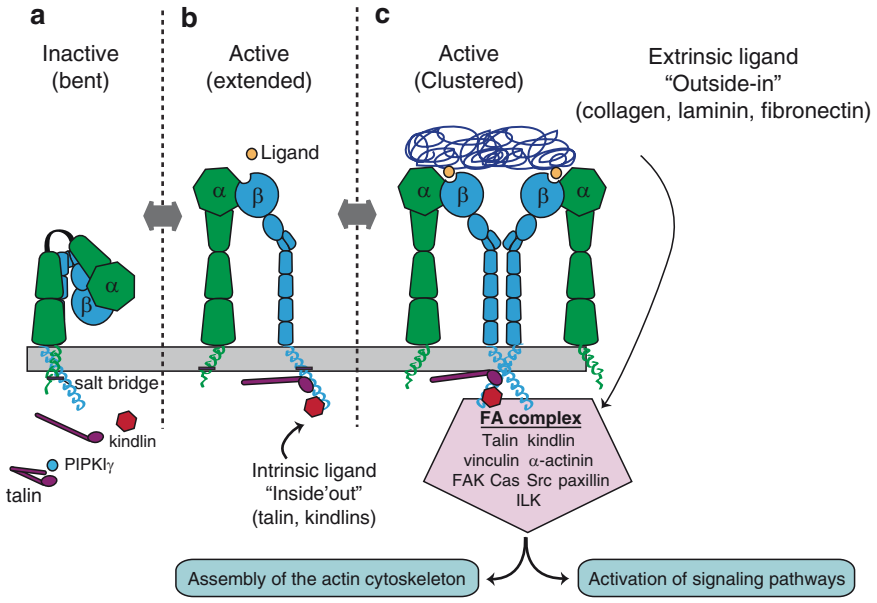

Fig. 2.2 Integrin structure of α subunits lacking the I-domain. **a** Integrins in their unbound, inactive resting state. In this state integrins are in a bent conformation and the transmembrane and cytoplasmic regions are closely associated. **b** Once activated by talins and kindlins, there is separation of the cytoplasmic and transmembrane subunits and extension of the integrins extracellular domains. Extracellular ligand binding can occur in this conformation. **c** When activated integrins bind to ligand, they cluster at the plasma membrane. Clustering is necessary to send intracellular signals to form tight focal adhesions (FA), important for actin cytoskeletal assembly and activation of further downstream signals to control various cellular functions

Transmembrane Domain

The transmembrane (TM) domains of integrins are single spanning structures comprised of ~25–29 amino acid residues that form α-helical coiled coils that either homo- or heterodimerize (Adair and Yeager 2002). Unlike integrin extracellular domains, no high-resolution experimental X-ray crystal structures are available for the TM domain of any integrin heterodimer, and much of the structural data are based on NMR analysis. Structural information from the αIIbβ3 heterodimer TM domains have only recently been solved in their entirety (Adair and Yeager 2002; Lau et al., 2008a, 2008b, 2009). The αIIb TM domain is a 24 residue α-helix followed by a backbone reversal that lacks a significant helix tilt (Lau et al. 2008a). The distal GFF motif (depicted in bold in Figure 2.3) is highly conserved in the 18 human integrin α subunits and likely plays an important role in the transition from the resting to active states. The β3 TM domain is a 30-residue linear α helix that is somewhat longer than the width of a typical lipid bilayer, implying that a pronounced helix tilt is present within the plasma membrane (Lau et al. 2008b).

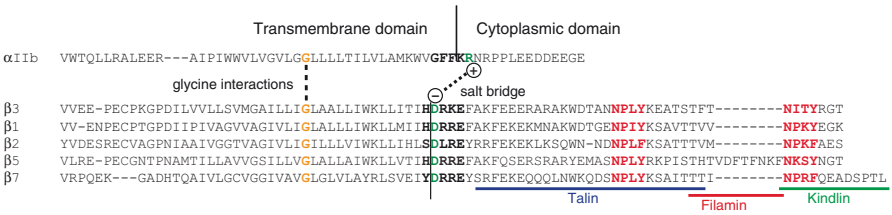

Fig. 2.3 Sequence homology for integrin transmembrane and cytoplasmic domains. The α and β subunits in the transmembrane domains are tightly packed through glycine–glycine interactions present within the sequence (gold). The putative salt bridge present at the membrane proximal region of the cytoplasmic domain is depicted in green. Talins and kindlins bind the proximal NpxY motif and more distal NxxY motifs, respectively, shown in red

Inactive integrin TM subunits are tightly packed and form a complex consisting of a coiled-coil interaction between canonical GxxxG dimerization motifs within the TM domains (depicted in gold in Fig. 2.3) (Gottschalk 2005).

Cytoplasmic Domain

Integrin cytoplasmic domains are generally short, largely unstructured and comprised of 10–70 amino acid residues, with the exception of the β4 subunit which contains >1,000 amino acid residues. Like TM domains, no high-resolution X-ray crystal structures of the cytoplasmic domains have been solved, and much of the structural information is also based on NMR data. Only the structures of the αIIbβ3 cytoplasmic tails have been characterized under heterodimeric conditions (Li et al. 2001; Ulmer et al. 2001; Vinogradova et al. 2002; Weljie et al. 2002; Vinogradova et al. 2004). β cytoplasmic tails are highly homologous, while α subunit tails are highly divergent. The conserved GFFKR and HDR(R/K)E sequences located in the membrane proximal regions of the α and β subunits, respectively, are proposed to form a salt bridge between arginine (R) from the α subunit and aspartic acid (D) from the β subunit (Adair and Yeager 2002; Vinogradova et al. 2000). In αIIbβ3 integrins, the salt bridge represents a physical interaction between αIIβ and β3 cytoplasmic tails (depicted in green in Fig. 2.3). Although not confirmed by structural data from other integrin heterodimers, the salt bridge is thought to be present in other integrin pairs and generally believed to function to maintain integrins in the inactive, low-affinity state. Disruption of the salt bridge in β3 integrins has been shown to play key roles in regulating integrin activation states (Hughes et al., 1995, 1996), however, in β1 integrins, disruption of the salt bridge via a point mutation in the aspartic acid residue of β1 did not result in a major phenotype in mice. These findings suggest that the salt bridge might not play a major role in β1 integrin activation (Czuchra et al. 2006).

Within the β integrin tails are two well-defined motifs: a membrane proximal NpxY and a membrane distal NxxY motif (depicted in red in Fig. 2.3). These motifs represent canonical recognition sequences for phosphotyrosine-binding (PTB) domains (Calderwood et al. 2003) and serve as binding sites for multiple integrin binding proteins, including talin and the kindlins.

Integrin Signaling

Integrins are able to transduce signals intracellularly following ligand binding ("outside-in" signaling). However, unlike most other cell receptors, integrins can shift between high- and low-affinity conformations for ligand binding ("inside-out" signaling). Depending on the cell type, integrins can be either basally activated, as with most adherent cells that are attached to a basement membrane, or basally inactive, as with platelets or leukocytes that freely circulate until activated to undergo platelet aggregation or mediate an inflammatory response, respectively. The αIIbβ3 integrin heterodimers located on the cell surface of platelets represent the best-characterized basally inactive integrins. In these cells, integrins exist in a low-affinity state with respect to extracellular ligand binding and rely on intracellular signaling to become activated.

Integrins themselves have no kinase activity but instead provide a connection between the extracellular matrix and the actin cytoskeleton. This connection allows integrins to regulate cytoskeletal organization and cell motility as well as to alter fluxes of many intracellular-signaling pathways including cell survival, cell proliferation, cell shape, and angiogenesis. The extracellular domains can bind a variety of ligands, whereas the intracellular cytoplasmic domains anchor cytoskeletal proteins. This linkage between the cell exterior and interior allows for bidirectional signaling across the plasma membrane.

Inside-Out Signaling

Inside-out signaling or integrin activation is important in physiological situations such as in the blood, where cells are in close proximity to their ligands, yet cell–ligand interactions occur only following integrin activation in response to specific external cues such as injury to the vasculature or the induction of inflammation (Ratnikov et al. 2005). This characteristic of integrin regulation is also important in the developmental processes when cells are required to migrate for specific periods of time during morphogenic processes.

In the normal resting, inactive state, integrin extracellular domains are unbound to ligands and exist in a bent conformation. Activation signals from within the cell induce straightening of the extracellular domains and stabilize the extended, active conformation. This conformational change exposes the external ligand-binding site to

which ligands bind, allowing the transmission of signals from the outside to the inside (Fig. 2.2). The exact changes that occur in the extracellular head domain once integrins undergo conformational change to the high affinity state are still unclear. Two models of conformational change have been proposed. In the "switchblade" model (Luo et al. 2007), only extended integrins are predicted to bind ligand. In contrast is the "deadbolt model" (Xiong et al. 2003), which suggests that integrin extension occurs only after ligand binding has occurred. In both models conformational changes within the head domain facilitate ligand binding (Luo et al. 2007; Arnaout et al. 2007).

The TM domains play key roles in integrin activation. Separation of integrin TM domains is generally believed to be a requirement for integrins to adopt the high affinity state. Several possible models exist for disruption of TM domains (Wegener and Campbell 2008). In the piston model, vertical movements of the TM domains cause a shift in the domains, while a scissors model suggests there is an increase in the angle of the TM domains. Finally there is a separation model that predicts a physical separation of the two TM subunits. Regardless of the model, each result in disruption of the interaction between the two subunits, facilitating conformational changes in both the extracellular or cytoplasmic domains and subsequent integrin activation (Wegener and Campbell 2008). Recent data have shown that the dissociation of the α and β subunits in the TM complex is central to the extracellular conformational changes associated with integrin signaling (Lau et al. 2009).

The role of integrin cytoplasmic tails in the regulation of integrin affinity has been extensively examined in the rapidly activated αIIbβ3 and β2 integrin families (Wegener et al. 2007). A large number of cytoskeletal and signaling proteins bind to integrin cytoplasmic tails, however, only two proteins, talin and the kindlins, which both bind to β integrin cytoplasmic tails, have been demonstrated to be important for separation of the cytoplasmic tails and subsequent integrin activation. The membrane proximal NpxY motif is required for the binding of the cytoskeletal protein talin (Calderwood et al. 2003). The membrane distal NxxY motif is required for the binding of kindlins, which have recently been described to play a role in integrin activation (Montanez et al. 2008; Moser et al. 2008). The following sections discuss how talin and kindlins are thought to regulate integrin affinity.

Talin and Kindlin Binding are Required for Integrin Activation

Talin is an actin-binding protein that binds the proximal NpxY motif on β cytoplasmic tails and plays important roles in integrin activation and linkage to the actin cytoskeleton. Talins are large ~270 kDa proteins comprised of an N-terminal head domain (~47 kDa) and a large tail rod region (~220 kDa). The head domain is comprised of a FERM (4.1, ezrin, radixin, moesin) domain containing three subdomains (F1, F2, F3) which binds integrin cytoplasmic tails. The tail region contains multiple binding sites for vinculin as well as an additional binding site for integrin cytoplasmic tails. Talin is an essential mediator of integrin activation and has been shown to induce activation of the normally inactive platelet integrin αIIbβ3

(Calderwood et al. 1999, 2002). Integrin activation by talin is thought to occur by competition with the αIIb tail for binding to the β3 tail on αIIbβ3 integrin. The F3 subdomain of talin resembles a phosphotyrosine binding (PTB) domain and binds the membrane proximal NpxY motif of β integrin tails, leading to destabilization of the putative integrin salt bridge. The net result is a change in the position of the TM helix, leading to a packing mismatch within the αIIbβ3-TM helix, and separation or reorientation of the integrin tails (Luo et al. 2004, 2005, 2007; Yin et al. 2006; Loh et al. 1996; Li et al. 2003, 2004, 2005; Partridge et al. 2005). These events result in conformational change of the extracellular domain, leading to increased ligand affinity and integrin activation. Although other PTB domain-containing proteins (Dok1, tensin) can also bind the proximal NpxY motif on β tails, only talin modulates integrin activation. This is thought to be because the talin F3 subdomain binds to an additional, more proximal site on β integrin tails, which induces displacement of α and β integrin tails (Wegener et al. 2007).

As integrin activation is strictly controlled, the binding of talin to integrins is tightly regulated. Under normal physiologic conditions, talin exists in an autoinhibited state, unable to bind integrins. The talin C-terminus interacts with the talin PTB domain, blocking the integrin-binding pocket. Additionally, talins also homodimerize (Goldmann et al. 1994) and the intermolecular interaction in turn also masks integrin-binding sites (Ratnikov et al. 2005). The mechanisms of talin activation are unclear but likely involve the phosphotidylinositol-4,5-bisphosphate (PIP2) lipid second messengers. Talin binds and activates PIPKIγ which results in increased production of PIP2. PIP2 regulates talin, vinculin and other focal adhesion (FA) proteins, which leads to integrin activation, reinforcement of the cytoskeleton, intracellular signaling, and FA formation. In addition talin–integrin interactions are also controlled through tyrosine phosphorylation of the NpxY motif of β integrin tails by src family kinases (Law et al. 1996; Sakai et al. 2001).

Kindlins have recently been shown to play an important role in integrin inside-out signaling. Kindlins are a novel family of evolutionarily conserved adaptor proteins named after the gene mutated in Kindler syndrome in humans, a rare disease caused by mutations in kindlin-1 and characterized by skin blistering. Kindlin-mediated integrin activation requires a direct interaction between kindlin and β integrin tails. Like talin, kindlins also contain a FERM domain that has high levels of sequence similarity to talin FERM domains (Kloeker et al. 2004) and also represents the site of binding to β integrin tails. Kindlins bind the distal NxxY motif on β1, β2 and β3 integrin tails (Montanez et al. 2008; Moser et al. 2008; Ma et al. 2008; Shi et al. 2007; Ussar et al. 2008; Moser et al. 2009) (Fig. 2.3) but additional sequences may also be involved in kindlin binding. Although the intervening sequence between the two NxxY motifs in the β1 and β3 integrin cytoplasmic tails are dispensable for talin binding, mutation of a double Thr or Ser/Thr within this sequence impairs kindlin binding (depicted in blue in Fig. 2.3) (Moser et al. 2008).

Both kindlins and talin bind distinct regions of the β integrin tails and together cooperate to regulate integrin affinity (Montanez et al. 2008; Moser et al. 2008;

Calderwood et al., 1999, 2002; Ma et al. 2008; Moser et al. 2009). Kindlins facilitate talin function, and expression of kindlin alone is insufficient to shift integrins to a high affinity state. In vitro studies have demonstrated that the amount of talin expressed in cells determines the efficacy of kindlins in promoting this function (Ma et al. 2008). Conversely, talin depends on kindlins to promote integrin affinity (Montanez et al. 2008). Thus both kindlins and talin are required to increase integrin affinity and promote integrin activation.

Outside-In Signaling

Integrins themselves lack intrinsic catalytic activity. Ligand binding to the extracellular domain of integrins results in signal transduction to the cytoplasm in the classical direction from the outside-in. These intracellular signals affect cellular growth, differentiation and apoptosis. Integrin signaling is complex and significantly influenced by crosstalk with growth factor receptors. Further, the intracellular signals generated lead to the assembly of the FA complex, a large, dynamic multiprotein complex involving over 150 intracellular proteins (Zaidel-Bar et al. 2007). FAs serve as the hub for transmission of intracellular signals. Within FAs, proteins are in constant flux, continually associating and dissociating with each other. The pathways following activation of multiple signaling cascades are complex and a schematic detailing of the complexities involved is shown in Fig. 2.4.

Integrins are Essential to FA Formation and Assembly of the Actin Cytoskeleton

Extracellular ligand binding leads to integrin clustering in the plane of the plasma membrane and the generation of tight FAs which promotes assembly of actin filaments. Actin filaments are reorganized into larger stress fibers leading to further integrin clustering and enhanced matrix binding (Giancotti and Ruoslahti 1999). In this manner, integrin linkage to the actin cytoskeleton allows regulation of FA growth as well as regulation of cell shape via the spatiotemporal control of cell protrusion and retraction during cell migration. Several key FA proteins are involved in establishing and maintaining the integrin–cytoskeleton linkage. These include (1) integrin-bound proteins that can directly bind to actin (talin, α-actinin, filamin); (2) integrin-bound proteins that indirectly bind the cytoskeleton (kindlins, integrin-linked kinase (ILK), paxillin, FAK); (3) non-integrin-bound actin-binding proteins (vinculin); and (4) adaptor and signaling molecules that regulate the interactions of the proteins from the above-mentioned groups. It is beyond the scope of this chapter to review in detail the complex machinery involved in the assembly of the actin cytoskeleton and the reader is referred to several recent excellent reviews (Geiger et al. 2009; Legate et al. 2009).

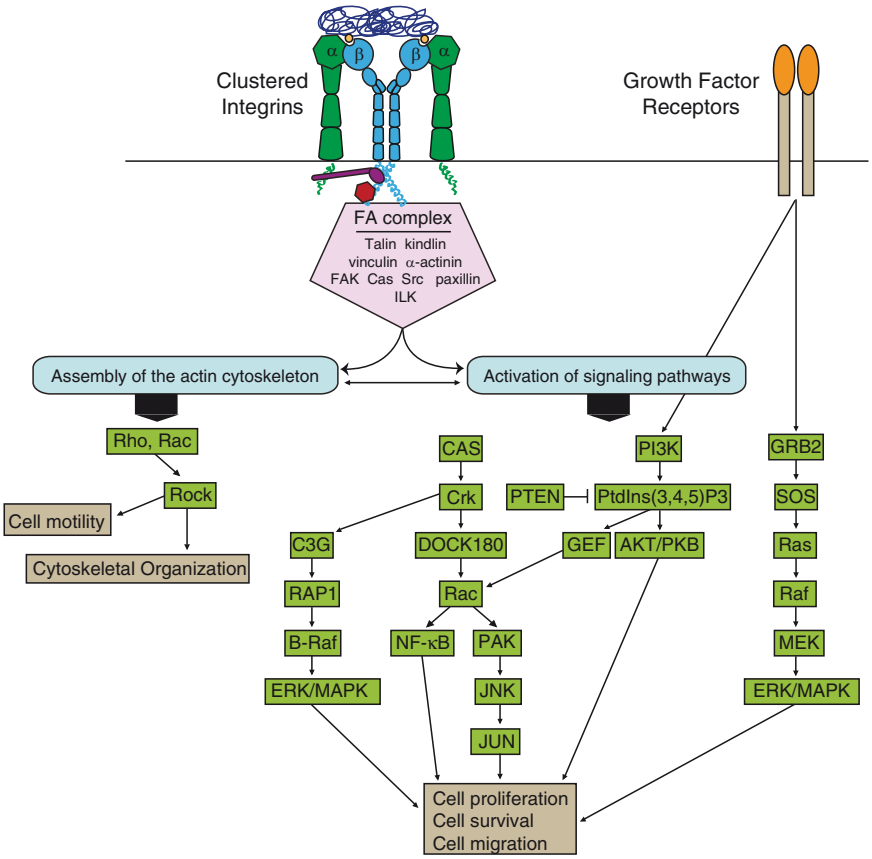

Fig. 2.4 Integrins cluster and play a role in formation of the FA complex which mediates downstream cell processes including assembly of the actin cytoskeleton and activation of downstream-signaling pathways. The signaling pathways are complex and roughly divided into the Src–FAK, Ras–MEK–MAPK, and Akt/PI3K signaling pathways. There is significant cross-talk at the level of integrin and growth factor receptors. There is also cross-talk present at the level of Src–FAK which regulates signals for actin cytoskeletal assembly and other downstream signaling pathways

Talin

The initial integrin–cytoskeleton linkage following integrin ligation involves the recruitment of talin to β integrins. In contrast to integrin activation, which requires only the FERM domain in the talin head region, integrin–cytoskeletal linkage requires both the head and rod domains of talins. Talin provides a crucial connection to the actin cytoskeleton as mice with deletions of talin-1 die during gastrulation due to a defect in cytoskeletal organization and cell migration (Monkley et al. 2000). Further evidence for the importance of talin in cytoskeletal organization comes from flies lacking talin. In these animals integrins are able to associate with the ECM but are unable to link to the cytoskeleton, thus resulting in muscle detachment (Brown et al. 2002).

Vinculin

Following talin binding, proteins such as vinculin are recruited to the nascent focal adhesion. Vinculin does not bind integrins directly; rather it binds to several sites on the talin rod and to actin and is thought to act as a crosslinker that stabilizes the talin–actin interaction (Gallant et al. 2005; Humphries et al. 2007). Vinculin knockout fibroblasts make fewer and smaller FAs which are unable to mature (Saunders et al. 2006), providing evidence that vinculin is required to reinforce the link between integrins and the actin cytoskeleton.

α-Actinin

α-Actinin, a binding partner of both talin and vinculin, is another critical protein that links integrins to the cytoskeleton. α-actinin can directly bind β integrins and operates as a close partner of actin and has been shown to have an essential role in adhesion strengthening (Brakebusch and Fassler 2003). Flies lacking α-actinin die due to defects in muscle structure and function (Fyrberg et al. 1998) and mutations of the 4 α actinin isoforms in mice result in abnormalities in different organs depending on the isoform deleted (Craig et al. 2007; Weins et al. 2007).

Integrin-Linked Kinase

Integrin-linked kinase (ILK) is another important scaffolding protein which links integrins to the actin cytoskeleton. ILK is a multidomain adaptor protein that directly binds β1 and β3 integrin tails and indirectly associates with actin through its main binding partner, parvin. ILK also binds to the cytoskeleton through its associations with paxillin, which also binds parvin and vinculin (Legate et al. 2006). Cells deficient in ILK demonstrate severe delays in focal adhesion formation resulting in defective cell spreading (Sakai et al. 2003). Deletion of ILK from the skeletal muscle results in detachment of basement membranes and accumulation of extracellular matrix, providing further evidence that ILK plays critical roles in stabilizing the integrin–actin interaction (Wang et al. 2008).

Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase signaling protein that most likely interacts indirectly with β integrins through its association with paxillin (Geiger et al. 2009; Brakebusch and Fassler 2003). Although not required for nascent FA formation or for the initial connection of integrins to the actin cytoskeleton, FAK is required to stabilize the linkage to actin by modulating the affinity of α-actinin to actin (Ilic et al. 1995), which is thought to occur via FAK-mediated phosphorylation of α-actinin. In addition to cytoskeletal stabilization, FAK also plays an essential role in promoting FA turnover (Ilic et al. 1995).

Kindlins

Kindlins, which were discussed earlier, directly bind β1 and β3 integrins and connect them to the actin cytoskeleton via a migfilin–filamin interaction as well as through the ILK–parvin complex (Larjava et al. 2008). Mouse platelets lacking kindlin-3 are unable to organize their cytoskeleton or establish stable lamellipodia (Moser et al. 2008), providing evidence for the importance of the kindlin interactions with the cytoskeleton. In addition loss of kindlin-2 results in early embryonic lethality and impaired actin polarization (Montanez et al. 2008), and loss of kindlin-1 results in shear-induced detachment of intestinal epithelial cells (Ussar et al. 2008).

Paxillin and Tensin

Paxillin and tensin are two important integrin–actin regulatory proteins. Paxillin is detected early in nascent adhesions at the leading edge of the cell. Paxillin is a structural protein that allows numerous simultaneous interactions, which are further modified by phosphorylation (Turner 2000). Interestingly, paxillin may also link talin to the cytoplasmic tail of α integrins, thereby increasing the stability of the integrin–talin–actin interaction (Alon et al. 2005). Tensin is recruited to FAs at a later stage than paxillin and couples integrins to actin (Zamir and Geiger 2001). Tensin also interfaces with signaling pathways through binding tyrosine-phosphorylated FAK, p130Cas, epidermal growth factor receptor (EGFR), the Akt kinase PDK1, and the RhoGAPs (Legate et al. 2009).

Integrins Play a Role in Actin Polymerization and Cytoskeletal Dynamics

The physical link between integrins and actin is required for local regulation of actin polymerization as well as for global control of cytoskeletal dynamics. The underlying mechanisms are not fully understood; however isolated integrin adhesions contain the complete machinery necessary for actin polymerization (Butler et al. 2006). This includes the Arp2/3 complex, which controls the assembly of a branched actin filament network in the lamellipodium through its actin nucleation function, and the Rho GTPases, which play key roles in the global regulation of actin dynamics.

Arp2/3 complex

The Arp2/3 complex is a seven-subunit protein that plays a major role in regulation of the actin cytoskeleton by stimulating actin polymerization. Arp2/3 complex has no endogenous actin nucleating activity and must be activated by the Wiskott–Aldrich Syndrome protein (WASP)/Scar family of activator proteins (Pollard 2007). Arp2/3 is recruited to nascent integrin adhesions through interactions with FAK and vinculin to promote actin polymerization, which in turn generates the protrusive force for the lamellipodium. FAK and vinculin are required for normal lamellopodium formation.

Rho GTPases

Rho GTPases play key roles in the global regulation of actin dynamics. The Rho GTPases are a family of ~20 signaling proteins that cycle between an active GTP-bound form and an inactive GDP-bound form (Jaffe and Hall 2005). Cycling is regulated by three sets of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). In addition to regulating actin dynamics Rho GTPase proteins also play a role in cell proliferation, apoptosis, gene expression, and other multiple common cellular functions. Recruitment and activation of GEFs and GAPs at FAs regulate and control the abilities of Rho GTPases to establish cell polarity and directional migration (Jaffe and Hall 2005). The most important GTPases for the regulation of actin dynamics at FAs are Rac, Cdc42, and RhoA, which are described below.

Rac

The small GTPase, Rac, is thought to be a principal regulator of lamellipodium formation. Ligation and clustering of integrins leads to membrane targeting of Rac and recruitment of multiple GEFs via signaling complexes such as the FAK–p130Cas–Crk–DOCK180 pathway and the paxillin–GIT–PIX pathway. Rac can regulate actin polymerization by activating the Arp2/3 complex via the Scar/WAVE proteins. Rac also increases the availability of free actin-barbed ends by the removal of capping proteins as well as increases the availability of actin monomers by regulating cofilin (Jaffe and Hall 2005). Rac also feeds back to integrins by promoting recruitment and clustering of activated integrins at the edge of the lamellipodium (Kiosses et al. 2001).

Cdc42

Cdc42 (cell division cycle 42) is a cell cycle protein primarily thought of as a regulator of cell polarity in epithelial cells and neurons but not in mesenchymal cells. Cdc42 activity has been shown to be regulated by Src activity and ligand binding to integrins can activate Cdc42 via the ILK–Pinch–parvin complex (Legate et al. 2006). Cdc42 regulates polarity by directly binding WASP, which then activates Arp2/3 and subsequent lamellipodium formation, as well as by orienting the microtubule-organizing center and Golgi apparatus in front of the nucleus, toward the leading edge (Jaffe and Hall 2005). It is thought that Cdc42-induced filopodia act as precursors for Rac-induced lamellipodia, establishing a temporal hierarchy of integrin-dependent Rho GTPase activation in the regulation of cell spreading and migration (Guillou et al. 2008).

RhoA

RhoA is another small GTPase which activates two key effector pathways and is primarily thought of as a regulator of cell contractility. RhoA activates the Rho kinase pathway, which acts downstream to activate ROCK and promote contractility through

the phosphorylation of the regulatory light chain of myosin II. RhoA also activates the diaphanous-related forming (DRF) protein family, which regulates actin bundling and microtubule stability (Fukata et al. 2003). Thus initial phases of cell adhesion or lamellipodial protrusion require suppression of RhoA activity and thus RhoA is activated only when contraction is required to retract the trailing edge of cells. Activation and inactivation of RhoA is regulated by integrin-dependent signaling.

Integrins Signal from the Outside-In and Crosstalk with Growth Factors to Control Downstream Intracellular-Signaling Pathways

Integrins are unique, for they can signal from localized stimuli captured from their interactions with ECM as well as integrate mechanical and chemical signals due to their direct association with the cytoskeleton. Furthermore, integrins are able to crosstalk with growth factor receptors and integrins are in fact required for many growth factor receptors to function (Legate et al. 2009). For example growth factor stimulation and integrin-mediated adhesion together increase the intensity and duration of extracellular signal-regulated kinase (ERK) activation. Furthermore phosphorylation of the epidermal growth factor (EGF) receptor is altered more when activated by cell adhesion compared to when stimulated by EGF ligand alone. This implies that integrin activation induces clustering of growth factor receptors including the EGF-, platelet-derived growth factor (PDGF)-, and fibroblast growth factor (FGF)-receptors.

Signaling pathways activated downstream of the integrins are extremely complicated and differ depending on the cell types and physiologic conditions. Nevertheless some key molecules are recognized to be important in the formation of signaling complexes that initiate transduction of integrin-dependent signaling.

Src–FAK-Signaling Complex

One of the key complexes required to initiate integrin-dependent signaling is the Src–FAK complex. Src is a nonreceptor tyrosine kinase constitutively associated with the cytoplasmic tail of β3 integrins via its SH3 domains (Harburger and Calderwood 2009). Src is a member of the src kinase family, which also includes the ubiquitously expressed Src, Fyn, and Yes proteins. All Src proteins are regulated by a C-terminal tyrosine residue that, when phosphorylated, binds to the SH2 domain to autoinhibit kinase activity. Clustering of integrins induces Src activation. The nonreceptor tyrosine kinase FAK is activated by integrin ligation to induce autophosphorylation on tyrosine residue 397. This induces an interaction with Src that stabilizes the active conformation of Src, leading to increased catalytic activity. Subsequently, additional tyrosines on FAK are phosphorylated, resulting in full activation of both kinases (Schlaepfer and Hunter 1996; Calalb et al. 1995). FAK can also be regulated by the receptors for growth factors including EGF and FGF.

Thus FAK also serves as an important intersection point for integrins and growth factor receptors. (Parsons 2003).

Ras–MEK–MAPK-Signaling Pathway

A major signaling pathway downstream from the Src–FAK complex is the Ras–MEK–MAPK-pathway, which is activated by integrated signals from integrins and growth factors. Phosphorylation of the downstream kinases, MAPK and ERK2, modulates the dynamics of the focal adhesions as well as cell proliferation, cell cycle progression, and survival (Walker et al. 2005). Integrin signaling can influence MAPK activation on multiple levels. The Src–FAK complex can activate PAK1, which in turn phosphorylates and activates MEK1. MEK1 is required for adhesion-mediated signaling to proceed to MAPK activation and thus serves as a critical convergence point between growth factor and integrin signaling (Slack-Davis et al. 2003). Another intersection point of these two pathways occurs at the level of Raf1. Thus there are multiple ways by which the Src–FAK complex regulates ERK and this regulation is cell-type-specific since deletion of FAK leads to proliferation defects in mammary epithelial cells (Nagy et al. 2007) and cardiomyocytes (Peng et al. 2006) but not in keratinocytes (Schober et al. 2007) or endothelial cells (Braren et al. 2006).

Akt/PI3K-Signaling Pathway

Another serine/threonine kinase that is often activated downstream of integrins is Akt, which is activated in a PI-3-kinase dependent manner. FAK activation leads to PI-3-kinase recruitment to focal adhesion resulting in elevation of local PtdIns-3,4,5-P3 levels which serve to catalyze various signaling reactions. Activation of PI-3-kinase leads to downstream Akt activation, which regulates integrin-mediated cell survival. Akt is initially activated by phosphorylation of Thr308 in the activation loop after which it is phosphorylated on different threonine and serine residues by other kinases such as mTOR. ILK may also represent a potential intersection point for growth factor receptor and integrin signaling as ILK is connected to growth factor receptors via a Nck2–PINCH–ILK interaction (Vaynberg et al. 2005; Velyvis et al. 2003) and ILK is connected to integrins and the cytoskeleton as described above.

In summary, integrin ligation leads to the activation of multiple signaling cascades with well-characterized outputs. Activation of these cascades occurs through multiple pathways which are spatiotemporally regulated, both through the assembly of signaling modules as well as through regulation of plasma membrane order and endocytosis. The question of how these processes are regulated in three-dimensional environments as well as in vivo requires detailed exploration. The use of real-time imaging to study the assembly, activity, and targeting of signaling complexes might provide valuable insights.

Perspective

In this chapter we have discussed some of the general features of the structure and function of the integrin family of receptors. Over the past 20 years, a vast amount of information has been discovered with regards to integrins and the crucial roles they play in biological cellular functions. Gene technology has allowed the ability to generate integrin knockout mice which have provided important insights into the functions of the various integrin heterodimers. We have highlighted the emergence of important integrin structural data which has helped elucidate some of the pathways by which integrins undergo conformational changes to become activated and attain the ability to signal bidirectionally. We have also highlighted how integrins are critically involved in extracellular matrix assembly and cell migration. Finally, we have provided a framework to show how integrins modulate many intracellular signaling cascades.

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Chapter 3
Cancer in Context: Importance of the Tumor Microenvironment

Barbara Fingleton and Conor Lynch

Abstract Our understanding of tumor biology has evolved from the consideration of only tumor cell autonomous changes to the realization of the plethora of interactions among various cells and molecules present within the locale of a growing tumor. Characterizing this so-called "tumor microenvironment" has opened a new window of opportunity for both diagnostic and therapeutic approaches. From a therapeutic perspective, an attractive feature of targeting noncancer cells is that they are not inherently genetically unstable and therefore drug resistance is less likely than in mutation-prone tumor cells. In this chapter, we consider the roles of different cell types, molecules, and environmental conditions that together constitute the unique topography of a tumor microenvironment.

Keywords Tumor-associated fibroblasts • angiogenesis • immune cells • mesenchymal stem cells • cellular fusion • proteases, glycosidases • pH • hypoxia

Introduction

"Location, location, location" is the principal rule not only in real estate but also in tumor biology. Considering a tumor as merely an uncontrolled mass of mutated epithelial cells (in the case of a carcinoma) is to miss the critical interactions that can profoundly influence tumor behavior. As demonstrated elegantly by Josh Fidler and his colleagues, tumor cells injected into different anatomical sites have altered invasive and metastatic capabilities (Gohji et al. 1997; Nakajima et al.

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_3,

1990), altered sensitivity to chemotherapeutic drugs (Fidler et al. 1994; Staroselsky et al. 1990), as well as altered proliferation programs (Gutman et al. 1995). The various anatomical sites represent vast differences with respect to the cell types, matrix proteins, and signaling molecules present. The contribution of the nonepithelial constituents of a carcinoma to its behavior has also been revealed by genomic profiling studies. A noteworthy example comes from Finak et al. (2008), who used laser capture microdissection combined with microarrays to demonstrate that a gene expression signature of stromal cells from within a tumor can be a robust prognostic indicator in breast cancer patients. Even in lymph node negative patients, the gene signature of stromal cells can predict clinical outcome with a relatively high degree of confidence, independent of typical tumor breast tumor characteristics such as hormone receptor status (Finak et al. 2008). Thus, the multifaceted behavior of epithelial tumors, as well as other types, can be dependent in large part on their environment. The purpose of this chapter is to survey the stromal composition of a tumor microenvironment and to outline the current thinking regarding the roles of different cell types, molecules, and environmental conditions.

Cellular Constituents of the Tumor Microenvironment and Their Functions

As stated earlier, the cellular constituents of a tumor depend on the specific location. For example, specialized cells such as osteoclasts and osteoblasts are important features of the bone, but are not players in other organs. Here, we will concentrate predominantly on cell types that contribute to a greater or lesser extent in most tumor sites, but will also briefly introduce some of the more specialized cell types.

Fibroblasts

Since a prominent feature of some tumors is the "desmoplastic response," whereby matrix molecules such as type I collagen are secreted in large amounts around tumor cells by adjacent fibroblasts, it has long been recognized that fibroblasts in the vicinity of tumor cells behave differently to "normal" fibroblasts. This has led to the terminology of tumor-associated fibroblasts (TAFs) or cancer-associated fibroblasts (CAFs). The matrix deposition phenotype in particular is similar to the function of fibroblasts in healing wounds and thus TAFs are regarded as "activated" similar to wound fibroblasts (Dvorak 1986). Indeed, one prognostic gene expression signature for breast cancers was based on the gene expression profile of wound-healing fibroblasts compared with quiescent counterparts (Chang et al. 2005). However, it is now clear that TAFs participate in tumor development in a myriad of ways, of which collagen secretion is only one. A seminal demonstration of the potency of TAFs came from a collaboration between the Tlsty and Cunha

laboratories when initiated nontumorigenic prostatic epithelial cells were admixed with either TAFs or normal fibroblasts and injected into mice. Malignant tumors only grew out when TAFs were included with the epithelial cells (Olumi et al. 1999). Even more surprising was the result of a study reported by Bhowmick et al. (2004) in which mice were generated that had a cell-specific deletion of transforming growth factor-beta (TGF-β) type II receptor from fibroblasts. These investigators used the promoter from fibroblast-specific protein-1 to target deletion of the receptor only in fibroblasts. No carcinogen or oncogene was used, yet these fibroblast-manipulated mice spontaneously developed invasive carcinomas in the foregut as well as hyperplastic lesions in the prostate, thus demonstrating that altering signaling pathways in fibroblasts alone appears sufficient to initiate tumor development in epithelial cells. One mechanism suspected to cause this phenotype is enhanced proliferation of fibroblasts in the absence of normal TGF-β growth-suppressive signals, resulting in increased levels of hepatocyte growth factor, thus stimulating excessive epithelial proliferation (Bhowmick et al. 2004). In the mammary gland, conditional ablation of the TGF-β type II receptor in fibroblasts resulted in aberrant ductal development primarily due to increased numbers of fibroblasts (Cheng et al. 2005). The ablated fibroblasts secreted increased levels of several growth factors such as hepatocyte growth factor, TGF-α, and macrophage-stimulating protein, and consequently promoted the growth of mammary tumor cells in vivo. Another way that TAFs support tumor development is via hormonal signaling. In the breast and prostate, stromal fibroblasts are recognized as key producers of estrogen (Yamaguchi 2007). In prostate cancers, androgen receptors on stromal cells have been demonstrated as driving the tumorigenesis in epithelial cells (Cunha et al. 2004).

Fibroblast activation protein (FAP), also known as seprase, appears to be a marker specifically of TAFs. In a study of human colon tumor sections from patients with metastatic disease, stromal FAP expression correlated with decreased survival (Henry et al. 2007). A vaccine generated against FAP was shown to be effective in mouse models of breast and colon tumors, both reducing tumor growth and increasing the efficacy of chemotherapeutic drugs (Loeffler et al. 2006). The chemotherapy-enhancing effect is thought to be related to reduced levels of type I collagen in the tumors, thus allowing greater penetrance of drug, the collagen-producing activated fibroblasts having been killed by CD8+ T cells following vaccine administration.

Endothelial Cells

The formation of capillaries and new blood vessels from the existing vasculogenic network is referred to as angiogenesis. Endothelial cells, which are a specialized form of epithelium, are intimately involved in the formation of new blood vessels (Risau 1997). The link between angiogenesis and tumor growth was first postulated in 1971 (Folkman 1971) and subsequently numerous studies have demonstrated that without the process of angiogenesis, growth of a tumor is restricted due to a

limitation of oxygen and other nutrients derived from the blood supply. Therefore, in order to grow beyond a certain size, tumors need to induce an angiogenic response from the blood vessels proximal to the tumor microenvironment. Hypoxic conditions in the core of the developing tumor and the resultant expression of hypoxia inducible factors (HIFs) in tumor cells are potent mediators of angiogenesis and are discussed under environmental conditions in this chapter.

Vascular endothelial growth factor (VEGF)-A is perhaps the best described factor involved in angiogenesis (Ferrara 2002). Several tumors express VEGF-A or have acquired the ability to liberate VEGF-A from the connective tissue surrounding the tumor. Tumor-derived VEGF-A mediates its effects via the VEGF receptor, VEGFR2 which is expressed by endothelial cells. VEGF-A has a tenfold higher affinity to VEGFR1 compared with VEGFR2, but to date, the role of VEGFR1 in angiogenesis remains poorly understood (Shibuya and Claesson-Welsh 2006). Activation of the VEGFR2 receptor on the endothelial cell surface results in migration, proliferation, creation of the blood vessel lumen, and fenestrations. Tumor cells rarely express VEGFR2 and therefore, the VEGF-A–VEGFR2 interaction is an elegant example of how cellular interaction in the tumor microenvironment can promote tumor progression, in this case leading to the generation of blood vessels that supply the tumor with necessary nutrients. Furthermore, VEGF-A has also been reported as chemotactic for macrophages, which also play an integral part in orchestrating angiogenesis (Lin and Pollard 2007), as well as other immune cells (Kerbel 2008). These contributions of VEGF-A and VEGFR2 have resulted in the development of several therapies designed to inhibit tumor angiogenesis, thus tumor growth. For example, bevacizumab, an anti-VEGF-A monoclonal therapy has been approved for the treatment of several cancers (Ruegg and Mutter 2007).

Understanding the process of angiogenesis has also led to the discovery of several other factors that are implicated in the process, such as platelet growth factor (PlGF) and Delta-like ligand (Dll)-4. PlGF binds to VEGFR1 and is implicated in tumor-mediated angiogenesis (Fischer et al. 2007). PlGF expression is mostly confined to the tumor microenvironment and is rarely found in normal nonpathological conditions, therefore making it an attractive therapeutic target. More recently, Dll4 has been demonstrated to play a pivotal role in endothelial cell function and angiogenesis in the tumor microenvironment (Noguera-Troise et al. 2006; Ridgway et al. 2006). The membrane-bound Dll4 interacts with Notch1 and Notch4 receptors on adjacent endothelial cells and disruption of this interaction in mice by the deletion of only one allele results in severe cardiovascular defects that are similar to those described for VEGF-A (Gale et al. 2004). Unexpectedly, monoclonal antibodies that block the interaction of Dll4 with Notch result in exacerbated angiogenesis, but the resultant blood vessels are poorly functional and therefore prevent tumor cells from access to nutrients from the blood supply.

In addition to stromal cells of the tumor microenvironment contributing to angiogenesis, the cancer cells themselves may also play a direct role in the process. Aggressive cancer cells such as those from a melanoma, breast, and prostate origin have been shown to take on endothelial like gene expression patterns in a phenomenon known as vascular mimicry (Hendrix et al. 2003). The endothelial like cancer cells

generate extracellular matrix (ECM)-rich vasculogenic networks that are thought to be involved in the sprouting, intussusception, and the bridging of endothelial cells derived from blood vessels surrounding the developing tumor and are also thought to provide a mechanism of conducting intratumoral fluid.

In contrast to blood vessels, the endothelial cells lining lymph vessels have large gaps to allow for fluid collection. The lymph vessels and lymphatic capillaries also differ in that they are not surrounded by mural cells, and therefore, cancer cell intravasation is not as difficult as intravasation into blood vessels. In a number of carcinomas, the lymph node is often the first site to exhibit signs of metastasis and this is thought to occur due to the entry of tumor cells into preexisting lymph vessels and the drainage of the cells into lymph nodes distal to the tumor site (Fisher et al. 1983). Understanding the role of the lymphatics in normal and pathological processes has been greatly limited by a lack of markers that delineate between the endothelial cell populations that comprise the blood and lymphatic vessels. However, recent studies aided by the discovery of new markers, such as VEGF-C, VEGF-D, and VEGFR3, that more readily identify lymphatic populations have shown how tumor-mediated lymphangiogenesis contributes to the dissemination of tumor cells via the lymphatics (Mandriota et al. 2001; Stacker et al. 2002).

VEGF-C and VEGF-D promote endothelial cell migration, proliferation, and lymph vessel permeability via the VEGF receptors, VEGFR2 and VEGFR3 (Joukov et al. 1997), with the latter receptor thought to be important in driving a lymphatic phenotype. VEGF-C and VEGF-D are subject to proteolytic processing, and shorter versions of these factors may have differential effects on endothelial cells perhaps explaining how these molecules contribute to both angiogenesis and lymphangiogenesis.

Pericytes

Blood vessels are typically supported by smooth muscle cells on the abluminal surface that facilitate architectural support and contraction of the blood vessels. In smaller blood vessels and capillaries, these cells are termed mural cells or pericytes but function in the same capacity and are thought to be derived from mesenchymal stem cells (MSCs) (Gerhardt and Semb 2008). The number of pericytes attached to the surface of the blood vessels varies with the requirements of different organs but appears to correlate with the permeability of the vasculature. For example, a large number of pericytes are key for the maintenance of the blood–brain barrier whereas in the glomeruli of the kidneys, the number of pericytes is far lower thereby allowing for the filtration of waste and toxins from the blood. Pericytes are intimately linked to the endothelial cells by cell adhesion molecules such as N-cadherin and Ephrin-B2 and are embedded within the same ECM (Foo et al. 2006; von Tell et al. 2006). The interaction between pericyte-derived factors such as angiopoietin-1 (Ang-1) and the endothelial cell surface receptor Tie-2 are also vital in stabilizing the relationship between the two cell populations (Uemura et al. 2002).

The close proximity between endothelial cells and pericytes is necessary for proper communication between the two cell populations and the integrity of the blood vessel.

During angiogenesis, pericytes play a critical role in the maturation of the blood vessel and in stabilizing the newly formed vessels. Given the role of pericytes in blood vessel function, it is not surprising that they also play an important role in tumor angiogenesis and potentially in the metastasis of tumor cells from the primary site. Tumor-induced vasculature is, however, generally "leaky" and this is due to the low numbers of pericytes surrounding the blood vessels feeding the tumor cells (Baluk et al. 2005). In contrast to Ang-1, tumor-derived Ang-2 is thought to have antagonistic effects on Tie-2 and therefore prevents pericyte incorporation into the newly forming blood vessels, resulting in leakier vessels (Hammes et al. 2004).

The role of pericytes in tumor angiogenesis make them an interesting target for therapeutics since interference in pericyte-endothelial cell interaction would destabilize the blood vessels and heighten the need of the endothelial cells for the survival factor VEGF-A. In turn, tumors would presumably be more susceptible to VEGF-A-based antiangiogenic therapies. In this regard, in vivo studies using the transgenic model of pancreatic tumorigenesis RIP-Tag, where the *r*at *i*nsulin *p*romoter drives *T a*ntigen expression, have shown that targeting pericytes with platelet-derived growth factor receptor inhibitors sensitizes the tumors to antiangiogenic treatment, thereby resulting in an inhibition of tumor progression (Bergers et al. 2003). An important caveat may be that while the therapies are successful in depriving the tumor cells of a blood supply, the resultant changes in the tumor cells due to hypoxia may invoke a more metastatic phenotype that in turn may be able to easily intravasate into the surrounding vasculature that has been made leaky due to the combined pericyte and endothelial cell targeting. However, this combination approach of targeting the pericytes and the endothelial cells in the tumor microenvironment could be extremely useful for the treatment of tumors that appear resistant to antiangiogenic therapies.

Immune Cells

Immunological surveillance, a term coined by Macfarlane Burnet, describes the concept of a patrolling immune system ready to identify and remove nascent tumors (Burnet 1964). The concept has now evolved to immunoediting, whereby tumor cells and immune cells interact and influence each other's behavior (Dunn et al. 2002). Nevertheless, the principle remains the same – that tumor cells and immune cells are in close contact, thus immune cells are critical components of the tumor microenvironment. Indeed the presence of tumor cells within tumors has long been recognized histologically, prompting Virchow to hypothesize that such cells contribute to tumor development (Balkwill and Mantovani 2001). There is now considerable evidence for both pro- and antitumorigenic contributions of immune cells to various cancers (Johansson et al. 2008). Both arms of the immune system – adaptive and innate – are involved in tumor biology and we will consider these separately.

Adaptive or acquired immunity involves specific responses to recognized antigens, mediated through B and T cells. Cytotoxic CD8+ T cells are considered as antitumor effectors that can be responsible for effective immunotherapy of cancers, whereas CD4+ helper T cells are required to prime the immune response. A recent study in colorectal cancer identified high levels of intratumoral T cells specifically of the cytotoxic and memory subtype, as significant indicators of good prognosis (Galon et al. 2006). Further, in the stromal cell breast cancer signature identified by Finak et al (2008), gene expression patterns indicative of a T-cell response were associated with good prognosis for this disease also. Harnessing the power of these cells has been the rationale for multiple efforts at developing vaccines for therapy of established tumors (Morris and Ribas 2007). Thus far, however, the majority of cancer vaccines have failed to produce spectacular results (Finke et al. 2007). Part of the problem is the presence within tumors of regulatory T cells (Tregs), which are normally important for attenuation of self-reactive cells, thereby preventing autoimmune disease (Cools et al. 2007). In cancer, however, the presence of such cells within tumors can interfere with antitumor immune responses. Hence, strategies that combine depletion of Tregs with stimulation of antitumor dendritic and T cells are now being explored (Beyer and Schultze 2008).

Antibodies can also be involved in effective antitumor responses, however within the tumor microenvironment, antibody-producing B cells have recently been shown to play a paradoxical protumorigenic role in a mouse model of skin tumorigenesis (de Visser et al. 2005). This study showed that immunoglobulin produced by B cells was responsible for recruitment of innate immune cells that then promoted tumor growth and progression. Using B cell-deficient mice could effectively abrogate tumor development in this model of squamous cell skin carcinoma.

The tumor-promoting role of several innate immune cell populations such as macrophages and neutrophils has been widely accepted and such cells are now considered targets for anticancer therapies. Tumor-associated macrophages (TAMs) in particular have been shown to promote angiogenesis, growth, and invasive behavior of several tumor types with breast cancer being the best-studied setting (Lin and Pollard 2007). Similarly, neutrophils can promote invasive behavior (Welch et al. 1989) and, through the release of various growth factors, cytokines and proteases, can promote angiogenesis. Chronic inflammation, per se, is a known risk factor for multiple tumor types including gastric, colon, ovarian, hepatic, and pancreatic carcinoma (Balkwill and Mantovani 2001). One associated mechanism may be the generation of DNA-damaging reactive oxygen or nitrogen species (ROS and RNS), released during the oxidative burst from activated macrophages and neutrophils (Josephy and Coomber 1998).

The contribution of other immune cells such as mast cells and eosinophils is less well defined. In mouse models, mast cells have been demonstrated to contribute to the progression of pancreatic islet tumors (Soucek et al. 2007) and squamous cell skin carcinomas (Coussens et al. 1999), but conversely to be protective against development of intestinal tumors (Sinnamon et al. 2008). Evaluation of tumor tissue from human cancer patients has shown that the level of mast cells within the tumor

correlates with prognosis, although such studies have generally comprised small sample numbers. Eosinophils are typically regarded as important players in allergic responses and little attention has been paid to their possible role in tumor development. Nevertheless, there is some evidence to suggest that, like other immune cells, the presence of eosinophils within the microenvironment can influence tumor behavior either positively as in gastrointestinal tumors or negatively as in oral squamous cell carcinoma (Lotfi et al. 2007).

Dendritic cells, which are themselves innate immune cells, can be regarded as bridging the gap between innate and acquired immunity. A major role for dendritic cells is sampling the environment and presenting possible antigens to T cells. In many cancer patients, dendritic cell function is suppressed thus disabling the antitumor response. A key molecule responsible for suppression of dendritic cell function appears to be the angiogenic protein VEGF, which is produced by tumor cells and also by TAMs and neutrophils (Ohm and Carbone 2001). In addition to VEGF-mediated suppression of dendritic cell maturation, T cells can also be inhibited by VEGF.

Mesenchymal Stem Cells

MSCs are nonhematopoietic pluripotent stem cells that typically reside in the bone marrow. MSCs can differentiate into a number of cell types including bone, cartilage, adipose tissue, pericytes, and fibroblasts (Stagg 2008). A number of reports have shown that MSCs are recruited in large numbers to the tumor stroma (Hall et al. 2007). However, the presence of MSCs in the tumor microenvironment and their role in tumor progression has only begun to be elucidated.

In a xenograft model, where MSCs were coimplanted with weakly metastatic breast cancer cells, the resultant tumor was found to have much higher metastatic potential than breast cancer cells xenografted alone (Karnoub et al. 2007). Evidence of cross communication between the tumor cells and the recruited MSCs was also demonstrated in this study. Tumor-derived factors were shown to stimulate the expression of chemokine ligand 5 (CCL5) by MSCs that in turn promoted the motility, invasion, and metastasis of the breast cancer cells (Karnoub et al. 2007). Therefore, the mobilization of MSCs from the bone marrow to the tumor microenvironment can potentially contribute to a more aggressive phenotype. MSCs may also play potentially pivotal roles in prostate tumor progression. CAFs share a number of similar surface markers and functions as MSCs and may originate from bone marrow-derived MSCs (Haniffa et al. 2007). The expression of TGF-β by CAFs induced the expression of chemokine receptor-4 (CXCR4) in an initiated but nonmalignant human prostate cancer cell line. The expression of the CXCR4 cognate ligand, chemokine ligand-12 (CXCL12), by the CAFs subsequently stimulated the migration of the CXCR4 prostate cells, thereby generating a more migratory phenotype (Ao et al. 2007). These studies support the rationale for therapies that target MSCs directly or MSC-derived signals in the tumor microenvironment as a means of preventing tumor growth and metastasis.

Cellular Fusion in the Tumor Microenvironment

While in the current section we have largely focused on the individual cell types, the concept of cell fusion within the tumor microenvironment has begun to gain momentum as an explanation as to how tumor cells can take on more metastatic or aggressive traits. Cancer cell fusion to other cell types, in particular those derived from the bone marrow, was first postulated by Aichel in 1911 based on his observations of anueploidy within tumor cells (Pawelek and Chakraborty 2008). Recent studies have found that the gene signature of highly metastatic cancer cells often resembles the expression profile of bone marrow-derived cells of a myeloid lineage with studies of macrophage–cancer cell fusions demonstrating that genes from both parental partners are expressed in the hybrid cells (Chakraborty et al. 2001). The first study to demonstrate that tumor–stromal fusion was occurring in human patients with multiple myeloma was recently reported (Andersen et al. 2007). Multiple myeloma is a hematological malignancy of B-lymphocytes and is largely confined to the bone. The malignant cells induce extensive bone destruction/osteolysis by activating osteoclasts, the cells primarily responsible for demineralization and resorption of the calcified bone matrix. Osteoclasts are large multinucleated cells that are generated via the fusion of stimulated monocytes. Delaisse and colleagues demonstrated that in human cases of multiple myeloma, 30% of the osteoclasts associated with the disease contained B-cell nuclei (Andersen et al. 2007). This fusion was also demonstrated in vitro using osteoclasts from normal female mice and male multiple myeloma cells and subsequently identifying the Y chromosome in the osteoclasts over time. Cellular fusion in the tumor microenvironment is therefore a distinct possibility and may be an important phenomenon during tumor progression.

Molecular Constituents of the Tumor Microenvironment and Their Functions

Matrix Molecules

One of the defining characteristics of a particular tissue is the composition of the proteinaceous “ground substance” or ECM. Type I collagen is the most abundant protein in the body; however, this does not mean that type I collagen is the dominant matrix constituent at all anatomic locations. In the basement membranes for example, type IV collagen together with laminin and heparansulphate proteoglycans are the principal matrix molecules. In the case of a tumor, the matrix composition can affect tumor biology in multiple ways. First, the free availability versus sequestration of various signaling factors is dependent on what binding partners are present. Heparan-containing proteoglycans and fibronectin can bind up VEGF (Mitsi et al. 2006), whereas TGF-β binds directly to the proteoglycan decorin (Macri et al. 2007), or through interactions with the latency complex, to fibrillin,

collagen, or fibronectin (Rifkin 2005). Second, the invasive program of tumors will be dependent on organizing a suitable proteolytic profile to match the protein barriers present. The barrier function of matrix proteins can affect not just invasion but also growth and expansion. Hotary et al (2003) have elegantly demonstrated that a colony of tumor cells confined in a 3-dimensional collagen matrix cannot increase in size unless the enzyme matrix metalloproteinases (MMP)-14 is present. Third, the matrix itself can change the behavior of tumor cells through signaling via the integrins and associated proteins (Eble and Haier 2006). Transformed cells acquire resistance to anoikis, the cell death program initiated once survival signals dependent on ECM attachment are lost, through changes in integrin signaling (Reddig and Juliano 2005). Furthermore, recent data have shown that matrix molecules, once thought to be indicators of a circumscribed and therefore contained tumor, actually promote tumor growth and progression. Recessive dystrophic epidemolysis bullosa (RDEB), a blistering skin disease caused by defects in the type VII collagen gene, is often associated with early-onset epidermal squamous cell carcinomas. Expression of a specific fragment of type VII collagen was shown to be required for the tumorigenicity of Ras-transformed keratinocytes from RDEB patients (Ortiz-Urda et al. 2005). Specific sequences within the collagen VII fragment promoted cell invasion and tumorigenesis. Similarly, pancreatic adenocarcinoma cells are stimulated to increase motility and metastasis by collagen type I, through downregulation of E-cadherin (Koenig et al. 2006) and expression of N-cadherin (Shintani et al. 2006). Since pancreatic cancers in particular are characterized by excessive type I collagen deposition, this tumor-promoting activity of collagen may partly explain the aggressive nature of pancreatic adenocarcinoma (Armstrong et al. 2004).Fourth, tumors can change the matrix for example by proteolytic processing that can then release cryptic fragments with their own signaling ability. These fragments, termed “matrikines,” include the various angiogenic regulators such as endostatin and tumstatin (Nyberg et al. 2005), as well as an EGFr-binding fragment of LM-332 that promotes cell proliferation, motility, and invasion (Schenk et al. 2003). More detailed discussions of the roles of different matrix molecules and their receptors are given in following chapters.

Proteases and Glycosidases

The human protease degradome contains 569 proteases that are divided into six classes based on the amino acid recognized by the catalytic site of the enzyme (serine, cysteine, aspartyl, threonine, and glutamic) or, as with metalloproteinases, the cofactor that is essential for their catalytic activity. Each class of proteases can be further broken down into secreted versus membrane bound, and by the domain structure of the protease (Lopez-Otin and Overall 2002). Proteases from different classes can interact and activate other proteases, thereby mediating a proteolytic cascade. Collectively, proteases are crucial for normal tissue homeostasis and therefore their regulation is usually tightly regulated at the gene and posttranslational level by endogenous inhibitors. However, in pathological scenarios such as cancer,

this tight regulation of protease expression is often perturbed leading to excessive protease activity that is associated with tumor progression.

Matrix Metalloproteinases

In the context of the tumor microenvironment, a family of 23 proteases known as the MMPs have received, perhaps, the most attention (Lynch and Matrisian 2002). MMPs collectively are capable of processing the entire repertoire of ECM proteins. In order for tumor cells to invade and metastasize to secondary sites, the barrier represented by the ECM must be broken down thereby necessitating MMP expression in the tumor microenvironment. The expression of individual MMP family members has been found to correlate with poor prognoses in a variety of cancers (Fingleton 2007). The apparent role of MMPs in tumor invasion and metastasis led to the generation of broad spectrum MMP inhibitors (MMPIs) as therapies for the treatment of cancer. However, although preclinical animal models indicated efficacy of these agents, clinical trials in humans were unsuccessful (Coussens et al. 2002). Perhaps the most compelling argument as to why the MMPIs failed was the lack of understanding of the myriad of functions that have subsequently been ascribed to the MMPs. Further research in the MMP field has found that in addition to their role in the processing of ECM components, the MMPs are also able to act in the processing of nonmatrix molecules such as growth factors and cytokines (Lynch and Matrisian 2002). For example, MMP-9 has been linked to angiogenesis in the tumor microenvironment by virtue of its ability to mediate the release of VEGF-A from the ECM, although the direct cleavage of VEGF by MMP-9 has not been shown (Bergers et al. 2000). Additionally, MMP-9 processing of kit ligand in the bone marrow is essential for the mobilization of hematopoietic progenitor cells to the tumor microenvironment (Heissig et al. 2002). While many of the substrates processed by MMPs can promote tumor progression by impacting proliferation, survival, angiogenesis, immunoevasion, migration, and invasion, more recent studies have also demonstrated that MMPs, such as MMP-3 and MMP-8, can protect or prevent tumor progression (Lopez-Otin and Matrisian 2007). These studies add to our understanding of MMP functions and demonstrate the complexity of this small group of proteases. Ultimately, the development of highly selective MMPIs that target individual MMP family members, whose contribution to the progression of multiple tumor types have been explored, may be of benefit in the treatment of human tumors.

Serine Proteases

The presence and contribution of serine proteases in the tumor microenvironment to tumor progression has been well established, in particular with respect to the plasminogen activation system. The system is comprised of urokinase plasminogen activator/urokinase plasminogen activator receptor and tissue plasminogen activator, which convert plasminogen to plasmin. Negative regulation of the system is

achieved by plasminogen activator inhibitors, PAI-1 and PAI-2. Plasmin activation leads to ECM degradation and the release of multiple growth factors from the matrix that in turn can stimulate angiogenesis, invasion, and metastasis (Andreasen et al. 2000). Several in vivo models have provided evidence as to the contribution of the plasminogen activation system in tumor progression. For example, genetic ablation of either plasminogen or urokinase plasminogen activator resulted in significant attenuation of metastasis in a mouse model of breast cancer (Almholt et al. 2005; Bugge et al. 1998).

Prostate-specific antigen (PSA) is a serine protease, also known as kallikrein III. High levels of PSA in peripheral blood are usually indicative of prostate cancer. PSA activity in the primary prostate tumor microenvironment can generate bioavailable TGF-β and insulin-like growth factors (IGFs) by processing latency binding proteins that sequester these growth factors. PSA has also been described as processing a number of ECM components such as laminin and fibrillin and therefore, is also implicated in primary prostate tumor progression (Balk et al. 2003). Furthermore, in the bone, a common site for prostate cancer metastasis, the proteolytic inactivation of parathyroid hormone-related peptide, an important humoral factor involved in tumor mediated bone destruction, can potentially explain why prostate-to-bone metastases are largely blastic or bone forming in nature.

Cysteine Proteases

Of the cysteine proteases, the best studied in the context of tumor progression are the cysteine cathepsins (Gocheva and Joyce 2007). This is a family comprised of 11 members that are expressed by many tissue types. Cathepsins are localized intracellularly to lysosomes and are thought to function in protein degradation but, within tumors, they are often secreted into the extracellular millieu. Hypoxia in the tumor microenvironment results in a more acidic pH, which is optimal for cysteine cathepsin activity.

A study of human pancreatic cancers from patients at varying stages of progression revealed that levels of cathepsin B and L expression correlate with tumor aggressiveness (Joyce et al. 2004). To test the role of cathepsin B and L in pancreatic tumor progression, Rip-Tag2 mice that were null for either enzyme were generated. In cathepsin B null Rip-Tag2 mice, a significant decrease in angiogenesis, tumor volume, survival, and invasion were observed compared with wild-type controls, whereas in cathepsin L null Rip-Tag2, a significant decrease in tumor survival was reported (Gocheva et al. 2006). Cathepsin B was also demonstrated to contribute significantly to tumor growth and metastasis in a murine model of breast cancer, where expression of the enzyme was localized to both tumor cells and TAMs (Vasiljeva et al. 2006).

Glycosidases

While enzymes capable of processing the protein components of the ECM have received much attention for their contributions to tumor progression, the role of

glycosidases that process heparan sulfate proteoglycans (HSPGs) has only recently begun to be explored. HSPGs are expressed by a large number of cell types and are located on the cell surface or within ECM, and function in the sequestration of inactive biomolecules such as basic fibroblast growth factor (bFGF) or heparin-bound epidermal growth factor (HB-EGF) (Bernfield et al. 1999). The enzymatic processing of the heparan sulfate chains containing these sequestered factors assists in promoting the bioavailability of these factors in the tumor microenvironment. Heparanase-1 expression is normally restricted to keratinocytes and immune cells but is commonly expressed in tumors at high levels. Heightened heparanase activity is associated with increased angiogenesis by virtue of the release of VEGF-A and bFGF (Ilan et al. 2006). Furthermore, the contribution of heparanase-1 to tumor progression and metastasis has been observed in several animals models. Perhaps the most striking of these used ribozymes to silence heparanase expression in B16-BL6 melanoma cells, resulting in a 90% decrease of detectable lung metastases (Edovitsky et al. 2004).

Growth Factors and Cytokines

Cytokine and growth factor expression by various cells is essential for cell–cell communication in the tumor microenvironment. The range of different factors is vast and defining the distinct contribution of individual cytokines to cell behavior and tumor progression would require an entire volume rather than a chapter. Instead, there are specific examples given in the various sections of this chapter that serve as indicators of the complexity of this class of molecule.

The Metabolic Microenvironment

The two major factors that describe the metabolic environment of a tumor are pH and oxygen tension. These factors are interlinked via molecules such as the transcription factor HIF-1 and one of its target genes, carbonic anhydrase IX.

Tumor pH

Tumors are generally considered to be acidic environments, meaning that the extracellular pH is lower than that in the normal tissue, although intracellular pH is approximately the same (Gerweck et al. 2006). The cause of the lowered pH has been the subject of much investigation and three major contributing factors have been identified. The first is lactic acid generated as an end-product of glycolysis, in an inefficient anaerobic respiration process (Kim and Dang 2006). Initially, glycolysis

may be a response to anaerobic conditions; however, as the transcriptional program associated with hypoxia is stabilized in tumor cells, aerobic glycolysis becomes standard in a process known as the Warburg effect (Gatenby and Gillies 2008). Even in lactate dehydrogenase-deficient tumors however, the pH is low (Yamagata et al. 1998), thus suggesting that mechanisms other than glycolysis contribute to this state. A second major cause then appears to be the production of carbonic acid from carbon dioxide, catalyzed by the tumor-associated enzyme carbonic anhydrase IX (Svastova et al. 2004). A third suggested mechanism is increased activity in tumor cells of vacuolar type (v-type) H+ ATPases that pump protons across the plasma membrane (De Milito and Fais 2005). One of the major consequences of the increased acidity is selection of aggressive tumor cells that can withstand the harsh environment (De Milito and Fais 2005; Gatenby and Gillies 2008). The aggressive nature manifests as enhanced migration, invasion, angiogenesis, and tumor growth (De Milito and Fais 2005; Rofstad et al. 2006). As mentioned previously, the acidic extracellular milieu allows activity of cysteine proteases such as cathepsin B that are normally found in the lysosomal compartment, and these can contribute to the aggressive behavior of tumors. Critically, tumor acidification can also significantly alter efficacy of chemotherapeutic drugs (Tredan et al. 2007). In particular, chemical entities that are weak acids such as chlorambucil will be nonionized in an acidic environment thus enhancing uptake by tumor cells. The more basic intracellular pH then causes increased levels of ionized drug, which is the active moiety. Conversely, drugs that are weak bases such as doxorubicin are less able to cross plasma membranes in an acidic environment and are therefore less effective (Gerweck et al. 2006). Another mechanism of drug resistance related to tumor pH is activity of the multidrug resistant protein, p-glycoprotein, which is enhanced in cells exposed to acidic medium (Thews et al. 2006).

Oxygen Tension

Within solid tumors, regions of low (hypoxic) or no (anoxic) oxygen develop as a function of diffusion distance from tumor vasculature. Tumor cells have evolved survival mechanisms in the face of such stresses that can profoundly alter the microenvironment around themselves. The best-characterized response to low oxygen is stabilization of the transcription factor subunit HIF-1α. HIF-1α is an ubiquitously expressed gene whose oxygen-sensitive protein product is usually degraded through binding of the von Hippel-Lindau (VHL) protein and proteasome activity (Semenza 2001). In the setting of low oxygen however, modifications of the HIF-1α protein that facilitate its binding by VHL do not occur and the protein is free to heterodimerize with HIF-1β. The heterodimer then binds hypoxia response elements on various genes leading to upregulation of important regulators of the tumor microenvironment, among others (Semenza 2007). These genes include VEGF, which promotes angiogenesis and, as described previously, can also be immunosuppressive; carbonic anhydrase IX, responsible for acidification of the microenvironment;

as well as the glucose transporter Glut-1, which allows increased glucose consumption by tumor cells thus, as discussed earlier, potentiating lactic acid production through aerobic glycolysis. The hypoxic/anoxic profile of a tumor is dynamic and can include areas of acute hypoxic conditions, followed by reoxygenation as well as chronic hypoxia (Bristow and Hill 2008). Chronic hypoxia acts, like pH, as a selection pressure for subpopulations of cells that manifest characteristics of tumor progression (Semenza 2000). Further, the hypoxic microenvironment can strongly affect chemotherapeutic drug efficacy as well as radiotherapy responses (Bristow and Hill 2008). Since HIF-1 is a central regulator of the response to hypoxia and of the various genes that then characterize the malignant phenotype, pharmacological inhibitors of HIF-1 have been proposed as novel anticancer therapeutics (Semenza 2007). Combination of these agents with standard chemotherapy and/or other agents that target aspects of the tumor microenvironment is being investigated.

Example of a Specialized Tumor Microenvironment – the Bone

The metastatic bone-tumor microenvironment represents an excellent example of how cytokines regulate cell–cell interaction and how different cell types present can affect the behavior of tumor cells.

While bone is a reservoir of hematopoietic progenitor cells, the cells primarily responsible for bone formation and resorption are the osteoblasts and osteoclasts, respectively. Osteoblasts are derived from MSCs and differentiate into bone forming cells when provided with cues from their surrounding environment such as bone morphogenetic protein-2. Osteoblasts secrete type I collagen and incorporate several growth factors such as TGF-β and IGF-I into the bone matrix as it is being synthesized (Guise et al. 2006). Osteoclasts are derived from myeloid cells and factors such as macrophage colony-stimulating factor and receptor activator of nuclear kappa B ligand are essential for the differentiation and fusion of monocytes to form giant multinucleated osteoclasts that subsequently resorb the bone (Guise et al. 2006).

Bone metastasis is a common event during the progression of several cancers, most notably, breast and prostate. Once established and growing in the bone environment, the metastatic tumor cells hijack the normal bone remodeling process, which results in areas of uncontrolled bone formation and resorption. The heightened activity of osteoblasts and osteoclasts leads to an increase in bone-derived growth factors such as TGF-β and IGF-I that in turn promote the growth of the tumor thereby leading to a positive feedback loop that has been described as the "vicious cycle" (Mundy 2002). While all bone metastases contain areas of osteoblastic and osteoclastic activity, the balance in metastatic breast cancer is typically tipped toward osteolytic type lesions while interestingly, prostate bone metastases are often blastic in appearance. The factors that govern the formation of lytic or blastic bone metastases are an excellent example of how cancer cells can manipulate the normal cells of the local microenvironment to support their establishment and growth in a foreign site (Fig. 3.1).

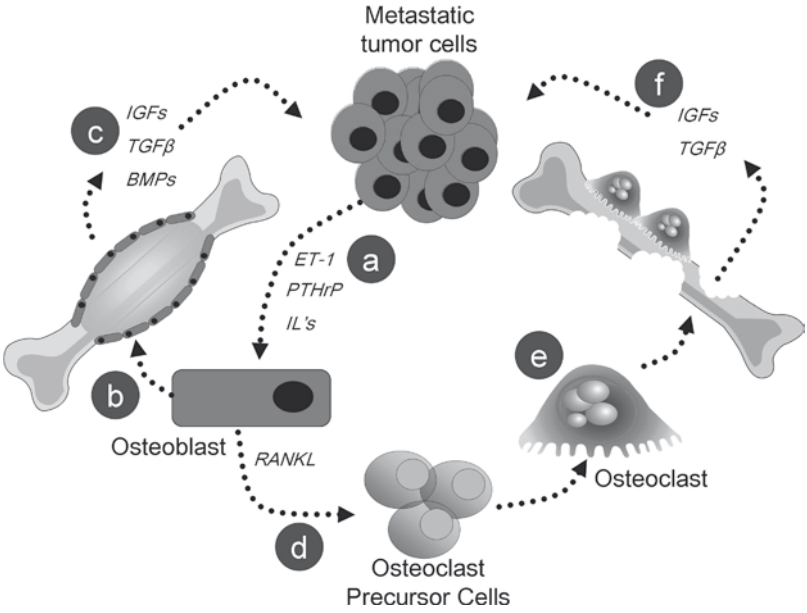

Fig. 3.1 Cytokine regulation of the metastatic bone-tumor microenvironment. (**a**) Metastatic tumor cells secrete factors that stimulate osteoblasts lining the endosteal bone. (**b**) The presence of high levels of tumor-derived factors such as endothelin-1 (*ET*-1) can stimulate osteoblast differentiation and proliferation leading to excessive bone formation. (**c**) While synthesizing the bone matrix osteoblasts generate factors such as bone morphogenetic protein-2 and transforming growth factor-beta (*TGF-β*) that in turn can stimulate the tumor cells to proliferate or become "osteomimetic." (**d**) Tumor-derived factors such as parathyroid hormone-related peptide (*PTHrP*) can also induce the expression of the osteoclast recruitment and activation factor, receptor activator of nuclear kappa B ligand (*RANKL*). (**e**) Fusion of osteoclast precursors leads to the formation of large multinucleated osteoclast cells. (**f**) Excessive osteoclast-mediated bone resorption leads to the release of bone matrix sequestered growth factors such as IGF and TGFβ that subsequently promote tumor growth and stimulate the expression of factors such as PTHrP, thereby ensuring the continuation of the vicious cycle of tumor-induced osteoblastic and osteolytic responses

Conclusion

Unlike the artificial conditions of a cell culture dish, tumor cells do not normally grow in isolation. Instead, multiple cell types, macromolecules, and signaling factors together contribute to the pathophysiological phenomenon that we call cancer. While our understanding of tumor biology has been derived from a reductionist approach toward analyzing the role of individual molecules, we also need to consider the complexity of the system and how each player fits in the grander scheme if we are truly to conquer cancer.

Acknowledgments We regret that limited space precludes citing much relevant literature. The authors are supported by Susan G. Komen for the Cure, grant # BCTR0600431 (to B.F.) and by

the Department of Defense Prostate Cancer Research Program New Investigator Program, award # W81XWH-07-1-0208 (to C.C.L.). Views and opinions of the authors do not reflect those of the US Army or the Department of Defense.

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Chapter 4
Basement Membrane Collagens and Cancer

Vadim Pedchenko and Ambra Pozzi

Abstract The development of cancer is a complex multistage event that requires tumor cell growth, adhesion, migration, and invasion. Tumor growth is strictly dependent on angiogenesis, the formation of new blood vessels from preexisting vasculature, which involves endothelial cell proliferation, migration, and tubulogenesis. The steps involved in angiogenesis and tumor cell dissemination include the destruction of basement membranes (BM), specialized extracellular matrix structures that separate epithelia from the surrounding stroma. Collagens represent the major component of all basement membranes. In addition to providing mechanical stability and scaffold for the assembly of other molecules, collagens also directly affect multiple aspects of cell behavior. In the last decades, our understanding of the events involved in tumor progression has increased considerably because of the discovery of a number of collagen cleavage products that strongly affect the behavior of tumor and endothelial cells, and the recognition that endothelial and tumor cells play an active role in the production and degradation of ECM components. In this chapter, we focus on the structure and role of BM collagen molecules in the control of tumor-associated angiogenesis and tumor progression. We describe: (1) the composition of the major types of collagen in basement membranes; (2) the interaction of these components with tumor and endothelial cells; (3) the role of intact collagen molecules and their cleavage products in the control of specific steps of tumor progression (migration, invasion, and recruitment of blood vessels); and (4) how collagen-derived fragments may potentially be used as therapeutic tools for the treatment of cancer.

Keywords Endothelial cells • cancer cells • basement membranes • collagen • cancer therapy

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_4,

Basement Membrane Collagens

The extracellular matrix (ECM) consists of a complex mixture of macromolecules that serves an important role in tissue and organ morphogenesis, as well as in the maintenance of cell and tissue integrity and function (Bosman and Stamenkovic 2003). In physiological condition, ECM deposition is regulated by a fine balance between synthesis and degradation. However, in pathological conditions, such as cancer, both tumor and stromal cells contribute to uncontrolled ECM deposition. The diverse morphology and composition of the ECM in tumors result in diverse effects on cancer cell properties and functions. In addition, the ECM can both exert a mechanical force and act as a reservoir for signaling molecules such as growth factors (Taipale and Keski-Oja 1997), thus facilitating the key events in tumor progression such as cell proliferation, migration, and invasion.

BMs are specialized structures that separate epithelial/endothelial elements from the surrounding stroma. They are composed of many constituents, including nonfibrillar collagens (i.e., collagens IV, XV and XVIII), laminins (i.e., LM-111, LM-332, LM-511, and LM-521), nidogens, agrins, and proteoglycans (i.e., perlecan) (reviewed in (LeBleu et al. 2007; Bix and Iozzo 2008; Ho et al. 2008; Khoshnoodi et al. 2008; Miner 2008)). Originally, BMs were thought to serve only as a selective barrier and scaffold for cell adhesion; today, it is well accepted that individual components of BM regulate various biological activities such as cell growth, differentiation, and migration, and influence morphogenesis and tissue repair (Miner 2008; Sudhakar and Boosani 2008; Suhr et al. 2009).

Collagen IV

Collagen IV is an obligatory component of all BMs, and belongs to a collagen super-family composed of at least 27 distinct types built of 42 different polypeptide α chains (Myllyharju and Kivirikko 2004). Collagens are the most abundant proteins in human body, constituting approximately 25% of total protein. They form diverse structures such as microfibrils, fibrils and networks, and based on this supramolecular architecture, collagens are classified into fibril-forming, fibril-associated (FACIT), network-, beaded filament-, anchoring fibril-forming, and transmembrane collagens (Ricard-Blum et al. 2005). A key structural feature common for all collagens is the presence of triple-helical domains formed by Gly-X-Y amino acid repeats from three identical or different α chains. There is an absolute requirement for glycine in every third position for the helix formation imposed by the absence of side chain in this amino acid. Frequently present proline at the X and hydroxyproline at the Y positions strongly stabilize the structure of triple helix through formation of hydrogen bonds, which contributes to both thermal stability and remarkable resistance of the collagen triple helix to degradation by most of the proteolytic enzymes (trypsin, chimotrypsin, pepsin).

Six genes have been identified in the type IV collagen (Hudson et al. 2003). They are located pair-wise with head-to-head orientation on human chromosomes 13 (*COL4A1-COL4A2*), 2 (*COL4A3-COL4A4*) and X (*COL4A5-COL4A6*), and encode the six distinct α chains, α1(IV) through α6(IV). Each 1,650 residues long α chain is composed from three domains: a short amino terminal 7S domain, a central collagenous domain (~1,400 residues) that form a triple helix, and a noncollagenous domain (NC1) of 230 amino acids at the carboxyl terminus (Fig. 4.1). The presence of cysteine and lysine residues in 7S domain is essential for the interchain cross-linking of triple helical molecules during collagen IV network formation. The unique distinctive feature of collagen IV is the ability to form specialized supramolecular structures called networks. In contrast to fibril-forming collagens that are characterized by continuous Gly-X-Y repeats, collagenous domain of collagen IV contains about 25 short interruptions that confer flexibility to the molecule necessary for the network formation.

Despite many possible permutations, the six α(IV) chains form only limited number of heterotrimer molecules (called protomers) designated as α1α1α2, α3α4α5, and α5α5α6 (Hudson et al. 2003) (Fig. 4.1a). Protomers were detected in the medium from cultured cells by electron microscopy as about 400-nm long flexible thread-like structures with NC1 globule on one end (Oberbaumer et al. 1982).

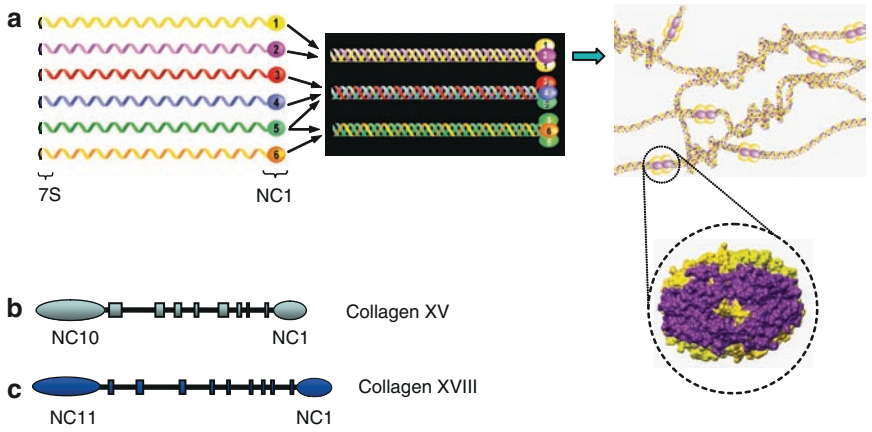

Fig. 4.1 Structure of some of the nonfibrillar BM collagens. **a** Collagen IV is a family of six homologous α chains (α1–α6), each composed of an amino terminal 7S, a central collagenous triple helix, and a carboxyl terminal NC1 domains. The α chains assemble within cells into heterotrimers (protomers), which are further assembled into collagen IV networks after secretion to the extracellular space (the α1α1α2 network is shown as an example on the right). The insert shows the structure of NC1 domain hexamer formed by head-to-heat association of two protomers as determined by X-ray crystallography (Sundaramoorthy et al. 2002). **B** and **c** Structure of α1 chains forming collagens XV. Each chain is composed by multiple collagenous domains (black lines) which are separated by 10 or 11 NC domains. Large amino and carboxyl terminal domains are represented by ellipses, while smaller internal NC regions are shown as rectangles. The exact homotrimer and supramolecular organization for XV and XVIII is currently unknown

Protomer assembly is initiated by interactions between NC1 domains of three α chains followed by the propagation of triple helix towards amino termini in a zipper-like manner (Dolz et al. 1988). Recent studies showed that NC1 domains also govern the specificity of α chains selection (Boutaud et al. 2000; Khoshnoodi et al. 2006). Upon secretion in extracellular space, protomers further assemble into collagen IV networks through association of NC1 trimers from two protomers to form dimers and four 7S domains to form tetramers. Lateral associations between triple helical domains of collagen IV protomers also contribute to the networks formation. NC1 domain hexamers and 7S tetramers could be isolated from various BMs after treatment with bacterial collagenase. Furthermore, the recently solved crystal structure of the NC1 hexamer revealed detailed information about interactions between individual NC1 domains (Sundaramoorthy et al. 2002; Than et al. 2002). Three distinct collagen IV networks are known based on the composition of constituent protomers: the α1α1α2 network, which is ubiquitously expressed in the majority of BMs (Timpl et al. 1981), the α3α4α5 network, which is restricted mainly to glomerular and alveolar BM of kidney and lung (Saito et al. 2000; Borza et al. 2002); and the α5α5α6 network, which is a feature of skin, smooth muscle and kidney Bowman's capsule BMs (Ninomiya et al. 1995; Borza et al. 2002). The expression of collagen IV chains is tightly regulated. For instance, in the glomerular BM, genes encoding the α1(IV) and α2(IV) chains are expressed during early embryonic development, but their levels gradually decrease as the expression of genes encoding the α3(IV), α4(IV), and α5(IV) chains starts. This developmental switch is critical for the maturation of the glomerular BM as a specialized plasma filtration barrier in the kidney, since most mutations in genes encoding the α3(IV), α4(IV), or α5(IV) chains leads to defects in α3α4α5 network assembly and renal failure in patients with Alport syndrome (Hudson et al. 2003).

Collagen IV plays an important role in the interaction of cells with underlying BMs. Cell culture studies have shown that collagen IV promotes the adhesion and spreading of many different cell types including endothelial cells (Underwood and Bennett 1993), breast cancer cells (Abecassis et al. 1987), prostate carcinoma (Dedhar et al. 1993), melanoma (Chelberg et al. 1989), fibrosarcoma (Aumailley and Timpl 1986), glioma (Knight et al. 2000), and neuroblastoma (Tzinia et al. 2002). Moreover, collagen IV also strongly activates cell migration and stimulates proliferation of endothelial cells.

Many cellular responses to collagen IV and other ECM proteins are mediated by members of the integrin family of transmembrane receptors. Integrins comprise a large number of structurally and functionally related glycoproteins that form noncovalently bound heterodimers of an α and a β subunit (Hynes 2002; Humphries et al. 2006; Askari et al. 2009). Each subunit has a large extracellular domain, a short transmembrane segment and a cytoplasmic tail with affinity for cytoskeletal proteins. Thus, the term integrins reflect the ability of these proteins to "integrate" cells into their immediate environment by connecting the cytoskeleton with the extracellular components. So far, 18 α and 8 β subunits have been identified in mammalian cells that form 24 distinct integrins (Hynes 2002; Humphries et al. 2006; Askari et al. 2009).

The major collagen IV receptors belong to the β1 subgroup of integrins, namely α1β1 and α2β1 (Paulsson 1992; Aumailley and Gayraud 1998; White et al. 2004). Binding of these receptors to collagen IV triggers specific pathways involved in cell survival, migration, and invasion, including activation of Shc, T-cell protein tyrosine phosphatase, Phospholipase Cγ, FAK, ERK, PI3K, p38 MAPK and PRL-3 (Wary et al. 1996; Pozzi et al. 1998; Loster et al. 2001; Vossmeyer et al. 2002; Mattila et al. 2005; Peng et al. 2006; Abair et al. 2008). Deletion of α1β1 integrin by homologous recombination resulted in a dramatic decrease in adhesion and migration of fibroblasts and smooth muscle cells to collagen IV (Gardner et al. 1996), as well as inability to activate selective cytoplasmic signaling (Pozzi et al. 1998; Chen et al. 2007; Abair et al. 2008). The importance of α2β1 integrin as collagen IV receptor was demonstrated by the fact that down regulation of the integrin α2 subunit leads to decreased collagen IV-dependent adhesion and motility (Keely et al. 1995). A major site for binding of α1β1 and α2β1 integrins to collagen IV has been identified within a triple-helical cyanogen bromide-derived fragment (CB3), located 100-nm away from the amino-terminus of the collagen IV (Vandenberg et al. 1991). Indeed, antibodies to CB3 fragment blocked cell adhesion to collagen IV by 80%. Later on, one binding site for α1β1 integrin and two for α2β1 integrin were suggested in different, but adjacent positions based on further proteolytic fragmentation of CB3 (Kern et al. 1993). Further refinement identified a conformational-dependent site formed by the two residues, Asp^{461} on α1 chain and Arg^{461} on α2 chain as critical for α1β1 integrin binding (Eble et al. 1993). In contrast, the precise structure of α2β1 binding sites remains unknown.

In addition to integrins α1β1 and α2β1, several other integrins may also participate in cell binding to collagen IV. For example, α3β1 integrin was involved in adhesion of small lung carcinoma cell line to collagen IV (Elices et al. 1991), and antisense oligonucleotide to the integrin α3 subunit decreased adhesion of prostate carcinoma cells to collagen IV (Leung-Hagesteijn et al. 1994). However, the role of α3β1 as a collagen IV receptor remains controversial, since integrin-specific antibody failed to block cell adhesion to collagen IV (Elices et al. 1991; Melchiori et al. 1995), and purified integrin α3β1 failed to bind collagen IV in vitro (Eble et al. 1998). Most surprisingly, genetic ablation of the integrin α3 subunit increases adhesion and migration of keratinocytes on collagen IV leading to the hypothesis that integrin α3β1 might act as a trans-dominant inhibitor of collagen IV binding integrins (Hodivala-Dilke et al. 1998).

Finally, binding of two new integrins, α10β1 and α11β1, to collagen IV has been reported (Tiger et al. 2001; Tulla et al. 2001). Their expression is spatially and temporally restricted to chondrocytes and fetal muscle cells, suggesting that they may play a specialized role in development.

During invasion and metastasis cancer cells extensively degrade both epithelial and vascular components of the BMs. Consequently, alterations in collagen IV integrity and/or assembly have been reported in various tumors. For example, in aggressive basal-cell carcinomas, down regulation of the α5(IV) chain and degradation of the α1(IV) chain have been observed (Quatresooz et al. 2003). Similar loss of α5/α5/α6 and α3/α4/α5 chains has been reported in invasive breast

and lung carcinomas (Nakano et al. 1999, 2001). In colorectal cancer, the α1 and α2 chains of collagen IV form a linear staining in the BMs, while the α5 and α6 chains present a discontinuous or negative staining, suggesting that the α5/α6 chain staining may be a diagnostic marker for the invasiveness of colorectal cancer (Hiki et al. 2002; Ikeda et al. 2006).

Collagens XV and XVIII

Two other nonfibrillar collagens expressed in BMs are collagens XV and XVIII. Collagens XV and XVIII are closely related and belong to multiplexin subfamily (for *multiple* triple heli*x* domains with *in*terruptions) (Myers et al. 1992; Rehn et al. 1994). Both collagens are encoded by single genes, and the corresponding α1 chains are assembled into homotrimers (Fig. 4.1b). Each α1 chain has a long noncollagenous amino terminal domain, multiple short triple-helical domains and, as in collagen IV, a carboxyl terminal noncollagenous (NC1) domain. Collagen XV contains 9 collagenous domains, separated and flanked by 10 noncollagenous regions. It is highly expressed in the adrenal gland, kidney and pancreas (Muragaki et al. 1994). It is associated with vascular, neuronal, mesenchymal and some epithelial BMs, indicating a function in adhesion between BM and the underlying connective tissue stroma. Closely related, collagen XVIII contains 10 collagenous domains, separated and flanked by 11 noncollagenous regions (Oh et al. 1994) (Fig. 4.1b). The large C-terminal NC1 domain consists of trimerization region (50 residues), a protease-sensitive hinge region (70 residues) and an endostatin domain (180 residues). Several splicing variants of this collagen have been described: three in mice, and two in humans (Saarela et al. 1998). Each of them shows a characteristic expression pattern, with the highest levels in liver, kidney and lung, where collagen XVIII forms a major component of vascular and epithelial BMs (Muragaki et al. 1995). Besides the typical collagen features, collagen XVIII also has properties of a heparan sulfate proteoglycan (HSPG), with several Ser-Gly dipeptides suitable for glycosaminoglycan attachment and with long heparitinase-sensitive carbohydrate chains. Together with collagen IV, important biological role of collagens XV and XVIII could be illustrated by the early evolutional conservation with homologues of all three types existing in *Drosophila* and even earlier in nematodes (Myllyharju and Kivirikko 2004).

In pathological conditions, such as mammary ductal carcinoma, complete degradation of collagen type XV precedes tumor cell migration and invasion, suggesting that the disappearance of collagen XV can be used as an early marker of aggressive breast cancer (Amenta et al. 2003). Furthermore, the expression of collagen XV is down regulated in the BM of human colonic adenocarcinomas, but increased in the surrounding interstitium suggesting a role for this protein in the invasive process and its usefulness as a sensitive indicator of tumor invasion (Amenta et al. 2000). Similar to this, low collagen XVIII expression by hepatocarcinoma cells correlates with large tumor size and invasiveness, while tumors expressing high levels of collagen XVIII are smaller and noninvasive (Musso et al. 2001).

Finally, studies performed on mice lacking collagen XVIII expression show that loss of collagen XVIII enhances neovascularization and vascular permeability in atherosclerosis, suggesting that collagen XVIII is a negative regulator of endothelial cell functions (Moulton et al. 2004). Although it is not clear what receptors bind intact collagen XV and XVIII and transduce signaling, integrins have been shown to bind cleavage products of collagen XVIII thus controlling cell functions (see below for details).

Collagen XIX

Collagen XIX is a poorly characterized member of the fibril-associated collagens with an interrupted triple helices (FACIT) class of collagen molecules (Khaleduzzaman et al. 1997). While this basement membrane-localized collagen is found in all embryonic tissues in an 18-day-old mouse embryo, only a few adult tissues seem to accumulate Col19a1 mRNA (Sumiyoshi et al. 1997). Type XIX collagen is composed of a chain with a 268-residue amino terminus, an 832-residue discontinuous collagenous region, and a 19-residue carboxyl peptide (Myers et al. 2003). This chain assembles to forms oligormers of various lengths (Myers et al. 2003). Light microscopy immunohistochemistry of adult human tissues demonstrated that type XIX is localized in vascular, neuronal, mesenchymal, and some epithelial basement membrane zones. In the normal breast, XIX form a continuous staining pattern in all BMs; however, complete degradation of collagen type XIX precedes tumor cell migration and invasion, suggesting that the disappearance of collagen XIX can be used as an early marker of aggressive breast cancer (Amenta et al. 2003).

Collagen-Derived Cleavage Products and Their Role in Angiogenesis and Cancer Progression

Fragments derived from BM by proteolytic cleavage raised a great interest in the scientific community after the finding that some ECM fragments exert potent anti-angiogenic and anti-tumorigenic activity. This discovery initiated extensive series of in vitro and in vivo studies to unravel molecular mechanisms and characterize effects of these fragments as potential anti-cancer therapies. In particular, several fragments of nonfibrillar collagens were shown to alter specific cell functions, such as tumor cell migration/invasion and endothelial cell proliferation/survival (reviewed in (Marneros and Olsen 2001; Mundel and Kalluri 2007)). Interestingly, effects of these fragments on tumor and endothelial cells are often opposite to those of parental intact collagen molecules. This indicates that binding sites which mediate these effects are sequestered or cryptic in the context of intact BM, therefore, these fragments

are sometimes referred to as matricryptins (Davis et al. 2000) or matrikines (Maquart et al. 2005). The anti-angiogenic and anti-tumorigenic properties of these cleavage products are summarized in Table 4.1.

Proteolytic cleavage within the hinge region of the NC1 domain of collagen XVIII leads to the generation of a potent anti-angiogenic carboxyl terminal fragment endostatin (Fig. 4.1). It was first discovered in 1997 by O'Reilly and co-workers as a 20-kDa protein secreted by murine hemangiothelioma tumor cell line that inhibits proliferation of endothelial, but not cancer cells (O'Reilly et al. 1997). Recombinant endostatin expressed and purified from insect cells inhibited growth of several tumors including lung carcinoma, melanoma and fibrosarcoma in mice via potent anti-angiogenic activity. Several proteases including cathepsin L, MMP-7 and elastase are able to release endostatin from collagen XVIII (Felbor et al. 2000; Ferreras et al. 2000; Lin et al. 2001; Heljasvaara et al. 2005). Moreover, endostatin was detected in mouse and human serum in concentrations from 100 to 300 ng/ml (Dhar et al. 2002). This led to hypothesis that under normal physiological conditions endostatin may play a role of endogenous inhibitor of angiogenesis. Although it is unknown how endostatin exerts its anti-angiogenic activity, a number of biologically important effects were described for this fragment. Endostatin can compete with b-FGF and VEGF for heparan sulfate chain binding thus preventing the storage of these pro-angiogenic growth factors in the ECM milieu (Eriksson et al. 2003). Endostatin treatment induces endothelial cell apoptosis through marked decrease of anti-apoptotic protein Bcl-2 (Dhanabal et al. 1999). It can also inhibit activity of the matrix metalloproteinase (MMP) 2 by binding to its catalytic domain (Lee et al. 2002) and prevent MMP2-mediated activation of membrane type-1 matrix metalloproteinase (Kim et al. 2000). Endostatin inhibits proliferation and migration of endothelial cells by interacting with integrin $\alpha5\beta1$ (Sudhakar et al. 2003) and, to smaller extent, with integrins $\alpha v\beta3$ or $\alpha v\beta5$ (Rehn et al. 2001; Wickstrom et al. 2002) which are primarily expressed by angiogenic microvascular endothelial cells (Friedlander et al. 1995) (Table 4.1). Finally, endostatin could exert its anti-angiogenic activity by binding the proteoglycan glypicans on the surface of endothelial cells (Karumanchi et al. 2001).

Another fragment derived from the NC1 domain of type XV collagen, restin, is highly homologous (70%) to endostatin (Ramchandran et al. 1999; Sasaki et al. 2000). Despite the high homology, binding properties to other ECM molecules as well as tissue distribution of restin differs from those of endostatin (Sasaki et al. 2000). Moreover, restin inhibits b-FGF induced migration, but not proliferation of endothelial cells (Ramchandran et al. 1999) (Table 4.1).

Recently, anti-tumorigenic and anti-angiogenic activities have been also attributed to the NC1 domain of collagen XIX (Ramont et al. 2007). In vivo treatment with this NC1 domain reduced melanoma growth and tumor-associated angiogenesis, while in vitro treatment with this domain inhibited both melanoma cell invasion and endothelial cell tubulogenesis. Although it is not clear which receptor(s) is required to mediate these effects, treatment with the NC1 domain of collagen XIX led to decreased expression of membrane type-1 matrix metalloproteinase (matrix metalloproteinase-14) and VEGF (Ramont et al. 2007) (Table 4.1).

Table 4.1 The NC1 domains of collagen IV, XV, XVIII and XIW collagen chains and their effect on cell functions

Collagen IV	α1(IV) NCI	Inhibition of cell growth, migration and tube formation in vitro Anti-angiogenic in vivo	Integrin α1β1	Inhibition of FAK, Raf, MEK, ERK, and p38 MAPK activation	Sudhakar et al. (2005)
Collagen IV	α2(IV) NCI	Promotion of cell apoptosis in vitro. Inhibition of cell proliferation migration and tube formation in vitro	β1 integrins as well as integrins αvβ3 and αvβ3	Stimulation of procaspase-9 cleavage	Petitclerc et al. (2000) Kamphaus et al. (2000) He et al. (2003); He et al. (2004) Magnon et al. (2005)
Collagen IV	α3(IV) NC1	Inhibition of cell protein synthesis in vitro Inhibition of cell tube formation in vitro Anti-angiogenic in vitro and in vivo	CD47/IAP Integrins α3β1, αvβ3, and αvβ5	Inhibition of protein synthesis. Inhibition of FAK and Akt phosphorylation Inhibition of hypoxia-induced COX2 expression Inhibition of COX2-mediated synthesis of angiogenic factors via the IkBα/NFkB axis	Petitclerc et al. (2000) Maeshima et al. (2002); Kawaguchi et al. (2006) Pasco et al. (2000a, b); Pedchenko et al. (2004); Borza et al. (2006); Miyoshi et al. (2006); Boosani et al. (2007) Shahan et al. (1999) Maeshima et al. (2000a, b)
Collagen IV	α4(IV) NC1	Unknown	Unknown	Unknown	
Collagen IV	α5(IV) NC1	Poor anti-angiogenic in vitro	Unknown	Unknown	Petitclerc et al. (2000)
Collagen IV	α6(IV) NC1	Inhibition of cell growth in vitro Anti-angiogenic in vivo	Integrin αvβ3	Unknown	Petitclerc et al. (2000) Mundel et al. (2008)
Collagen XV	Restin	Inhibition of cell migration in vitro	Unknown	Unknown	Heljasvaara et al. (2005)

(continued)

Table 4.1 (continued)

Collagen XVIII	Endostatin	Inhibition of cell migration and growth in vitro Anti-angiogenic in vivo	Glypican-1, β1 integrins, integrins α5β1 αvβ3 and αvβ5	Competition with b-FGF and VEGF for heparan sulfate chain binding. Binding to and inhibition of the catalytic domain of MMP2 Inhibition of the FAK/c-Raf/p38/ERK1 pathway Disassembly of actin stress fibers and focal adhesions	Kim et al. (2000); Karumanchi et al. (2001); Lee et al. (2002); Eriksson et al. (2003); Sudhakar et al. (2003) Rehn et al. (1994) Rehn et al. (2001); Rehn et al. (2004) Wickstrom et al. (2002)
Collagen XIX	NC1 domain	Inhibition of melanoma cell invasion and cell tubulogenesis in vitro Anti-angiogenic and anti-tumorigenic in vivo		Decreased expression of membrane type-1 matrix metalloproteinase and VEGF	Ramont et al. (2007)

The NC1 domains of different collagen IV α chains have been also associated with inhibition of angiogenesis and tumor cell growth (Petitclerc et al. 2000). The α1(IV) NC1 domain acts as an anti-angiogenic molecule by inhibiting endothelial cell proliferation, migration and tube formation in vitro, as well as primary and metastatic tumor growth in vivo (Colorado et al. 2000) (Table 4.1). The anti-angiogenic properties are mediated by binding to integrin α1β1, the major collagen IV binding receptor, although the molecular mechanism has not been yet determined (Sudhakar et al. 2005). The α2(IV) NC1 domain also displayed anti-angiogenic and anti-tumorigenic activity (Table 4.1) via αv and β1 integrin-dependent mechanisms (Kamphaus et al. 2000; Petitclerc et al. 2000). Further studies identified two different subdomains of α2(IV) NC1, the N-terminal (residues 1–89) and the C-terminal (residues 157–277). Although both of them decrease tumor angiogenesis in vivo, the amino terminal subdomain enhances endothelial cell apoptosis (He et al. 2003), whereas carboxyl terminal subdomain seems to exert its functions by specifically inhibiting endothelial cell proliferation (He et al. 2004). Increased endothelial cell apoptosis is mainly attributed to inhibition of Akt, FAK, and eukaryotic initiation factor-4E-binding protein-1 phosphorylation, induction of Fas ligand expression, as well as cleavage of procaspases 8 and 9 (Panka and Mier 2003). In addition, recombinant α2(IV) NC1 domain exhibits anti-tumorigenic activity by inhibiting in vivo growth of melanoma and ovarian carcinoma, and inducing senescence in tumor cells presumably through integrin α1β1-, αvβ3-, and αvβ5 -dependent mechanism (Magnon et al. 2005; Roth et al. 2005).

Particular attention has been given to the biological effects of the NC1 domain of the α3(IV) chain, after the observation that recombinant α3(IV) NC1 (a) potently inhibited bFGF-induced angiogenesis in chick chorioallantoic membrane (Petitclerc et al. 2000); (b) prevented protein synthesis in endothelial cells by inhibiting FAK, PI3K and Akt phosphorylation, as well as dissociation of eukaryotic initiation factor 4E protein from the 4E-binding protein 1 (Maeshima et al. 2002); and (c) directly prevents tumor cell growth in vitro and in vivo in an Akt/mTOR-dependent manner (Kawaguchi et al. 2006). Since then, two distinct peptides within the α3 NC1 domain have been identified with specific anti-angiogenic (Maeshima et al. 2000a, 2000b) and anti-tumorigenic properties (Han et al. 1997) (Table 4.1). The peptide encompassing residues 185–203 of the α3 NC1 inhibits proliferation of melanoma cells without affecting proliferation of normal dermal fibroblasts or endothelial cells (Han et al. 1997; Shahan et al. 1999). Two serine residues in SNS triplet within peptide 185–203 are important for inhibition of melanoma proliferation (Han et al. 1997). This peptide also decreases melanoma cell migration in vitro by preventing binding and activation of MMP2 at the cell surface, down regulating the expression of membrane type-1 metalloproteinase and inhibiting expression of the integrin β3 subunit (Pasco et al. 2000a). In contrast, the peptide corresponding to residues 54–132 of the α3 NC1 domain inhibits endothelial cell proliferation without any effect on tumor cell proliferation (Maeshima et al. 2001a). Anti-angiogenic activity of this region was found to be associated with T7 peptide (residues 74–98) (Maeshima et al. 2001b), and the critical role of leucine, valine and aspartic acid residues was shown recently by the site-directed mutagenesis

(Eikesdal et al. 2008). Interestingly, both peptides bind to the αvβ3 integrin, but their binding sites on this integrin seems to be different (Maeshima et al. 2000a; Pasco et al. 2000b). In contrast to these findings, we demonstrated that the α3(IV) NC1 domain binds to endothelial cells via integrin α3β1 and the binding site for this integrin is within residues 185–203 (Borza et al. 2006). Consistent with this data, the α3(IV) NC1 domain binds both integrins α3β1 and αvβ3 on endothelial cells (Boosani et al. 2007); however, only integrin α3β1-dependent binding of this fragment is crucial to prevent hypoxia-mediated cycloxogenaase (COX)2 expression and COX2-mediated synthesis of angiogenic factors via regulation of IkBα/NFkB axis (Boosani et al. 2007) (Table 4.1). Finally, we demonstrated that the binding site for αv-containing integrins is not within the α3(IV) NC1 domain itself, but rather within an RGD site, which is part of a short collagenous sequence at the N terminus adjacent to the NC1 domain (Pedchenko et al. 2004). The physiological relevance of the RGD containing α3(IV)NC1 domain is demonstrated by the finding that this collagen IV-derived fragment prevents tumor-associated angiogenesis in vivo and inhibits endothelial cell tube formation in vitro (Miyoshi et al. 2006).

A key question is how and why the α3(IV) NC1 domain is generated in vivo. If generation occurs during collagen IV turnover and/or remodeling, it is conceivable that this fragment might act as endogenous inhibitor of tumor cell growth. Interestingly, mice deficient for α3(IV) chain show accelerated tumor growth associated with increased angiogenesis (Hamano et al. 2003). Moreover, treatment of these mice with recombinant α3(IV) NC1 domain reduced tumor growth (Hamano et al. 2003). Although proteolytic pathways releasing this NC1 domain from BM are currently unknown, cleavage of collagen IV by MMP9 released α3(IV) NC1-containing fragments in vitro suggesting involvement of this metalloprotease in the generation of anti-angiogenic fragments (Hamano et al. 2003).

Finally, the α6 NC1 domain also reduces angiogenesis and tumor growth, although underlying mechanism remains unknown (Petitclerc et al. 2000; Mundel et al. 2008). In contrast to the α1(IV), α2(IV), α3(IV) and α6(IV) NC1 domains, homologous NC1 domains of two other collagen IV α chains, namely α4(IV) and α5(IV), seem to lack any anti-angiogenic or anti-tumorigenic activity (Petitclerc et al. 2000).

Anti-angiogenic and Anti-tumor Therapies Using Collagen Fragments: Pros and Contras

The requirement of a blood supply for tumors to grow and metastasize has made tumor angiogenesis an attractive therapeutic target for the treatment of numerous cancers. However due to the primary importance of vascular system in the normal homeostasis of the body, use of anti-angiogenic drugs has both advantages and disadvantages that we will be highlighted below.

Although ECM-cleavage products strongly inhibit tumor-associated angiogenesis in multiple animal models, phase II clinical trials for endostatin demonstrated rather limited success in human cancers. As emphasized in a recent review by Xu

and colleagues, the greatest benefit of these cleavage products occurs when they are administered at early stages of tumor growth, thus explaining why some of the fragments are ineffective in patients with advanced cancer (Xu et al. 2007). Another major problem with these fragments is their short half-life in human body, a phenomenon that could be circumvented by fusing these fragments with Fc fragment of immunoglobulin, as recently done with endostatin (Lee et al. 2008). Finally, the anti-tumor properties of some matrix-derived products are strictly conformation-dependent (Floquet et al. 2004). Thus, it is conceivable that while naturally derived matrix products might have desirable selective effects, exogenously administered recombinant fragments might fail due to inappropriate conformation.

Another possible explanation for the failure and/or unsatisfactory action of these ECM derived compounds is that their effects could be restricted to certain tumor types. Endostatin, for example, has been shown to block the migration and invasion of colon cancer and head and neck squamous carcinoma (Dkhissi et al. 2003; Wilson et al. 2003). Therefore, it is conceivable that the effects of endostatin are tumor specific and maximum beneficial effects can be achieved when both endothelium and tumor cells are targeted simultaneously. Another explanation is that tumors might "counter-act" the anti-angiogenic or anti-tumorigenic effects of these fragments. In this context, it has been recently shown that despite an initial beneficial effect of endostatin and collagen IV-derived fragments, tumors ultimately are able to escape angiogenesis inhibition. This effect is due to the ability of tumor cells to up regulate the expression of potent pro-angiogenic factors such as VEGF, PDGF-A, PDGF-B (Fernando et al. 2008).

Finally, some of the matrix fragments might also act as "double edge sword" as they exert both angio-suppressive and angio-stimulatory effects, as observed for endostatin (Morbidelli et al. 2003). In addition, given that they bind receptors ubiquitously expressed, such as β1-containing integrins, they might have undesirable side effects on normal cells by interfering with cell motility and/or proliferation. As an example, in addition to blocking tumor angiogenesis, the α3(IV)NC1 domain also inhibits the activation of leukocytes (Monboisse et al. 1994), which could result in altered responses to infection and/or injury. Moreover, it is conceivable that collagen-derived fragments based anti-angiogenic therapies alone might not be sufficient to fully prevent and/or inhibit the growth of various tumors, and they might only be used as adjuvant therapies. Their beneficial effects could be significantly improved by optimizing formulation, dose regiment, time of treatment, and by using them in combination with other anti-tumorigenic drugs (Yokoyama et al. 2000; Abdollahi et al. 2003).

Conclusions

Four collagen types, IV, XV, XVIII and XIX are major components of various BMs and play a role in the homeostasis of different cell types by stimulating proliferation, migration, survival, and inducing polarization. In contrast, their cleavage products

act in opposite ways, by inhibiting cell proliferation and migration or inducing apoptosis. In cancer progression, degradation of surrounding BM is considered a key event for the promotion of tumor invasion, migration into the blood and lymphatic vessels, and dissemination to distant sites. Generation of collagen fragments can slow cancer progression by restricting blood supply to the tumor and blocking tumor cell migration. The observation that some of these collagen fragments have anti-angiogenic and anti-tumorigenic activity has raised a great interest in the field of cancer making these fragments attractive candidates for anti-cancer therapies. Treatment of tumor-bearing mice with recombinant collagen fragments or synthetic peptides have led to such promising results that several fragments are now being tested in patients. Although these treatments induce tumor stabilization or even regression in some cases, the preliminary results of clinical studies suggest that these fragments alone may not be sufficient to completely abolish cancer growth. As mentioned above, suboptimal regiments, potential side effects, and selectivity to limited number of tumor types might all contribute to the poor efficacy of these fragments for the cancer therapy. Nevertheless, the discovery of these fragments has led to the hope that they could be administered in combination with other anti-cancer drugs to reduce and ideally prevent tumor progression.

Acknowledgments This work was supported by 2P01DK065123 (AP) and a Veteran Administration merit award (AP).

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Chapter 5
Laminins and Cancer Progression

Jerome Jourquin, Manisha Tripathi, Cherise Guess, and Vito Quaranta

Abstract Laminins ($\alpha\beta\gamma$ heterotrimers) form the basement membrane. They are important for many cell processes, through adhesion and signaling. There are 17 known laminins with different properties, depending on their subunits. Among the many functions fulfilled by laminins, they actively contribute to all stages of cancer progression, from the onset of the disease to the life-threatening development of metastases. At the cellular level, laminins are crucial in helping cells adhere, migrate, and differentiate.

Following the six hallmarks of cancer (self-sufficiency in growth signals, sustained angiogenesis, evading apoptosis, insensitivity to antigrowth signals, limitless replicative potential, and the ability for tissue invasion and metastasis), this chapter will outline available data involving laminins and their receptors in cell proliferation, death, angiogenesis, and cancer invasion and metastasis.

In light of those evidences, there is no doubt that laminins are able to regulate all stages of cancer progression, either directly or in partnership with receptors and coreceptors. Two laminins (laminin-111 and -332) are particularly active in carcinogenesis, while others seem to be more specific to angiogenesis (laminin-211, -213, and -221) or motility (laminin-511). Furthermore, laminins can activate different receptors, which induce different signaling pathways. Some are redundant to laminin functions (PI-3K, FAK, ERK), and others are only activated in a particular context, such as the laminin-induced relocation to the nucleus of transcription factors.

Overall, this chapter updates our knowledge on the role played by laminins in cancer. By integrating the latest developments in the field, this review helps elucidate how these proteins can be at the center of new diagnostic tools, prognostic power, and therapeutic strategies in the fight against cancer.

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_5,

Abbreviations

3-D	Three-dimensional
67LR	67 kDa laminin receptor
ADPKD	Autosomal dominant polycystic kidney disease
AE-2	Alveolar epithelial type II cells
BM	Basement membrane
BP	Bullous pemphigoid antigen
CDKI	Cyclin-dependent kinase inhibitor
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
Grb2	Activation of growth factor receptor-bound protein 2
HGF	Hepatocyte growth factor
IGCA	Intestinal-type gastric carcinomas
IGF	Insulin-like growth factor
IL	Interleukin
IRS	Insulin receptors
JNK	Jun N-terminal kinase
LCNC	Large-cell neuroendocrine carcinoma
LG	Laminin G domain-like
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MT-MMP	Membrane type matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor-kappa B
NGF	Nerve growth factor
NSCLC	Non-small cell lung carcinomas
PI-3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SH2	Src homology 2 domain
STAT	Signal transducers and activators of transcription
TGF	Transforming growth factor
tPA	Tissue plasminogen activator
TUNEL	Terminal transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor.

Introduction

Laminins are glycoproteins present in the basement membrane (BM), which is a specialized extracellular matrix (ECM) separating all epithelial and endothelial cells from the underlying stromal cells, and providing both structural support and vital signaling cues from the microenvironment (LeBleu et al. 2007). They regulate adhesion and signaling through their receptors, which are classified in two categories: integrin and non-integrin types (Table 5.1). They adhere to other ECM molecules, including other laminins, to form a network that essentially strengthens the BM by allowing it to resist tensile force. Laminins also fulfill key functions both during embryonic development, as well as in the adult organism, generally related to tissue maintenance and turnover (Tzu and Marinkovich 2008).

All members of this family of 17 identified proteins are αβγ heterotrimers formed by one of each of the 5α, 4β, and 3γ chains, bound together to form a triple helical coiled-coil domain (long arm) stabilized by disulfide bonds. Portions of each of the three chains form the short arms on each side (β and γ) and at the top of the structure (α) (Tzu and Marinkovich 2008). There is an intracellular pool of β and γ subunit heterodimers in excess of α chain, making α chain expression the limiting factor in laminin synthesis (Church and Aplin 1998). Depending on the heterotrimer formed,

Table 5.1 Laminin heterotrimers and their known receptors

Name		
New	Old	Known receptors
111	1	α1β1, α2β1, α6β1, α6β4, α7β1, 67LR, dystroglycan, LAR receptor phosphatase, heparan sulfates, sulfatides, syndecans
121	3	Unknown
211	2	α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, 67LR, dystroglycan, heparan sulfates, syndecans
213	12	α6β4, 67LR
221	4	Similar to laminin-211
311	6	67LR
321	7	Unknown
323	13	Unknown
332	5	α3β1, α6β1, α6β4
333	None	α6β1
411	8	α3β1, α6β1, α7β1, αvβ3, 67LR
421	9	αvβ3
423	14	αvβ3
511	10	α3β1, α6β1, α6β4, α7β1, 67LR
521	11	α3β1, α6β1
522	None	Unknown
523	15	Unknown

This table presents the relationship between the new and old laminin nomenclatures (Aumailley et al. 2005), as well as the known receptors for each laminin heterotrimer (Givant-Horwitz et al. 2005; Colognato and Yurchenco 2000)

laminins have different properties and bind different receptors (Tzu and Marinkovich 2008). For example, due to the inclusion of a truncated α3 chain, laminin-332 is the most stable laminin with three short arms, but it cannot self-assemble in the BM (Engvall and Wewer 1996). Nonetheless, this laminin is essential for the formation of hemidesmosomes, structures of cell-matrix adhesion characterized by the interaction of laminin-332, α6β4 integrin, collagen-VII, collagen-XVII, and other molecules (Jones et al. 1994). This is one of the many examples of the wide-spectrum of properties of the laminins. They are named according to their subunits. As this nomenclature is fairly recent (Aumailley et al. 2005), we provide a table featuring the relationship between the new and the old (Table 5.1).

Laminins have long been associated with cancer (Fig. 5.1), starting from the discovery of the first member of the family – laminin-111 – which was purified three decades ago from the Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al. 1979). Since then our laboratory and others have demonstrated the importance of laminins in tissue homeostasis and pathology, specifically involving them in cell adhesion and migration (Tzu and Marinkovich 2008). Although it is widely accepted that these two processes may participate in the onset of cancer and its progression, cancer is best described as unregulated cellular growth. The six hallmarks of cancer were defined as: self-sufficiency in growth signals, sustained angiogenesis, evasion of apoptosis, insensitivity to antigrowth signals, limitless replicative potential, and ability for tissue invasion and metastasis (Hanahan and Weinberg 2000). In

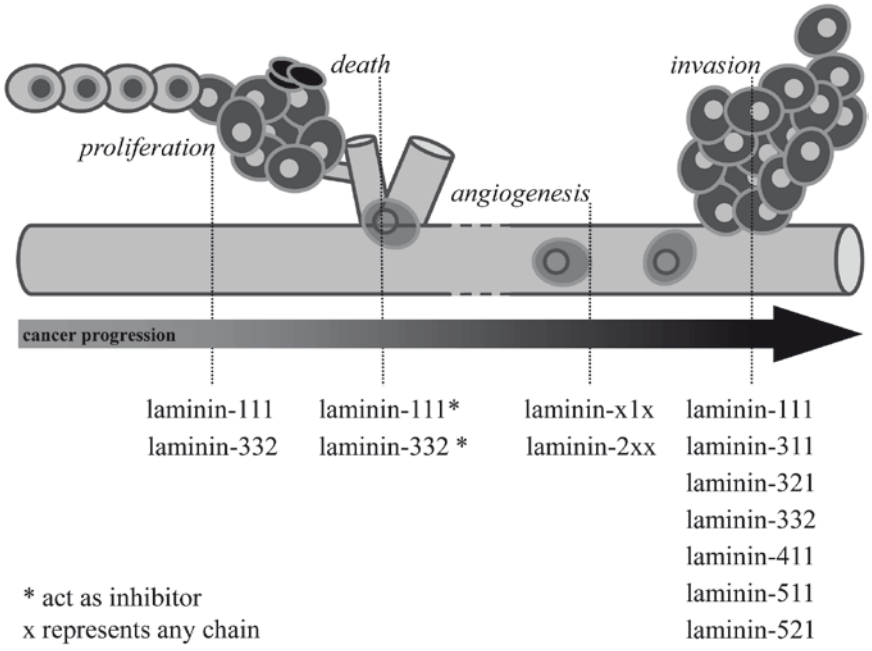

Fig. 5.1 Laminins and cancer progression. Laminins involved in the four main cell processes of cancer progression (proliferation, death, angiogenesis and invasion), as reported in this review

this chapter, we will explore what is known about the involvement of laminins in different cancer hallmark areas. Namely, we will review the literature on laminins, their receptors, and the signaling pathways that they activate in cell proliferation, death, angiogenesis, and cancer invasion and metastasis (Fig. 5.2).

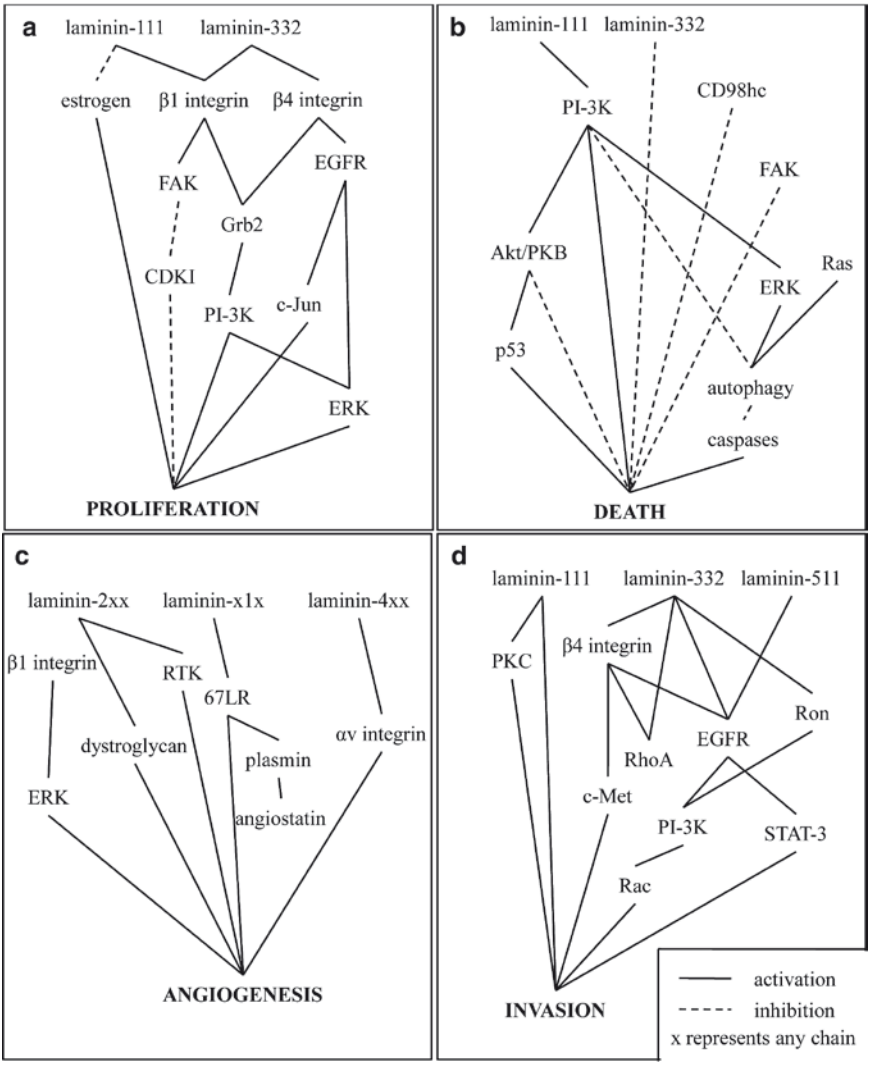

Fig. 5.2 Laminins, their receptors, and the signaling pathways involved in cancer progression. These four diagrams represent the relationships between laminins and cell proliferation (**a**), cell death (**b**), angiogenesis (**c**) and cancer invasion (**d**), respectively, as reported in this review. *Solid lines* denote activation, whereas *dot lines* represent inhibition

Laminins and Cell Proliferation

Proliferation is a well-orchestrated process in which positive and negative regulators act in cooperation. Along with cell death, proliferation is also crucial during development, and important in tissue maintenance and repair. In the case of cancer, it is accepted that normal proliferation mechanisms are *hijacked* by tumors to promote cancer progression (Hanahan and Weinberg 2000). In addition to growth factors, ECM macromolecules, including laminins, may promote or inhibit proliferation (Tzu and Marinkovich 2008).

A good example is the involvement of laminins in the central nervous system plasticity, neuronal death, glial scar/peripheral nerve regeneration, and cancer, mainly through laminin-111, -411, and -511 (Ljubimova et al. 2006). Major sources of laminin associated with neural tissue have been identified to be the Schwann cells and astrocytes (Ljubimova et al. 2006), cells reminiscent of tumor stroma cells since they support neuron functions, as well as blood-brain barrier formation and function. Integrin receptors $\alpha1\beta1$ and $\alpha3\beta1$ have been identified in neuronal cells, including neuroblastoma cell lines SY5Y and IMR32. Only $\alpha1\beta1$ seems involved in neurite outgrowth on a laminin substrate after treatment with nerve growth factor (NGF), making laminin-111 a good candidate for inducing neurite outgrowth (Rossino et al. 1991).

In the past 15 years, several reports have linked laminins to growth and development of breast, prostate and colon cancer cells (Givant-Horwitz et al. 2005). In pituitary tumors, laminin-111 was reported to be differentially expressed, as compared to normal pituitary cells, and to be involved in regulating proliferation and hormone secretion by corticotroph tumor cells. This effect of laminin-111 is dependent upon integrin $\beta1$ activation and GTPases (Kuchenbauer et al. 2001). In human melanoma, laminin-111 has been reported to exert mitogenic activity, apparently in association with neoplastic transformation (Mortarini et al. 1995). Nonetheless, laminin-111 can also behave as a negative regulator of proliferation, like in the rat pituitary tumor cell line GH3 and medullary thyroid carcinoma cell line CA-77 (Lekmine et al. 1999; Kuchenbauer et al. 2003). This inhibitory effect of laminin-111 on proliferation is intriguing because normal pituitary cells attach to a laminin matrix, whereas prolactinoma cells seem less exposed to laminin. This lack of cell-laminin interaction may explain why these cells hyperproliferate, and thus, how these cancers have increased hormone secretion (Kuchenbauer et al. 2003).

The involvement of laminin-111 in cell proliferation is further supported by in vitro three-dimensional (3-D) cultures. Primary mammary epithelial cells, and certain cell lines, cultured in the presence of laminin-111 arrest growth and reorganize to form rounded cell clusters, which function like normal mammary acini (Debnath and Brugge 2005). This type of 3-D cultures lends itself to investigating mechanisms responsible for cell polarization and proliferation (Shaw et al. 2004), and has become a model with good potential to distinguish normal and tumorigenic mammary tissue (Debnath and Brugge 2005). For example, $\beta1$ integrin has been found particularly important in mammary epithelial cells forming 3-D acini in

response to a matrix mainly composed of laminin-111 (Matrigel), which is known to support differentiated mammary cell functions (Streuli et al. 1995). Accordingly, the lack of β1 integrin impairs the formation of 3-D acini in vitro, whereas, in vivo, β1 integrin-null cells were reported to shed into the luminal space without undergoing cell death (Li et al. 2005). Furthermore, 3-D acinar structures derived from the mouse mammary cell line T4-2 (Shaw et al. 2004) undergo a complete reversal of the transformed phenotype, including proliferative arrest, assembly of adherens junctions, and restoration of membrane polarity, when treated with β1 integrin blocking antibody (Wang et al. 2002). These findings strongly support a role for laminin-111 and β1 integrin in organizing of 3-D acinar structures, and perhaps setting ranges for cell survival (Fig. 5.2a).

Integrin α subunits too are important for modulating signals induced by ECM, since they confer receptor transduction specificity by the nature of the ECM macromolecule with which they can associate. However, the specificity of α subunits in laminin-binding integrins is somewhat promiscuous, i.e., there is significant cross-binding to any or all laminin isoforms, and for practical purposes, effects of laminin on cell proliferation are driven by β integrin subunits, the most frequent being the β1 subunit, found in the laminin receptors α1β1, α2β1, α3β1, α6β1, and α7β1 (Stupack and Cheresh 2002; Table 5.1). Laminin-integrin interactions activate many intracellular downstream effectors along signaling networks supporting cell survival, proliferation and migration, such as focal adhesion kinase (FAK), protein kinase C (PKC), phosphoinositide 3-kinase (PI-3K), the small GTPase Rac, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NFκB) (Nguyen et al. 2000). Ligand binding alone is not sufficient for inducing integrin signaling, and it is generally thought that, in the case of laminin-binding integrins receptor, clustering is a prerequisite (Miyamoto et al. 1995). Among the different proteins that can interact with the cytoplasmic tail of β1 integrin, FAK has been linked to proliferation (Stupack and Cheresh 2002). In the absence of β1 integrin, there is a decrease in the level of phosphorylation of FAK. It has been shown that FAK regulates cell proliferation by repressing $p21^{Cip1}$ (cyclin-dependent kinase inhibitor 1A or CDKI-1a) or $p16^{Ink4a}$ (cyclin-dependent kinase inhibitor 2A or CDK4I) (Li et al. 2005). Although another study reports a positive regulation of proliferation by $p21^{Cip1}$ (Bill et al. 2004), the difference might reflect different effects in response to different matrices (collagen and fibronectin, respectively), leading to different signaling pathways mediated by the same β subunit (Li et al. 2005). Abnormal expression of β1 integrin has been involved with hyperproliferation because of altered cell-BM and/or cell–cell interactions (Li et al. 2005). Moreover, β1 integrin is linked to the maintenance of progenitor cells by promoting stem cell proliferation as some progenitors start differentiating (Zhu et al. 1999). Finally, Zhu et al. showed that integrin α11β1, a receptor for interstitial collagen, can influence tumor progression through upregulation of insulin-like growth factor 2 (IGF2) (Zhu et al. 2007). This is consistent with other studies linking β1 integrin to a similar upregulation of IGF2 in fibroblasts and PC3 prostate cancer cells (Goel et al. 2006). This upregulation effect may depend on cell adhesion on laminin-111, activation of growth factor receptor-bound protein 2 (Grb2), Src

homology 2 domain (SH2), and the PI-3K signaling pathway, resulting in overall increased proliferation (Goel et al. 2006). Interestingly, Goel et al. involved the integrin β subunit, whereas Zhu et al. involved the α subunit (Goel et al. 2006; Zhu et al. 2007).

Integrin α6β4 – the only integrin containing the β4 subunit – is a receptor specific to laminin. This integrin is unique because of the length of the β4 cytoplasmic tail, over 1,000 amino acids, whereas the tails of all other β subunits are ~50 residues or less (Wilhelmsen et al. 2006). The β4 tail can be phosphorylated on tyrosine Y1526, which is reported to activate ERK via the recruitment of Shc and Grb2 (Dans et al. 2001). However, phosphorylation of this site does not usually participate in EGF-induced mitosis under normal conditions, indicating that this mechanism might be important in enhancing EGF effects for cells already under strong EGF stimulation, such as carcinomas (Hintermann et al. 2001). Another way integrin α6β4 becomes involved in cell proliferation is by its ability to promote translocation of NFκB to the nucleus, a step inducing progression of the cell cycle in response to laminin-332 (Dans et al. 2001; Nikolopoulos et al. 2005) (Fig. 5.2a). Activation of NFκB in keratinocytes is reported to inhibit proliferation (Takao et al. 2003), whereas its inhibition triggers Ras and β4 integrin-dependent tumorigenesis in human epidermis (Dajee et al. 2003). Another interesting player that gets translocated to the nucleus following laminin stimulation is nucleolin, a cell surface protein. It is speculated that a three-way interaction between nucleolin, hepatocyte growth factor (HGF), and integrins – probably α6β1 or α6β4 – can counter HGF effects, at least in spreading of cells (Tate et al. 2006). Integrin α6β4 can be strongly activated by excessive binding to overexpressed laminin-332 – which is not integrated in the BM for the most part – leading to signaling pathways usually dormant in normal keratinocytes (Davis et al. 2001). This relationship between the amounts of laminins and the proliferative behavior of cells in contact deserves to be investigated quantitatively in more detail. Another example is laminin-111 driven inhibition of estrogen response, whereby a decrease in this laminin can lead to an increase in estrogen-induced proliferation (Woodward et al. 2000). Overall, α6β4 integrin works more as an adaptor protein in the context of normal cell proliferation. Nonetheless, most cancer cells show enhanced tyrosine kinase activity and phosphorylation of β4 integrin (Wilhelmsen et al. 2006). Moreover, tyrosine kinase receptors can be transactivated by integrins, either directly or through downstream effectors (Stupack and Cheresh 2002). This cross-activation is linked to a defect in hemidesmosomes—structures promoting strong and static adhesion. When hemidesmosomes are disrupted, α6β4 is no more localized on the basal side but spreads cell-wide, making it more available for tyrosine kinases to phosphorylate (Wilhelmsen et al. 2006). Hemidesmosome disassembly is also associated with decreased expression of bullous pemphigoid antigens BP180 and BP230, other proteins involved in hemidesmosome formation (Mercurio et al. 2001b), adhesion to BM and anchorage of intermediate filaments, respectively (Jones et al. 1994). α6β4 also regulates ErbB3, which can associate with ErbB2 and promote breast cancer progression and metastasis by activating STAT-3 (disruption of cell polarity) and c-Jun (induction of hyperproliferation) transcription factors

(Guo et al. 2006). Moreover, removing ErbB3 results in inhibition of α6β4-dependent PI-3K activation (Folgiero et al. 2007), key signaling pathway in α6β4-dependent regulation of cell proliferation.

Laminin-332 also strongly supports growth signaling, mainly through its γ2 chain. This laminin chain contains EGF-like repeats (domain DIII) which, after proteolysis by matrix metalloproteinases (MMP), can be released (Giannelli et al. 1997; Koshikawa et al. 2000). Domain DIII has been shown to bind and activate EGF receptor (EGFR), subsequently activating the downstream pathway of EGFR (PI-3K and ERK) (Hintermann et al. 2001; Schenk et al. 2003). Overexpression of laminin-332, specifically in its proteolytically processed form, is commonly associated with tumor progression (Giannelli et al. 1997; Giannelli and Antonaci 2000). Laminin-332's ability to promote cell proliferation is supported by integrins α6β4 and α3β1, leading to the activation of the mitogen-activated protein kinase (MAPK) pathway (Mainiero et al. 1997; Hintermann et al. 2001). Blocking γ2 chain is sufficient for preventing EGF-stimulated proliferation of endothelial cells (Joly et al. 2006). Moreover, the γ2 chain fragments have been found at the invasive front of tumors, where they may support cancer invasion (Koshikawa et al. 1999). Whole γ2 chain protein was also detected in several tumor types like esophageal, cutaneous, oral, laryngeal, colon, tracheal and cervical carcinomas, with or without concurrent detection of laminin-332 heterotrimers, raising the possibility that γ2 is present as a monomer in many cases (Guess et al. 2009). No γ2 has been reported in prostate cancer tissue, in which expression of laminin-332 is generally extinguished (Giannelli and Antonaci 2000; Marinkovich 2007). Laminin-332 heterotrimers, however, are consistently highly expressed at the invasive edge of many tumors, specifically hyperproliferative glioma, colon, stomach, tongue, cervical carcinomas or squamous epithelial cells. In some cases, this expression appears to be under the control of tissue plasminogen activator (tPA) and EGF (Giannelli and Antonaci 2000). Laminin-332 can also be overexpressed in nonneoplastic diseases, such as autosomal dominant polycystic kidney disease (ADPKD). This overexpression is functionally significant, since ADPKD cells—normally negative for laminin-332—form cysts and are hyperproliferative presumably because they are in contact with a thickened, laminin-332-containing BM (Joly et al. 2006).

Laminins and Cell Death

Apoptosis is thought of as a mechanism by which many cells in a tissue or organ can be controlled. Cancer cells acquire the ability to evade this mechanism, and can survive or proliferate in tissue or organs in spite of the presence of pro-apoptotic conditions or signals. In this context, laminin-111 adherent cells show increased proliferation and reduced apoptosis when compared to cells not adhering to laminin-111 (Kim et al. 1999). Accordingly, ovarian cancer cells are protected from radiation- and taxol-induced apoptosis when they are assembled as spheroids (Filippovich et al. 1997; Frankel et al. 1997), a process that can be promoted by

laminin-111 or reconstituted BM (Matrigel). Among the different pathways activated by laminin-binding integrins, PI-3K/Akt/protein kinase B (PKB)/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase kinase (MEK)/ERK pathways are involved in the regulation of apoptosis and survival, as it relates to tumorigenesis and resistance to therapy (Stupack and Cheresh 2002; Givant-Horwitz et al. 2005).

Interestingly, removal of laminin-332—or any of the integrin subunits composing its receptors (α3, α6, β1 or β4)—results in cell de-adhering and undergoing apoptosis (DiPersio et al. 2000), through a process called anoikis (Frisch and Screaton 2001). In this context, cell death is not countered by addition of soluble ligand (DiPersio et al. 2000). However, it is prevented by expression of activated downstream integrin effectors, such as active FAK or Rac (Frisch and Screaton 2001), thus linking integrins to cell survival signaling networks. This is confirmed by several studies showing that integrin-mediated signaling in cell survival is sustained by Ras, ERK, or JNK (Stupack and Cheresh 2002). Indeed, β1 integrin has been described as an anti-apoptotic agent in primary mammary cells, as well as when over expressed in mice (Faraldo et al. 1998). In apparent conflict, β1 integrin was also reported to prevent cells from evading apoptosis. In a recent study, Li et al. showed that, in the absence of β1 integrin, luminal alveolar cells detach and yet they evade apoptosis, as indicated by the absence of terminal transferase dUTP nick end labeling (TUNEL) or cleaved caspase-3 stainings (Li et al. 2005). Therefore, β1 integrin might well be pro- and antiapoptotic depending on the context (ligand, co-factors, and/or activated signaling pathways). Nonetheless, in tumor cells, the highest impact consequence of integrins loss may be acquired insensitivity to growth and differentiation signals in response to ECM molecules (Stupack and Cheresh 2002).

The relay of survival signals from ECM to cells is also affected by integrin-associated molecules, such as tetraspanins and CD98hc (Berditchevski 2001; Kolesnikova et al. 2001; Sterk et al. 2002). Removal of CD98hc was reported to induce anoikis, and the anti-apoptotic effect of CD98hc appears to require direct interaction with β1 integrin and activation of Akt, Rac, FAK and PI-3K signaling pathways (Feral et al. 2005). It was proposed that CD98hc might be able to regulate the expression of β1 integrin, but this is not the case because normal cells and cells null for CD98hc show the same amount of expressed surface integrins, especially β1 (Feral et al. 2005). An intriguing possibility is that this CD98hc connection with β1 integrin may have some role in tumor progression, because of the increased expression of CD98hc in many tumor cells (Campbell and Thompson 2001; Feral et al. 2005).

Cells can also die by autophagy, a process that does not require caspases, the regulators of apoptosis, but rather reactive oxygen species (Degenhardt et al. 2006). Melino et al. demonstrated that cells in the center of acini grown in vitro in 3-D laminin-rich BM undergo death by autophagy (Melino 2005). However, many reports agree that autophagy is indeed a mechanism of cell survival under metabolic stress (Malorni et al. 2007). In the context of prostate epithelial cells, blocking autophagy induces caspase activation and programmed cell death. Thus, autophagy might protect cells from apoptosis, possibly by circumventing

the release of agents such as cytochrome c from mitochondria (Edick et al. 2007). Although there is no evidence of a direct involvement of laminin in autophagy, effectors usually activated by their integrin-type receptors can induce (Ras and ERK) or inhibit (PI-3K) autophagy, a process depending on EGFR activation by integrins. Moreover, it seems that adhesion to and signaling from laminin-332 (mainly through α3β1) are necessary for sustaining autophagy in primary prostate epithelial cells, a mechanism also dependent on activation of EGFR and Src (Edick et al. 2007). In addition, it was shown that caspase-independent melanoma cell death could be driven by inhibition of Raf, or overexpression of Ras (Chi et al. 1999; Panka et al. 2006). Therefore, it seems that perturbations of ERK or Src pathway induce caspase-independent death involving reactive oxygen species. That would put laminin-332 and its receptors as negative regulators of the formation of reactive oxygen species, perhaps through an additional interaction with α1 integrin and the ERK pathway, as suggested recently (Chen et al. 2007). Nonetheless, contradictory results as to whether this regulation will enhance or inhibit autophagy will have to be reconciled (Scherz-Shouval et al. 2007).

α6β4 can also activate Akt, leading to a dual role in apoptosis, depending on the context in which the cell evolves: proapoptotic in cooperation with p53, or anti-apoptotic if p53 function is impaired (Clarke et al. 1995; Bachelder et al. 1999). Amplification of myc and inactivation of either p53 or p16 are commonly observed in immortalized mammary epithelial cells (Yaswen and Stampfer 2002). So α6β4 mainly transmits an anti-apoptotic signal to the cell. Of course, because α6β4 associates with growth factor receptors (Hintermann et al. 2001), it can also indirectly promote survival of endothelial cells via vascular endothelial growth factor (VEGF) expression and activity (Lipscomb et al. 2005) and of epithelial tumor cells via the laminin-332-driven formation of hemidesmosomes and activation of NFκB (Weaver et al. 2002). The pro-survival effect of α6β4 might be carried out through the ability of this integrin to promote cell cycle entry, which involves the translocation of ERK and NFκB to the nucleus (Dans et al. 2001; Nikolopoulos et al. 2005). Note, however, that the latter was also reported to inhibit proliferation, at least in keratinocytes (Takao et al. 2003).

Another facet of laminin roles in cell death (Fig. 5.2b) is the fact that laminins are transcriptionally regulated in response to fibroblast growth factor (FGF), in direct relation to Akt/PI-3K activation (Li et al. 2001). This regulation might act as an amplifier of all the roles discussed above, of laminins in apoptosis. Considering that tumors are usually over-stimulated with respect to growth factors and activated signaling pathways, laminins are of prime interest in understanding cancer progression mechanisms. As an example, Li et al. showed that, following FGF stimulation, Akt and PKB were activated, resulting in the expression of laminin and collagen-IV (Li et al. 2001). This may initiate a reinforcing loop downstream of laminin-332-activated α6β4 integrin, which also activated Akt that together with PKB, have been reported to inhibit apoptosis through their downstream targets, which include NFκB (Degenhardt et al. 2006).

Laminins and Angiogenesis

Angiogenesis is the process by which blood vessels are formed to maintain oxygen supply to cells in tissues. This process is also harnessed by cancer cells to maintain their oxygen supply as their growth expands (Hanahan and Weinberg 2000). Laminin-111 is present in endothelial BM. It was found that more than 20 peptides from laminin-111—from α1 and β1 chains—can promote endothelial cell adhesion, tube formation, and aortic sprouting in vitro (Malinda et al. 1999). BM of tumors and normal tissues also express other laminin chains, mainly α4, α5, β1 and γ1. These subunits can be combined into laminin-411 and -511, which were first described as specific to endothelial cells (Tzu and Marinkovich 2008). Laminin α4 chain G domain-like (LG) domains associate with integrins α3β1 and αvβ3 to induce blood vessel formation (Gonzalez et al. 2002), supported by the fact that laminin α4-null mice develop leaky blood vessels (Zhou et al. 2004). Laminin-411 expression is also increased in glioblastoma, switching from laminin-421 expression in normal cells. This upregulation is associated with angiogenesis and tumor progression (Gonzalez et al. 2002; Ljubimova et al. 2006). Vitolo et al. reported that some squamous cell carcinoma (SCC) and large-cell neuroendocrine carcinoma (LCNC) cell lines were positive for laminin β1 chain—which is present in laminin-211, -411, and 511—as well as for α6β1 integrin (Vitolo et al. 2006). Laminin β1 chain was thus proposed as a new breast carcinoma angiogenic marker (Fujita et al. 2005).

The laminin α2 chain seems to give a migratory advantage to endothelial cells that express it. The laminin α2 chain positive vessels may favor adhesion and trans-endothelial migration of neoplastic cells (Vitolo et al. 2006). For example, migration on laminin α2 in response to EGF is stronger for alveolar epithelial type II cells (AE-2) and MDA-MB-231 cell lines than if the cells are plated on laminin β1 or fibronectin (Vitolo et al. 2006). Moreover, EGF upregulates expression of α6β1 and α2β1 integrins, which could sustain or enhance the laminin α2 effects (Bello-DeOcampo et al. 2001). A few studies linked up regulation of laminin α2 chain to the early stages of neovascularization, and proposed it as an early marker of angiogenesis (Vitolo et al. 2006). Indeed, this chain is strongly expressed in endothelial cells, and in all neuroendocrine carcinomas, where it localizes in the stroma, nearby cells producing VEGF (Vitolo et al. 2006). These findings translate to breast cancer and non-small cell lung carcinomas (NSCLC) where the laminin α2 chain is present around stromal cells producing VEGF, FGF2, and transforming growth factor (TGF)-β1. It is possible that α2-containing laminins use integrin α6β4 to promote these effects because α6β4 is reported to regulate the onset of tumor angiogenesis in mature blood vessels (Zhou et al. 2004).

Laminin-binding integrins also play a role in angiogenesis (Fig. 5.2c.), in addition to RGD-binding integrins, such as the αv integrins (Gonzalez et al. 2002). The α3β1 and α6β1 integrins are reported to retard endothelial cell morphogenic and proliferative events, while promoting BM assembly, pericyte association, and tube stabilization (Zhou et al. 2004). Inhibition of integrin β1 results in changes in laminin

staining localization: in cysts, cells with mutant integrin β1 misplace laminin at the lateral surface of cells, instead of at the basal or the luminal space, which can lead to inversed polarity (Wang et al. 2002). Dystroglycan also cooperates with β1 integrin in laminin polymerization at the cell surface and BM assembly, dependent on Rac1 activation and the actin cytoskeleton (Colognato et al. 1999). Dystroglycan acts as a co-receptor for laminins, and may influence the function of other laminin receptors at the cell surface. Dystroglycan binds with high affinity to laminin α1 and α2 chains, mainly to the LG domains but it does not bind laminin α4 (Talts et al. 2000). Moreover, BM assembly by dystroglycan is required for correct signaling through α6β4 or β1 integrins (Muschler et al. 1999).

It seems that α1β1 and α2β1 integrins are also involved in all steps of angiogenesis: endothelial cell morphogenesis, proliferation, and survival (Stupack and Cheresh 2002; Vitolo et al. 2006). This would point to a role of interstitial collagen-I, for which direct evidence is currently lacking. α1β1 is also a receptor for collagen-IV and laminin-111. As α2β1 can compensate for α1β1 functions in vascularization (Senger et al. 2002), it seems like α1β1 and α2β1 might be promising targets for anti-angiogenesis therapy. This is intriguing because both of these integrins promote angiogenic cell processes specific to activated endothelial cells and tumor progression (Senger et al. 2002; Giancotti 2007). Moreover, α1β1 and α2β1 integrins function in VEGF-induced angiogenesis and is dependent upon their ligand concentration; high levels induce robust activation of ERK1/ERK2 MAPK by either integrin, whereas low levels of ligand require that the two integrins cooperate to induce a strong VEGF effect (Senger et al. 2002). α1 integrin-null mice exhibit enhanced MMP expression accompanied by a higher concentration of plasma angiostatin, which is responsible for reduced vascularity and cancer progression (Pozzi et al. 2000).

Interestingly, a recent study reported a potential role for laminin and its 67 kDa laminin receptor (67LR) in this process. Moss et al. found that 67LR binds to a cystein rich domain on the short arm of laminin β1 and can stimulate vascular growth, possibly after shedding of and binding to laminin-111 in malignant cells (Moss et al. 2006). Other studies have involved this receptor in angiogenesis on the surface of vascular endothelial cells (Donaldson et al. 2000) and, more generally, in tumor invasion and metastasis. Evidence in favor of a role of laminin in angiogenesis through 67LR is the positive correlation between the levels of 67LR expression and endothelial cell proliferation. It seems that 67LR prevents endothelial cell migration specifically by targeting proliferative endothelial cells, leaving quiescent cells unperturbed (Donaldson et al. 2000). 67LR seems to work by inducing conformational changes to laminin, apparently crucial for cell adhesion and migration (Ardini et al. 2002). Earlier studies by Grant et al. showed that the interaction of endothelial cells with laminin through 67LR induces differentiation, whereas laminin binding to high molecular weight receptors (e.g., integrin) promotes cell adhesion (Grant et al. 1989). Nonetheless, it is possible that 67LR effects are independent of laminin binding. Phylogenetic trees show that the laminin-binding function of 67LR appeared late in evolution, and its main function being ribosomal as it enhances protein synthesis during tissue repair (Ardini et al. 1998).

Laminins and Cancer Invasion and Metastasis

Due to the involvement of laminins and their receptors in numerous cell processes, it is not surprising laminins are also involved in cancer progression. Usually, laminin expression is associated with poor prognosis (Giannelli and Antonaci 2000; Mercurio et al. 2001a), but several patterns are observed. For instance, in intestinal-type gastric carcinomas (IGCAs), laminin-111 and laminin-332 are linked to invasion, whereas expression of both laminins are decreased in later stages of breast cancer, nurturing the possibility that these laminins, specifically laminin-332, might be tumor suppressors (Giannelli and Antonaci 2000; Sathyanarayana et al. 2003; Liu and Quaranta, personal communication).

It has been proposed that aggressive tumor cells may adhere to laminin and that, in turn, laminin promotes their motility and ultimately metastasis (Tzu and Marinkovich 2008; Fig. 5.1). For example, SCLC cells become motile on laminin and this interaction seems to confer drug resistance (Fridman et al. 1990). A role for laminins in chemotactic and haptotactic tumor cell migration has been described repeatedly (Tzu and Marinkovich 2008; Georgescu and Quaranta, personal communication). Laminins are expressed at the invasive front of many carcinomas, making them appealing candidates as promoters of spatial invasion. However, Goldberg et al. reported that there was no correlation between the expression of ECM proteins, including laminin and fibronectin, and the pathological grade of tumors they investigated, leading to the hypothesis that cells might have a potential to invade surrounding tissues but not necessarily a potential to metastasize, depending on the combination of ECM proteins and receptors available (Goldberg et al. 1998). Therefore the relationship between local invasion and metastasis remains a subject of investigation. In the case of laminin-332, local invasion may be promoted by disruption of cell–cell junctions and promotion of cancer cell motility, e.g., by engaging cell migration machinery (Marinkovich 2007), while a more direct role in metastasis is supported by experimental ex vivo evidence that laminin-332 in exposed *subendothelium* may participate in the binding of tumor cells to extravasion sites (Wang et al. 2004). Laminin-332 function in local invasion may be regulated by MMPs, so that upon cleavage, laminin-332 substate goes from being pro-adhesive to pro-migratory (Giannelli et al. 1997). From this initial observation, proteolytic processing of laminin-332 has been extended to include the cleavage of laminin α3 by plasmin, MMP-2, membrane-type MT1-MMP, and BMP1 (Tzu and Marinkovich 2008), of laminin β3 chain by MMP-7, MT1-MMP and hepsin (Udayakumar et al. 2003; Tripathi et al. 2008), and of laminin γ2 chain by MMP-2 and MT1-MMP (Koshikawa et al. 2004). While these various types of processing have all been shown to increase cell migration, it may be helpful to think of the possible biological role of laminin-332 proteolytic processing in the context of biological functions and/or molecular interactions that have been assigned to various domains of laminins. For instance, the LG4/5 domains of laminin α1, α2 and α3 chains promote binding of cell surface non-integrin receptors, such as heparin, syndecans and glycolipids (Tzu and Marinkovich 2008). Interestingly, the LG4/5

domain of laminin α3 is liberated by serine protease cleavage, and was reported to be involved in SCC tumorigenesis (Tran et al. 2008). The domains that support formation of a network between laminin-332 and collagen-VII may also be disrupted by laminin-332 proteolysis, leading to the hypothesis that integrity of this network is important as a whole in tumor cell invasion (Ortiz-Urda et al. 2005). The γ2 chain has been found to be specifically (over)expressed in aggressive car-cinomas (Giannelli and Antonaci 2000; Marinkovich 2007), which justifies the interest laminin γ2 chain has elicited as a possible marker for cancer prognosis. The γ2 chain is expressed in most carcinomas (Giannelli and Antonaci 2000), but its association with aggressiveness might have been confounded by the variability in reagents used to detect it, since most studies are by necessity immunohistological. A crucial point is whether the γ2 chain is expressed in the context of a laminin-332 heterotrimer, or as a monomer (see above). In a recent study, Guess et al. addressed this issue by analyzing gene expression data for laminin-332 individual chains (Guess et al. 2009). A colon cancer dataset was sufficiently large to show a significant statistical correlation between the β3:γ2 mRNA ratio and prognosis. In brief, the lower the ratio, the worse the outcome. A low β3:γ2 mRNA ratio suggests that monomeric γ2 chain is secreted, since it is known from studies on the biosynthesis of the laminin-332 heterotrimer that a dimer of β3 and γ2 is the first step in the biosynthetic pathway of heterotrimer assembly, prior to association to α3. Larger studies are needed to extend the association between low β3:γ2 mRNA ratio and poor cancer prognosis, as well as confirm the possibility that the high level of γ2 mRNA results in secretion of monomeric γ2 protein. If so, these conclusions will be in agreement with at least one previously proposed correlation between high γ2 monomer secretion and aggressiveness in melanoma (Seftor et al. 2001).

The human colon carcinoma cell line T84 also deposits laminin-332 in response to wounding, along with the deposition of laminin-311 and -321 (Lotz et al. 1997). These increases in laminin expression lead to a relocation of integrins from the lateral to the periphery of the cell, a redistribution required for morphological changes leading to cell migration (Lotz et al. 1997).

The α6 integrin was reported as one of the best predictors of breast cancer survival in women, moreso than the estrogen receptor (Friedrichs et al. 1995), though this does not seem to have reached clinical applications. Inhibition of tumor size and metastases correlates with the absence of α6β1, possibly linking this integrin to breast cancer, fibrosarcoma, and prostate carcinoma progression. Since α6 integrin inhibition blocks melanoma and fibrosarcoma metastasis (Yamamoto et al. 1996), and α6 integrin preferentially complexes with the β4 subunit (Hemler et al. 1989), results on breast cancer cell lines might seem counter-intuitive. Shaw et al. reported that one cell line they used, MDA-MB-435, only expresses α6β1, but not α6β4 (Shaw et al. 1996). However, recent developments revealed that MDA-MB-435 is not a breast cancer derived cell line but instead a melanoma cell line (Rae et al. 2007), which means that, after all, α6β1 might be important in melanoma progression whereas α6β4 remains the integrin of choice for breast cancer invasion.

The α6β4 integrin has been involved with cell migration in colorectal cancer cell lines (Mercurio et al. 2001b) and in keratinocytes (Raymond et al. 2005). Although

it cannot be excluded that α6β4 might simply enhance cell migration by facilitating adhesion of invasive cells to the different matrices encountered (Wang et al. 2004), it seems that α6β4 may be directly involved in invasion, through ErbB2/PI-3K signaling, activation of Src and Fyn (Wilhelmsen et al. 2006; Fig. 5.2d). A role of β4 integrin in cancer progression has been linked to its C-terminus domain (from residue 1355) (Mercurio et al. 2001a) because its deletion directly impacts migration (Nikolopoulos et al. 2005). β4 integrin has been known for its unique and long cytoplasmic tail, which can associate with intermediate filaments. Inhibition of interaction between β4 integrin and laminin-332 or intermediate filaments causes enhanced migration of keratinocytes (Geuijen and Sonnenberg 2002). This effect is attributed to the disruption of hemidesmosomes, making those structures a promoter of anti-migratory static adhesion (Wilhelmsen et al. 2006). Relocalization of β4 from hemidesmosomes to lamellipodia is accompanied with a reassociation of β4 integrin from linking intermediate filaments to linking actin and promoting migration (Rabinovitz et al. 1999; Hintermann and Quaranta 2004). Although laminin-332 is involved in hemidesmosomes, it has been shown that it can also activate Ron receptor—a transmembrane tyrosine kinase receptor involved in inflammatory response to cytokines—which will act with other proteins to prevent α6β4 integrin from forming hemidesmosomes (Santoro et al. 2003). PKC, in particular, is reported to be activated in response to laminins-111 and -332 (Rabinovitz et al. 1999). Growth factors seem to stimulate hemidesmosome disassembly which might explain why cells overexpressing growth factor receptors—many cancer cell lines such as A431—cannot form hemidesmosomes (Wilhelmsen et al. 2006). Indeed, EGF can disrupt hemidesmosomes and promote cell migration by activating PKC and Rac1, which will serine-phosphorylate the cytoplasmic tail of β4 integrin (Rabinovitz et al. 1999; Wilhelmsen et al. 2006). Rac1—downstream from PI-3K—is also involved in localizing α3β1 to lamellipodia to strengthen cell migration, which may be a secondary response to the early relocalization of α6β4 away from hemidesmosomes (Choma et al. 2004). α3β1 has also been associated with cell migration and invasion of melanomas and gliomas (Choma et al. 2004). Nonetheless, the effect of α3β1 on cancer invasion might be dependent on α6β4, necessary to/for the activation of PI-3K signaling pathway (Hintermann et al. 2001). However, α3β1 also promotes migration by binding unprocessed laminin-332 and inducing RhoA-dependent upregulation of laminin-332 (Nguyen et al. 2001). Overall, one can think of laminin-332 as an active player in redistributing integrins on the cell surface to sustain migration, specifically as laminin-332 gets cleaved (Giannelli et al. 1997). In turn, laminin-332 deposited by these migrating cells can form a gradient, and participate in haptotaxis of other cells (Frank and Carter 2004). When α6β4 integrin, associates with actin in the lamellipodia, it leads to activation of PI-3K and RhoA, and promotion of cancer invasion (Shaw et al. 1997). α6β4 can physically associate with Fyn, EGFR, ErbB2, c-Met and Ron (Rabinovitz et al. 1999; Hintermann et al. 2001; Wilhelmsen et al. 2006). Moreover, it has been shown that adhesion of cell protrusions to ECM through α6β4 by itself can generate enough traction to support migration (Rabinovitz et al. 2001). An interesting theory is that α6β4-dependent contractions of the BM create gaps and tears which would allow tumor cells to evade and invade. This is supported by the fact that some cancer cell

migration cannot be blocked by proteinase inhibitors (Rabinovitz et al. 2001), even if, as reported above, proteinase activity enhances tumor invasion (Giannelli et al. 1997; Koshikawa et al. 2000).

In vitro, laminin-511 enhances EGF-stimulated motility of colon carcinoma cells via integrins α3β1 and α6β4 (Pouliot et al. 2001). EGF and laminin α2 chain-positive endothelial cells may independently promote chemokinetic activity of MDA-MB-231 cell line (Vitolo et al. 2006). Growth factor receptors also have such an effect, including insulin receptors (IRS) 1 and 2 (Shaw 2001) or c-Met, the receptor for HGF (Trusolino et al. 2001). Interestingly, HGF induces hemidesmosome disruption and loss of polarity in squamous cells but not in pancreatic cancer cells (Matsumoto et al. 1994), underlining the fact that different types of cells may depend on different growth factors for cell migration.

Finally, several reports outlined the possible importance of laminins-511 and -521. Laminin-511 appears to be highly expressed in different cancers, including breast and brain cancer, ductal carcinoma in situ, tubular carcinomas, atypical medullary carcinomas, and fibroadenoma (Chia et al. 2007; Tzu and Marinkovich 2008). Laminin α5 expression is correlated with metastasis in vivo, at a level of expression greater that laminin-111 or -332 in breast cancer, prostate cancer, and NSCLC (Brar et al. 2003; Chia et al. 2007). Laminin-511 can promote cell motility by interacting with α3β1, α6β1, α6β4 integrins, and possibly with other postulated α3 heterodimers (Coppolino et al. 1995; Pouliot et al. 2001). Interestingly, Chia et al. reported that metastatic cells seem more motile on laminin-511 than non-invasive cells (Chia et al. 2007), potentially linking this laminin to metastatic behavior.

Concluding Remarks

We realize that, in writing this chapter, a large amount of information regarding the role of laminins in cancer has been inevitably left out. We tried to be comprehensive, but apologize for these inadvertent omissions. We also had to limit ourselves to laminins and laminin-binding integrins, and did not cover laminin-binding integrin associated molecules, which nonetheless appear to play crucial roles, including the tetraspanins (CD151, CD9 and CD81) and other cell surface proteins such as CD98hc. While tremendous progress has been made in clarifying the role of laminin and its integrin receptors in cancer, a large amount of work still remains. We hope to have given a sense for the exciting findings that lay ahead.

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Chapter 6
Fibronectins and Their Receptors in Cancer

Christie J. Avraamides and Judith A. Varner

Abstract The extracellular matrix protein fibronectin and its receptors (integrins α5β1, α4β1, α9β1, αvβ3, αvβ1, and αvβ6) play an active role in tumor growth and metastasis. Numerous tumors exhibit increased expression of fibronectin and its receptors, thereby promoting proliferation, survival, and motility of tumor cells as well as their subsequent metastasis. Additionally, fibronectin and integrins on endothelial cells promote tumor angiogenesis and lymphangiogenesis, thereby contributing to tumor metastasis. Antibodies against fibronectin are under development for cancer imaging and for targeted therapy. Antibodies directed against select fibronectin-binding integrins are also under clinical investigation, as these integrins are expressed on tumor cells and tumor neovessels.

Keywords Fibronectin • integrins • angiogenesis • lymphangiogenesis • cancer • extracellular matrix

Introduction

The extracellular matrix (ECM) plays a critical role in tumor growth and spread. The matrix can control tumor growth as well as tumor metastasis by promoting the growth of neoplastic cells, the development of new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis), the invasion of stromal cells, the intravasation of tumor cells into the circulation, and the arrest of tumor cells in capillary beds of distant organs. Tumor cells can then proliferate at these distant sites and the cycle repeats, producing additional metastases. In this review, we focus on the role of the extracellular matrix protein fibronectin and its receptors in cancer.

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_6,

Extracellular matrices and their receptors regulate a number of cellular processes, including cell proliferation, differentiation, and migration. ECM remodeling occurs during embryonic development, wound repair, inflammation, and disease, including cancer. Remodeling of the matrix occurs when cells express or activate enzymes such as metalloproteinases that degrade matrices and synthesize new matrix proteins (Kaspar et al. 2006). As oncofetal matrices differ from those of normal tissues, changes in ECM components during tumor development and progression form the focus of novel tumor imaging and therapeutic applications.

In order for any tissue to grow beyond a volume of about one cubic millimeter, it requires a blood supply. Angiogenesis, the development of new blood vessels from pre-existing vessels, is critical for embryonic development, and wound healing but is also critical for tumor growth and tumor metastasis (Carmeliet 2005). Growth factors that induce angiogenesis, such as vascular endothelial growth factor (VEGF), are secreted by tumor cells, macrophages, and fibroblasts within tumors (Adams and Alitalo 2007; Lin and Pollard 2007; Schmid and Varner 2007). Integrins also play key roles in regulating the endothelial cell migration and survival and are critical in regulating tumor angiogenesis (Avraamides et al. 2008).

Lymphangiogenesis, the growth of new lymphatic vessels, promotes tumor metastasis (Dadras et al. 2005; Hirakawa et al. 2005; Roma et al. 2006; Adams and Alitalo 2007). Tumor-secreted factors stimulate the growth of new lymphatic vessels within tumors and draining lymph nodes (Dadras et al. 2005; Hirakawa et al. 2005; Roma et al. 2006). This increased lymphatic vessel density in tumors is associated with increased metastasis to lymph nodes (Dadras et al. 2005; Hirakawa et al. 2005; Roma et al. 2006). Select growth factors and fibronectin-binding integrins can modulate lymphangiogenesis and affect tumor metastasis (reviewed in Avraamides et al. 2008).

Cancer treatments range from surgery to radiation therapy and chemotherapy. The central goals of cancer therapies are removal of tumors and prevention of metastases, with minimal damage to healthy cells. However, resistance to therapy develops in many patients and alternative strategies are under investigation. Fibronectin and its receptors may provide focal points for novel therapeutic strategies. The interaction of ECM molecules such as fibronectin with their receptors can favor drug resistance by suppressing apoptosis, modulating cell cycle progression, and inhibiting drug-induced DNA damage. In particular, β1 integrins can mediate resistance to cytotoxic chemotherapy and radiation (Yao et al. 2007). Since fibronectin and its receptors can provide tumor cells with survival signals and mediate drug resistance, treatments blocking either integrin signaling or fibronectin expression may be of therapeutic importance.

Fibronectin

Fibronectin is a large molecular weight extracellular glycoprotein which is present at low concentration in basement membranes, at high concentration in remodeling extracellular matrices, and at 300 μg/ml in plasma (Mosher 1984). Its receptors are the integrins, a large family of heterodimeric type I membrane protein receptors (Hynes 2002).

Fibronectin is expressed by endothelial cells during embryonic and tumor development but is poorly expressed by quiescent endothelium. This protein plays a key role in wound healing and angiogenesis. Fibronectin is a dimer of two subunits (220–250 kDa) that are disulfide bonded near the carboxy terminus. In the insoluble form, the dimer associates into disulfide bonded oligomers and fibrils, while the soluble form is dimeric. Each monomer contains twelve type I, two type II, and 15–17 type III protein domains (Fig. 6.1). Type I domains are 45 amino acid domains and are found at the amino and carboxy terminals of the molecule. These form antiparallel beta pleated sheets. Two Type II domains also form beta pleated sheets that comprise collagen binding domains. The type III repeats form beta barrels with extended protein interaction loops. Two type III domains form the ED-A and ED-B alternatively spliced domains of cellular fibronectin. These domains are not found in plasma fibronectin. The type III V (CS) domain is present in both subunits of cellular fibronectin but only in one subunit of plasma fibronectin (Magnusson and Mosher 1998) (Fig. 6.1).

Fibronectin is encoded by a single gene on human chromosome 2 (Ayad et al. 1994). However, alternative splicing can generate multiple isoforms. Endothelial cells secrete fibronectin that contains the alternatively spliced EIIIA/EDA (extra domain A), EIIIB/EDB (extra domain B) and V or IIICS domains. Transformation alters the splicing pattern of fibronectin-pre-mRNA, leading to augmented expression of oncofetal fibronectin isoforms that contain IIICS, EDA, and EDB sequences. The EDA domain is located between the type III domains 11 and 12 and the EDB domain is inserted between domains 7 and 8. Transforming growth factor β (TGFβ) promotes the inclusion of the EDA domain into oncofetal fibronectin (Muro et al. 2008). EDA is present during embryogenesis, while in adult tissue it is present in areas of tissue remodeling, such as wound healing or tumor growth.

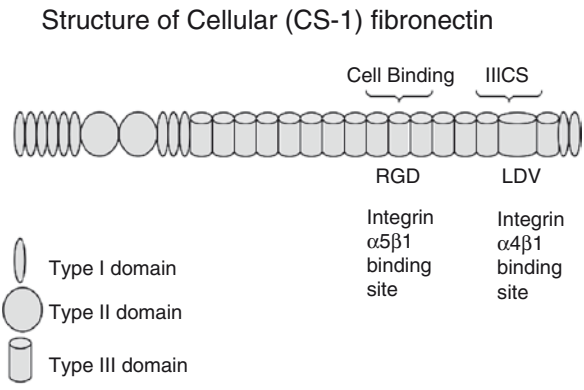

Fig. 6.1 Fibronectin structure. Fibronectin is a large (220–250 kDa) extracellular matrix protein comprising two chains that are disulfide bonded near the carboxy terminus. Shown is a cellular fibronectin isoform. Fibronectin subunits are comprised of type I, II and III structural units. The integrin α5β1 and αvβ3 binding Arginine-Glycine-Aspartic Acid (RGD) peptide is located in an extended loop in the tenth type II repeat, while the integrin α4β1 binding Leucine-Valine-Aspartic Acid domain is located in the alternatively spliced IIICS domain

Table 6.1 Fibronectin-binding integrins

Integrin		Ligands
β1	α3	laminin, collagen, fibronectin, entactin, epiligrin
	α4	Fibronectin (CS-1), VCAM
	α5	Fibronectin (RGD), L1-CAM, fibrinogen
	α8	Fibronectin
	α9	Fibronectin (CS-1), VCAM, tenascin
β3	αIIβ	Fibrinogen, fibronectin, von Willebrand's factor
αv	β1	Fibronectin
	β3	Vitronectin, fibronectin, von Willebrand's factor, denatured Collagen, thrombospondin, Del1, Cyr61
	β6	Fibronectin, TGF-beta

There are several subfamilies of the fibronectin-binding integrins, based on the pairings of integrin alpha and beta chains. A number of beta 1 family members bind fibronectin and can be found on many cell types of the body. Several alpha v family members found mainly on stably adherent cells also bind fibronectin. The platelet integrin αIIbβ3 and the leukocyte integrin α4β7 also bind fibronectin

The EDA domain may facilitate cell adhesion and spreading by enhancing binding of fibronectin to one of several integrins, including α4β1, α5β1, and α9β1 (Manabe et al. 1997; Liao et al. 2002). Fibronectin isoforms that contain the EDB domain are present around neovascular structures, on invasive ductal breast carcinoma and in brain tumors (Kaspar et al. 2006). This isoform is generally absent in plasma and tissues of healthy adults (Kaspar et al. 2006). Fibronectin containing the EDB domain is secreted by both tumor and endothelial cells (Kaspar et al. 2006).

Fibronectin binds to a number of integrins including α4β1, α5β1, α9β1, αvβ6 and αvβ3 (Komoriya et al. 1991; Ayad et al. 1994; Plow et al. 2000; Liao et al. 2002) (Table 6.1). Integrin α5β1 and integrin αvβ3 recognize the fibronectin Arg-Gly-Asp (RGD) sequence, which is located in an extended loop in the tenth type III repeat. The integrin primary binding site is flanked by the synergistic sequence PHSRN, which is located in the ninth type III repeat. This domain is required for firm binding to integrin α5β1 but is not required for binding to αvβ3 (Kaspar et al. 2006). The RGD sequence is also present in other ECM proteins such as osteopontin, Del-1, vitronectin, and others; these sites interact with many integrins, including αvβ5, αvβ6 and αvβ3 (Ayad et al. 1994; Aoka et al. 2002). Other integrins recognize alternative short peptide sequences. For example, integrin α4β1 recognizes EILDV and REDV in alternatively spliced CS-1 fibronectin. Some integrins, such as α4β1, can also bind cell surface receptors, such as VCAM-1, to promote cell-cell adhesion (Jin and Varner 2004).

The Integrin Family of ECM Receptors

Integrins are a large group of structurally related receptors for extracellular matrix proteins and immunoglobulin superfamily molecules (Hynes 2002). Integrins are divalent cation-dependent heterodimeric membrane glycoproteins comprised of

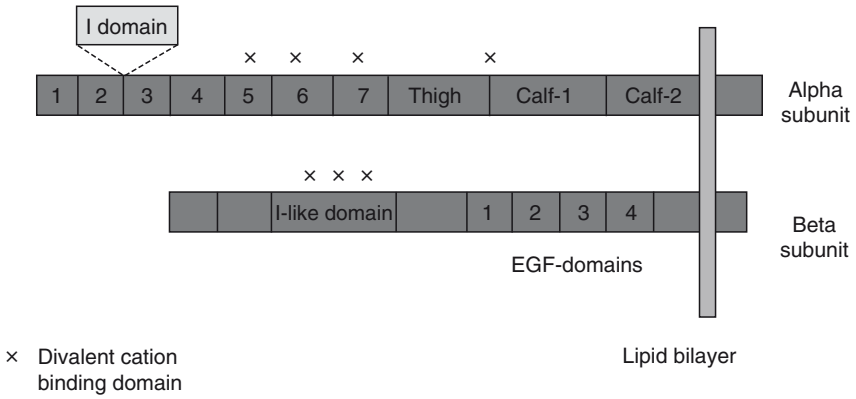

Fig. 6.2 Integrin subunit primary structure. The primary structure of the integrin alpha and beta subunits are shown. Integrin alpha subunits are composed of a large extracellular domain and short (about 30 amino acids) intracellular domain. Three membrane proximal domains recently named the "Thigh", "Calf-1" and Calf-2" domains support the integrin headpiece away from the membrane. The alpha subunit N-terminus contains seven repeats of 60 amino acids, which fold into a beta propeller ligand binding domain, a structure also found in trimeric G-protein beta subunits. Each integrin alpha subunit also has several divalent cation (Ca^{2+} and Mg^{2+}) binding sites, identified by "x" which play roles in forming salt-bridges with the ligand. Some alpha subunits have an additional inserted domain known as the "I domain" which directly binds ligand in those integrins. The integrin beta subunits are smaller than the alpha subunits and exhibit four EGF-like repeats. Ligand binding occurs via an I-like domain in integrins without an alpha chain I domain

noncovalently associated α and β subunits that promote cell attachment and migration on the surrounding extracellular matrix (Fig. 6.2). Eighteen α and eight β subunits associate to form 24 integrin heterodimers. Each integrin subunit consists of an extracellular domain, a single transmembrane region, and a short (approximately 30–40 amino acids) cytoplasmic region (Hynes 2002). Some integrins, such as α5β1, primarily recognize a single ligand, while others, such as αvβ3, can bind several ligands (Avraamides et al. 2008) (Table 6.1).

Integrin activity can be regulated by integrin expression or by growth factor or chemokine receptor signal transduction events that alter integrin conformation ("inside-out" signaling). Combined electron microscopy, crystallography, and nuclear magnetic resonance studies suggest that the globular region formed by the N-termini of the α and β chains is bent toward the membrane and the cytoplasmic regions of the two subunits are closely associated with one another in an inactive integrin (Lu et al. 2001; Beglova et al. 2002; Vinogradova et al. 2002; Arnaout et al. 2005). Integrin activation is believed to result from an unbending and elongation of the dimer and separation of the cytoplasmic domains (Lu et al. 2001; Beglova et al. 2002; Vinogradova et al. 2002; Arnaout et al. 2005), allowing interaction of integrin cytoplasmic domains with intracellular adaptor and effector proteins. Once activated, integrins bind ligands, cluster, and initiate their own signaling cascades that lead to cell migration and survival.

The activity of many integrins is regulated primarily at the transcriptional level. The expression of integrins α1β1, α2β1, α4β1, α5β1, and αvβ3 is strongly induced by growth factors or chemokines (Brooks et al. 1994a; Friedlander et al. 1995). However, integrins can also be activated by receptor-mediated signaling. Integrin αvβ5 is constitutively expressed on carcinoma cells, yet is only functionally activated by insulin like growth factor mediated signal transduction (Brooks et al. 1997). In circulating cells such as monocytes and other leukocytes, integrins are generally inactive until cells are stimulated by chemokines, hormones, or other factors. Integrin activity on leukocytes can be stimulated by chemokine signaling (Grabovsky et al. 2000). Additionally, integrin αIIbβ3 is inactive on resting platelets but becomes activated from within when an external stimulus such as thrombin or epinephrine binds a cell surface receptor and induces the conformational change in the integrin cytoplasmic domains. It then binds its ligand fibrinogen, leading to platelet aggregation. Thus, integrins roles in cancer may be controlled either by expression or by intracellular signaling (inside-out signaling). In tumors, the continuous expression of growth factors may continuously stimulate integrin expression and activity and thereby promote tumor growth and invasion.

The β1 Integrin Subfamily

The β1 integrin subfamily contains 11 members: αvβ1 α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1, and α11β1. β1 integrins activate many signaling pathways, including FAK, PI3 kinase, and Akt (Hazlehurst et al. 2000; Lee and Juliano 2000), which promote cell proliferation, migration, invasion, and survival (Giancotti and Ruoslahti 1990; Boudreau et al. 1996; Yao et al. 2007). Several β1 integrins can serve as fibronectin receptors, including α3β1 (Takada et al. 1988), α4β1 (Elices et al. 1990), α5β1 (Pytela et al. 1985), α8β1 (Schnapp et al. 1995), α9β1 (Weinacker et al. 1995), and αvβ1 (Vogel et al. 1990). Key roles in tumor angiogenesis and growth for integrins α4β1, α5β1, and α9β1 have been identified (Kim et al. 2000a, b; Garmy-Susini et al. 2005; Staniszewska et al. 2007; Vlahakis et al. 2007) while roles for integrins α3β1 (Morini et al. 2000; Tang et al. 2008), α4β1 (Gosslar et al. 1996; Matsuura et al. 1996; Okada et al. 1999), and α5β1 (Caswell et al. 2007) in tumor invasion and metastasis have also been identified.

The αv Subfamily

The αv integrin subunit can combine with several different beta subunits (β1, β3, β5, β6 and β8) to achieve unique ligand-binding profiles. Three alpha v integrin serve as fibronectin receptors: αvβ1 (Vogel et al. 1990), αvβ3 (Cheresh 1987) and αvβ6 (Busk et al. 1992; Weinacker et al. 1995). Integrin αvβ3 is a receptor for RGD containing proteins such as vitronectin, fibronectin, fibrinogen, and osteopontin and was the first of the alpha v integrins to be characterized (Cheresh 1987).

It was also the first integrin to be found to regulate angiogenesis (Brooks et al. 1994a). Integrin αvβ3 expression is upregulated on invasive melanoma and plays a role in metastasis to lymph nodes in melanoma, breast carcinoma, and pancreatic carcinoma (Albelda et al. 1990; Seftor et al. 1992; Hosotani et al. 2002). The fibronectin-binding integrin αvβ6 has been shown to promote invasion and metastasis in ovarian cancer (Ahmed et al. 2002) and oral carcinoma (Ramos et al. 2002). Increased integrin αvβ6 expression has been reported in carcinomas of the lung, breast, pancreas, stomach, colon, ovary, and salivary gland. As this integrin binds to latent TGF-beta, thereby promoting its activation (Munger et al. 1999), it has the potential both to inhibit early stage tumor growth and invasion and to promote late stage invasion and metastasis, as TGF-beta can promote an epithelial to mesenchymal transition (Derynck et al. 2001).

Altered Expression of Fibronectin and Its Integrin Receptors in Cancer

Fibronectin is poorly expressed by normal tissues but can be highly upregulated in transformed tissues; hence, it has been called an oncofetal antigen. The CS-1 and ED-A and ED-B isoforms are associated with provisional matrices expressed by tumor cells and endothelial cells undergoing angiogenesis. In fact, the expression of these isoforms serves as the basis for novel experimental methods to image and treat solid tumors. Most studies indicate that fibronectin plays a role in driving tumor angiogenesis and tumor invasion. However, some studies indicate that loss of fibronectin can occur in head and neck cancers, resulting in loss of contact inhibition and tumor metastasis (Beier et al. 2007). This loss of fibronectin is associated with expression of human papilloma virus early protein-2, a transcription factor that suppresses fibronectin expression (Beier et al. 2007).

Increased β1 integrin expression levels are found in a significant proportion of human tumors (Yao et al. 2007). Importantly, poorer overall survival rates are observed in early stage invasive breast cancer patients with high β1 integrin expression levels (Yao et al. 2007). High integrin β1 levels also correlates with poor prognosis in cancers of the lung, pancreas, and cutaneous melanoma. β1 integrins likely promote malignant progression by enhancing tumor cell survival and invasion, as integrin β1 inhibitory antibodies lead to selective apoptosis and decreased proliferation in three-dimensional cultures and in xenograft models of breast cancer in vivo (Yao et al. 2007).

Fibronectin and Integrins in Cancer Cell Proliferation and Survival

Fibronectin promotes primary tumor growth by providing a substratum that is permissive for cell survival, invasion, and growth. How does fibronectin promote tumor progression? Augmented fibronectin levels cause increased tissue rigidity, loss of

epithelial cell polarity, and enhanced proliferation, features that are hallmarks of tumor development. Under normal conditions, the interstitial ECM of mammary tissue does not contain fibronectin and the tissue is soft. During oncogenesis, the basement membrane is altered and matrix proteins such as elastin, tenascin-c, collagen III, and fibronectin are deposited within tumors. This tumor tissue is stiffer, preventing the cells from differentiating into acini (Williams et al. 2008). Proliferating mammary epithelial cells deposit fibronectin around the basal cell surface, while growth arrested cells do not express fibronectin (Williams et al. 2008). As addition of fibronectin to mammary epithelial cultures stimulates cell proliferation, these studies demonstrate that fibronectin promotes growth and suggest that transformed mammary epithelial cells in vivo upregulate fibronectin expression and contribute to breast cancer progression by stimulating proliferation and loss of tissue architecture.

Integrin ligation by fibronectin promotes integrin clustering and subsequent integrin-mediated intracellular signal transduction and invasion. Unlike growth factor receptors, integrins have no intrinsic enzymatic or kinase activities, but activate complex signaling pathways by coclustering with kinases and adaptor proteins in focal adhesion complexes. Focal adhesion complexes are comprised of integrins, focal adhesion kinase (FAK), Src, adaptor proteins such as Shc, signaling intermediates Rho, Rac, and Rap1, actin binding cytoskeletal proteins such as talin, α-actinin, paxillin, tensin, and vinculin (Mitra et al. 2006), and other signaling proteins. Integrin signaling promotes cell migration, proliferation, and survival. Loss of integrin ligation inhibits these events and unligated integrins can actively initiate apoptosis, even without loss of cell attachment. This form of death is stress response- and death-receptor-independent, but caspase 8-dependent, and has been called “integrin mediated death” (Stupack 2005).

Fibronectin and Integrins in Invasion and Metastasis

The majority of cancer-related deaths are due to metastases that became resistant to conventional therapies (Fidler 2003). Fibronectin plays a role in metastasis, not only by providing a substrate for the cells to attach, but also by providing signals that promote cell migration and invasion. In normal tissues, fibronectin is poorly expressed. However, transformed cells often express transcription factors such as *Twist* that increase metastasis (Yang et al. 2004). *Twist* promotes the epithelial-mesenchymal transition (EMT) by downregulating expression of epithelial genes, such as E-cadherin, and upregulating expression of mesenchymal genes, such as vimentin, collagen, and fibronectin. Yang et al. (2004) were the first to show that metastatic breast tumor cells express *Twist* and that *Twist* drives both EMT and metastasis. *Twist* expression has been shown to promote fibronectin expression and to drive EMT and metastasis in several tumor types.

Fibronectin expression has been implicated in metastasis of many tumor types, including ovarian carcinoma. During ovarian carcinoma cell (OvCa) metastasis, metastatic cells initially attach to the peritoneum and omentum in a matrix

metalloproteinase (MMP) dependent manner. Downregulation of MMP2 expression reduces attachment of OvCa cells to the mesothelial lining of the peritoneum (Kenny et al. 2008). Using xenograft models, pretreatment of OvCa cells with an MMP2 neutralizing antibody prevented metastatic cell adhesion and reduced the number of metastases, thereby prolonging survival (Kenny et al. 2008). Importantly, MMP2 may enhance peritoneal adhesion of OvCa cells by cleaving fibronectin and vitronectin into small fragments. Additionally, adhesion to cleaved fibronectin is inhibited by the suppression of integrin α5 or β3 expression with siRNAs. These studies suggest that fibronectin fragment binding to integrin α5β1 promotes ovarian carcinoma metastasis (Kenny et al. 2008).

Several fibronectin-binding integrins, including integrins α3β1 (Morini et al. 2000; Tang et al. 2008), α4β1 (Gosslar et al. (1996); Matsuura et al. (1996); Okada et al. 1999), α5β1 (Caswell et al. 2007), αvβ3 (Albelda et al. 1990; Seftor et al. 1992; Hosotani et al. 2002), and αvβ6 (Ahmed et al. 2002) have been found to play roles in tumor invasion and metastasis. Recent studies also show that integrin interacting proteins, such as Rab1 can play important roles in the invasion process. Rab proteins are members of the Ras superfamily of GTPases involved in membrane trafficking events; Rab proteins have been shown to increase the aggressiveness of ovarian and breast tumors both clinically and in mouse models (Caswell et al. 2007). Rab 25 binds β1 integrin cytoplasmic tails and localizes vesicles that deliver the integrin to plasma membrane at pseudopodial tips. Tumor cell invasion in a three-dimensional extracellular matrix is dependent on ligation of fibronectin by α5β1 integrin and the capacity of Rab25 to interact with β1 integrin (Caswell et al. 2007). Rab25 contributes to tumor progression by directing the localization of integrin-recycling vesicles and retaining a pool of α5β1 at the invading pseudopodial tips, enhancing the ability of tumor cells to invade the ECM.

Fibronectin and Integrins in Angiogenesis and Lymphangiogenesis

Angiogenesis plays a major role in tumor growth, while inhibition of angiogenesis may suppress tumor progression and metastasis. Fibronectin is essential for early vascular development, as early embryonic lethality occurs in mice in which fibronectin has been deleted. Fibronectin deficient embryos exhibit yolk sac and other mesodermal tissue defects (George et al. 1997). Selective deletion of the EDA and EDB alternatively spliced variants also causes embryonic lethality due to defects in yolk sac vessel and heart formation (Astrof et al. 2007). β1 integrins are also critical for angiogenesis and vascular development. While β1 integrin null embryos die in utero due to implantation defects, animals with an endothelial cell specific deletion of β1 integrin (Tie2-Cre1 floxed mice) die by E10.5 with severe vascular defects (Tanjore et al. 2007). Furthermore, endothelial cell proliferation and vessel branching is absent in β1 *(Itgb1)*-null embryoid bodies (Bloch et al. 1997).

Integrin α5β1 in Tumor Angiogenesis

Integrin α5β1 is upregulated on endothelium during tumor angiogenesis in both mice and humans (Kim et al. 2000a, b) and is poorly expressed on quiescent endothelium. A number of angiogenic growth factors including bFGF, IL-8, and TNFα, as well as, the ECM protein Del-1 (Kim et al. 2000a, b; Boudreau and Varner 2004) induce integrin α5β1 expression, which is not regulated by VEGF. A homeobox family transcription factor, HoxD3, regulates expression of α5β1 in endothelial cells (ECs) and Hox D3 antisense suppresses while Hox D3 overexpression upregulates α5β1 expression (Boudreau and Varner 2004).

Antagonists of integrin α5β1 inhibit tumor (Kim et al. 2000a, b), corneal (Muether et al. 2007) and choroidal (Umeda et al. 2006) angiogenesis in chicks and mice and suppress tumor growth. These antagonists induce endothelial cell apoptosis during angiogenesis. Integrin α5β1 mediated adhesion suppresses Protein Kinase A (PKA) (Kim et al. 2000a, b; Kim et al. 2002) activity, promoting endothelial cell migration and survival in vivo and in vitro. Expression of the catalytic subunit of PKA or exposure of cells to cAMP or forskolin to activate PKA inhibits cell migration and stimulates apoptosis in vitro and in vivo (Kim et al. 2002).

Early mesenchymal abnormalities result from embryonic deletion of the integrin α5 subunit, leading to embryonic lethality of α5-null embryos (Yang et al. 1993). These embryos have a truncated posterior, lack posterior somites, have abnormal organization of the emerging extra embryonic and embryonic vasculature, and reduced complexity of the emerging vasculature (Yang et al. 1993). Embryoid bodies cultured from ES cells that lack α5 integrins exhibited delayed and reduced formation of the early vascular plexus and complex vascular structures (Francis et al. 2002). Teratocarcinomas arising from α5 null ES cells exhibit decreased proliferation, increased apoptosis, and decreased vascularization when compared with controls (Taverna and Hynes 2001). These data indicate a key role for integrin α5β1 in vasculogenesis and angiogenesis.

Integrin α4β1 in Tumor Angiogenesis

Integrin α4β1 also plays important roles in angiogenesis; this integrin can bind both CS-1 fibronectin and VCAM-1, a member of the immunoglobulin superfamily, both of which are expressed on proliferating endothelium. Integrin α4β1 promotes adhesion and extravasation of leukocytes by binding to VCAM expressed on inflamed endothelial cells. However, loss of integrin α4 expression during embryonic development leads to defects in placentation and heart development, causing lethality between E10.0 and E12.0 (Yang et al. 1995). Recent studies showed that in response to VEGF, bFGF, IL-1, and TNFalpha, integrin α4β1 expression is induced on neovessels in murine and human tumors (Garmy-Susini et al. 2005). In chick and murine models of tumor growth, antagonists of integrin α4β1 blocked tumor neovascularization and decreased tumor growth. This integrin also supports

close association of endothelium with VCAM-1 expressing vascular smooth muscle during blood vessel formation and antagonists of α4β1-induced cell death of both endothelial cells and pericytes (Garmy-Susini et al. 2005). Thus, integrin α4β1-VCAM dependent cell-cell attachment promotes the survival of both endothelial cells and pericytes during angiogenesis.

Integrins on bone-marrow derived immune cells are also involved in angiogenesis by facilitating myeloid cell homing to tumors. Circulating, bone marrow derived cells migrate into tumors in response to secreted chemokines and cytokines. Monocytes then can differentiate into proangiogenic secreting macrophages. Integrin α4β1 plays the key role in promoting the homing of both monocytes and endothelial progenitor cells to neovascular tissue (Jin et al. 2006a, b). Human CD34+ and murine Lin-Sca1+ progenitor cells as well as human and murine bone marrow-derived myeloid cells (CD14+ CD11b+) adhered to endothelial cells in vitro and to tumor endothelium in vivo via integrin α4β1 (Jin et al. 2006a, b). Treatment of mice bearing Lewis lung carcinoma tumors with antagonists of integrin α4β1 significantly suppressed the number of monocytes and progenitor cells within tumors and reduced blood vessel density (Jin et al. 2006b). These studies suggest that the suppression of monocyte and progenitor cell homing to tumors by integrin α4β1 antagonists could be a useful supplementary approach to suppress tumor angiogenesis and growth.

Integrin α9β1 in Tumor Angiogenesis

Another β1 integrin that has a role in angiogenesis is integrin α9β1 (Staniszewska et al. 2007; Vlahakis et al. (2007)). This integrin is structurally similar to integrin α4β1 but, unlike α4β1, it is a receptor for several extracellular matrix proteins and cell surface receptors including tenascin-C, thrombospondin, osteopontin, IIICS fibronectin, VCAM, and other ligands (Marcinkiewicz et al. 2000; Liao et al. 2002). Integrin α9β1 is expressed on epithelia, osteoclasts, smooth muscle cells, and endothelial cells. The direct binding of VEGF-A by integrin α9β1 promotes VEGF-A stimulated angiogenesis and blocking antibodies to α9β1, suppressed VEGF-A induced angiogenesis (Vlahakis et al. 2007). Integrin α9β1 may also bind directly to the N-terminus of thrombospondin, thereby promoting angiogenesis (Staniszewska et al. 2007). However, integrin α9β1 null mice do not exhibit obvious defects in development of blood vessels but do die 8–12 days after birth due to lethal defects in development of the lymphatic system (Huang et al. 2000).

Alpha v Integrins in Tumor Angiogenesis

Integrin αvβ3 is important in endothelial cell survival and migration during angiogenesis. This integrin is widely expressed on tumor blood vessels in human tumor biopsies but not on vessels in normal tissues. Its expression on endothelial

cells is stimulated by angiogenic growth factors such as bFGF, TNFalpha, and IL-8 and it is upregulated on endothelium in tumors, wounds, and sites of inflammation (Brooks et al. 1994a). Experimental evidence indicates that αvβ3 plays a key role in endothelial cell survival and migration during angiogenesis (Brooks et al. 1994a, b). It is expressed in response to angiogenic growth factors and tumors in chick chorioallantoic membrane, mouse, rabbit, and human models of angiogenesis and tumor growth (Brooks et al. 1994a, b; Brooks et al. 1995) but is not expressed on resting vessels. Antagonists of αvβ3 inhibit angiogenesis and tumor growth in a variety of animal models of cancer, as well as choroidal angiogenesis in animal models of ocular disease (Brooks et al. 1995; Friedlander et al. 1995, 1996, 2007). Inhibition of αvβ3 function in quail embryos also disrupted vasculogenesis by disturbing vascular patterning and blocking lumen formation (Drake et al. 1995). In vivo angiogenesis assays showed that basic FGF or TNF-α depend on αvβ3 to initiate angiogenesis (Brooks et al. 1994a; Friedlander et al. 1995).

Ligation of integrin αvβ3 promotes endothelial cell survival, while antagonists of αvβ3 induce caspase 8-dependent cell death, thereby suppressing angiogenesis by inducing endothelial cell apoptosis. Furthermore, these antagonists induce apoptosis in the vasculature, leading to tumor regression. Integrin αvβ3 antagonists increase p53 activity, increase levels of the cell cycle inhibitor p21WAF1/CIP1, and decrease levels of bax (Strömblad et al. 1996). Signal transduction pathways activated by endothelial αvβ3 integrin include MAP kinase, focal adhesion kinase (FAK), and Src, resulting in cell proliferation, differentiation, and migration (Eliceiri et al. 1998). The ability of endothelial cells to degrade and remodel the ECM during invasion is also mediated by αvβ3, which binds the matrix metalloproteinase-2 (MMP-2) and localizes the enzyme on the surface of blood vessels (Brooks et al. 1996, 1998).

Embryonic deletion of the β3 subunit indicates that this integrin is not absolutely required for vascular development (Hodivala-Dilke et al. 1999). Even though a large percentage of β3 knockout mice die in utero due to placentation defects, the surviving mice exhibit normal vessels in the brain and gut, and normal postnatal retinal neovascularization, suggesting either that β3 integrins are not required for normal vascular development or that compensatory changes occurred (Hodivala-Dilke et al. 1999) in these mice. However, β3−/− mice exhibit enhanced tumor angiogenesis compared to normal mice (Reynolds et al. 2002), with strongly enhanced VEGFR2 expression and signaling during angiogenesis, suggesting that compensatory changes in angiogenic signaling may play a role in the survival of β3 deficient animals (Reynolds et al. 2004). Knockin mutations in the integrin β3 cytoplasmic tail (β3Y747F/Y759F) impair integrin αvβ3 signal transduction, suppress cell migration, and tube formation. Although these animals exhibit normal developmental angiogenesis, they do not undergo growth factor or tumor induced angiogenesis (Mahabeleshwar et al. 2006). Together, these studies suggest that integrin αvβ3 plays an important role in angiogenesis and that complete loss of this integrin in development is compensated by upregulation of additional proangiogenic mechanisms.

Genetic ablation of the alpha v subunit reveals that most mice lacking αv integrins die in utero at E9.5, although 20% survive and die soon after birth (Bader et al. 1998). These mice have severely abnormal vessels in the brain and intestines although other organs appear normal. In support of these findings, blood vessels in mice with a Tie2Cre mediated endothelial cell specific deletion of αv integrins develop normally (McCarty et al. 2005). However, genetic deletion of alpha v integrins on embryonic central nervous system neural cells, particularly glia, results in perturbed brain blood vessel formation and nervous system defects (McCarty et al. 2005). Similar results are observed in alpha v null animals (McCarty et al. 2002) and in integrin β8 deficient animals (Zhu et al. 2002) which lack the integrin heterodimer αvβ8, suggesting that glial αv integrin is needed for blood vessel development.

Integrins and Lymphangiogenesis

The lymphatic system consists of thin-walled lymphatic vessels, collecting ducts, lymph nodes, lymph, and lymphocytes. Lymphatic vessels drain fluid (lymph) that has diffused through the capillaries into the interstitial space. The lymphatic system not only drains fluid back into circulation, but is critical for immune regulation. The lack of lymphatic markers made it difficult to analyze the role of lymphatic vessels in tumor growth and metastasis. Recently, lymphatic vessel markers have been discovered including transcription factor Prox-1 (Wigle et al. 2002) and the CD44 homolog lymphatic vessel hyaluronan receptor-1 (LYVE-1) (Banerji et al. 1999). Lymphatic vessels arise from the venous vessel network during embryogenesis and differ structurally from vascular endothelium. The growth factors VEGF-C and VEGF-D, which can be expressed by tumor cells or macrophages in tumors, promote growth of the lymphatic vessel network by activating the lymphatic endothelial cell receptor VEGFR-3 (Kaipainen et al. 1995; Joukov et al. 1996; Jeltsch et al. 1997; Achen et al. 1998; Makinen et al. 2001). VEGF-C expression in the tumor periphery induces lymphangiogenesis and promotes tumor metastasis (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001).

Integrins that regulate lymphangiogenesis include α9β1 and α4β1. Quiescent lymphatic endothelial cells express integrin α9β1. α9β1 null mice die 6–12 days after birth due to chylothoraces, an accumulation of lymph in the pleural cavity, suggesting a role for α9β1 in developmental lymphangiogenesis (Huang et al. 2000; Vlahakis et al. 2005). α9β1 has a role in growth factor mediated lymphangiogenesis as PROX-1 a lymphatic endothelial cell selective transcription factor coordinately upregulates integrin α9β1 and VEGFR3 expression and endothelial cell motility in vivo. α9β1 promotes VEGF-C and VEGF-D stimulated cell migration by directly binding these growth factors. Antagonists of α9β1 suppress VEGF-C induced motility. However, the role of these integrins in tumor lymphangiogenesis remains to be determined. Integrin α4β1 is highly expressed on tumor lymphatic endothelium and antagonists of this integrin can block lymphangiogenesis

and tumor metastasis. Further studies of the adhesion molecules that regulate lymphangiogenesis in tumor should provide novel methods to suppress tumor spread via the lymphatics.

Fibronectin Binding Integrins in Resistance to Chemotherapy

Signals provided by the attachment of cells to the ECM can promote cell survival and impede apoptosis leading to drug resistance. Tamoxifen, a selective estrogen receptor modulator, is used in breast cancer treatment and some patients who initially respond to tamoxifen eventually develop resistance. A number of genes associated with resistance to first-line tamoxifen are extracellular matrix proteins; among these is fibronectin (Helleman et al. 2008). These results suggest that ECM plays a role in chemotherapy resistance. In fact, fibronectin mediated cell attachment stimulates survival signals that counteract apoptosis induced by drugs such as docetaxel (Xing et al. 2008). Docetaxel, a microtubule directed drug, exerts its effects via the induction of apoptosis. However, fibronectin mediated adhesion of cancer cells can promote Akt2 phosphorylation and protect tumor cells against docetaxel-induced apoptosis. PI3 kinase is activated by cell attachment to fibronectin; PI3 kinase promotes cell proliferation and inhibits cell death by activating Akt and inducing Akt to sequester the proapoptotic BCL2 member BAD, thereby suppressing apoptosis. Thus, the extracellular matrix can play a role in chemoresistance in cancer (Xing et al. 2008).

Other mechanisms by which fibronectin may promote chemoresistance include (1) protecting against Fas/CD95-mediated program cell death, (2) affecting cell cycle progression and (3) inhibiting drug induced DNA damage. Fibronectin regulates the localization of c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (c-FLIP), an inhibitor of pro-caspase 8 during death-inducing signaling complex (DISC) formation and blocks Fas-mediated programmed cell death. Apoptosis is inhibited after CD95 crosslinking due to inhibiting procaspase activation allowing tumor survival (reviewed by Vincent and Mechti 2005). Fibronectin adhesion is associated with increased p27kip1 protein level and the inhibition of cyclin A and E-dependent CDK2 kinase activity, a known mechanism of drug resistance. Fibronectin adhesion by β1 integrins can also protect U937, human monocytic leukemia cells, from drug-induced DNA damage by reducing topoisomerase II activity and modifying its subcellular distribution. It is speculated that FN adhesion reduced the availability of topoisomerase IIb to form drug induced cleavable complexes (reviewed by Vincent and Mechti 2005). Thus adhesion to fibronectin activates numerous pathways to drug resistance.

Glucocorticoids such as dexamethasone are used to treat patients with multiple myeloma and produce rapid reduction in tumor mass. Prolonged treatment however, can result in chemoresistance. Fibronectin promotes survival of plasma cells and contributes to de novo drug resistance. In vitro, it has been observed that myeloma cells that adhere to fibronectin via β1 integrins have significant advantage

when exposed to cytotoxic drugs. Multiple myeloma cells that exhibit drug resistance reveal significant increase in α4β1 expression (reviewed by Vincent and Mechti 2005).

Integrin α4β1 is involved in retention of normal hematopoietic progenitor cells and leukemic blast cells within the bone marrow. Integrin α4β1 is abundantly found on leukemic cells and induces resistance to cytostatic agents by interacting with fibronectin on BM stromal cells. Integrin α4β1 also protects leukemic cells from drug-induced apoptosis via PI3K/Akt2/BCl2 pathway. Integrin α4β1 expression is associated with bone marrow minimal residual disease which causes relapse after chemotherapy in patients with acute myelogenous leukemia (AML) (Mahlknecht and Schonbein 2008). The absence of α4β1 reduces leukemic blast cells on the BM. Using primary cell lines derived from patients with AML and treating them with histone deacetylase inhibitors (suberoylanilide hydroxamic acid and valproic acid) downregulates α4β1 and decreases adhesion of cells to mesenchymal stroma (Mahlknecht and Schonbein 2008). These results suggest that histone deacetylase inhibitor treatment may be useful in preventing minimal residual disease from AML.

Potential Clinical Applications

Integrins α4β1, α5β1, and αvβ3 on endothelial cells are not expressed on quiescent vessels but are expressed on angiogenic endothelium, making them excellent targets for imaging and excellent candidates for targeting of drugs to angiogenic vessels in tumors. A number of diagnostic strategies directed at detecting integrin αvβ3 have shown that integrin αvβ3 is expressed at greater levels on tumor vasculature than normal vasculature. Furthermore, integrin αvβ3 expression has been shown to correlate strongly with tumor stage and outcome (Gladson 1996; Bello et al. 2001; Vonlaufen et al. 2001).

Antibody Inhibitors of Integrins

Integrins αvβ3 and α5β1 have been implicated in tumor angiogenesis and antibody inhibitors of αvβ3 and α5β1 are in clinical trials for the inhibition of angiogenesis in cancer. Of the several integrin antagonists undergoing clinical evaluation for cancer treatment, all have proved to be nontoxic, including MEDI-522, a humanized anti-αvβ3 antibody, M200, a chimeric mouse-human anti α5β1 antibody, cilengitide, a cyclic peptide inhibitor of αvβ3 and αvβ5 and ATN-161, a non-RGD based peptide inhibitor of α5β1.

Peptide and antibody antagonists of αvβ3 block tumor angiogenesis and growth (Brooks et al. 1994b). Use of the anti-αvβ3 monoclonal antibody revealed that αvβ3 is a marker of human breast tumor associated blood vessels (Brooks et al.

1995) and is expressed on a majority of vessels in several human colon, pancreatic, and lung carcinomas (Max et al. 1997). Vitaxin/MEDI-522 is a humanized version of the anti-integrin αvβ3 monoclonal antibody LM609, which blocks tumor angiogenesis by inducing apoptosis in newly formed endothelial cells (Gutheil et al. 2000; McNeel et al. 2005). A Phase I study showed that Vitaxin had very low toxicity and is well tolerated. When tested on patients with metastatic cancer who had failed other treatments, Vitaxin led to disease stabilization without toxicity. In 2001, Medimmune began clinical trials using Vitaxin and in 2003 initiated Phase II trials in patients with advanced metastatic melanoma and in patients with metastatic prostate cancer (Hersey et al. 2005). A Phase II study on metastatic melanoma showed that 53% patients treated with Vitaxin survived longer than 1 year versus 27% of patients receiving standard therapy. MEDI-22 works by reducing FAK activity in blood vessels (Hersey et al. 2005).

A humanized anti-α5β1 antibody, M200 (volociximab), developed by Protein Design Labs and now partnered with Biogen-Idec Pharmaceuticals, has shown low toxicity in Phase I studies and was evaluated in Phase II trials for metastatic melanoma, renal cell carcinoma, and nonsmall cell lung cancer (Figlin et al. 2006; Kuwada 2007). In renal cell carcinoma studies, M200 was well tolerated and stable disease was noted in 87% of patients. In a melanoma trial in combination with DTIC, the antibody was well-tolerated and anti-tumor activity was noted in 62% of patients. Volociximab has also been used to image tumors in rabbit models of cancer (Bhaskar et al. 2008). Antibodies against integrin α5β1 have been shown to decrease angiogenesis in vitro and in vivo (Kim et al. 2000a, b; Bhaskar et al. 2008) and Volociximab inhibits endothelial cell growth in vitro. A mouse antibody against α5β1 antibody 339.1 inhibits murine EC migration and tube formation and promotes cell death (Bhaskar et al. 2007). In xenograft models it inhibited the growth of tumors 60% and this correlates with a decrease in vessel density and slow tumor growth in vivo (Bhaskar et al. 2007).

Peptide Inhibitors of Integrins

Another group of integrin antagonists with potent antiangiogenic function includes peptide inhibitors. The cyclic RGD-peptide cilengitide (EMD 121974) is an αvβ3/αvβ5-specific antagonist. This drug in Phase I clinical trials showed a favorable safety profile and no dose-limiting toxicities (Eskens et al. 2003). Currently this drug is in phase II trials for glioblastoma, nonsmall cell lung cancer, melanoma, and pancreatic cancer (Albert et al. 2006; Beekman et al. 2006; Bradley et al. 2007). ATN-161, a peptide inhibitor of α5β1, suppresses tumor angiogenesis. When combined with chemotherapy, ATN-161 reduced metastases and improved survival in the colon cancer model (Stoeltzing et al. 2003). In animal models of colon cancer, ATN-161 reduced metastases and improved survival when combined with chemotherapy. In Phase I safety trials, ATN161 was well tolerated and several patients exhibited stable disease. ATN-161 is currently in Phase II clinical trials for multiple myeloma and other tumors (Cianfrocca et al. 2006).

Antibodies against Fibronectin in Therapy and Imaging

The EDA domain of FN can be used as a marker of tumor angiogenesis as it accumulates around blood vessels of certain tumors (Villa et al. 2008). F8, B7, and D5 are human monoclonal antibodies that have been developed against the EDA domain. EDA domain disrupts restricted pattern of expression in plasma and normal human tissue and is overexpressed on neovasculature and stroma structures of some aggressive solid tumors. The F8, B7, and D5 antibodies selectively stain neovascular structures on freshly isolated frozen tumor sections and do not stain normal tissue (Villa et al. 2008). Anti-EDA antibodies can target tumors in vivo, as shown by radiolabeling them with I^{125} and intravenously injecting them in tumor bearing animals. All three antibodies were able to accumulate in the tumor, although F8 has more impressive tumor targeting selectivity in scFv format and the homodimeric small immune-protein (SIP) format. The F8 antibody may serve as a useful building block for the development of antibody based targeted anti cancer therapeutics (Rybak et al. 2007).

Extensive studies have been conducted with antibodies that recognize the EDB domain of fibronectin. L19 is a human antibody that has high affinity and specificity for the EBD domain and L19 based biopharmaceuticals have been produced and tested in preclinical animal models (Kaspar et al. 2006). L19 has been shown to target lung, colorectal, or brain cancer and is able to distinguish between quiescent and growing lesions. Many fusion proteins and chemical derivatives based on L19 have been produced. Fusion proteins with IL2 (L19-IL2) had striking anticancer activity in orthotopic murine models of hepatocellular carcinoma and pancreatic cancer (Kaspar et al. 2006). L19 antibody can be used for cancer imaging using near-infrared fluorescent dyes, gamma emitting radionuclides, PET application with suitable radionuclides, and ultrasound imaging using antibody coated microbubbles (Kaspar et al. 2006).

Another avenue to explore in cancer therapeutics is the evaluation of the role of inhibitors of protein tyrosine kinases in the fibronectin signaling cascade. Neuroblastoma is a neural crest cell malignancy with high metastatic potential. Signals from fibronectin-binding integrins promote neural crest cell motility in development via protein tyrosine kinase activation. Integrins alpha 4 and alpha 5 are present in late stage neuroblastoma tumors. The motility of integrin $\alpha5\beta1$ dependent neuroblastoma is dependent on FAK, Src, and p130 Cas while integrin $\alpha4\beta1$ dependent neuroblastoma motility is dependent on Src and p130Cas but not FAK (Wu et al. 2008). Integrin $\alpha4$ mediated Src activation is mechanistically distinct from FAK mediated Src activation during $\alpha5\beta1$ mediated neuroblastoma migration, and support the evaluation of inhibitors to $\alpha4$, Src and FAK in the control of neuroblastoma tumor progression.

Integrin Targeted Tumor Imaging

Vascular integrin targeting has been used to develop novel diagnostic tools for the detection of tumors. Both peptide and antibody based diagnostic agents targeting RGD binding integrins or alpha v integrins have been evaluated in animal models.

A cyclic RGD peptide (cyclic RGDyK) labeled with (18)F was used to image brain tumors in an orthotopic U251 model. MicroPET analysis showed significant accumulation in tumors with very low uptake in normal brain (Haubner et al. 2001; Chen et al. 2004b). To minimize liver uptake, the 18F-cRGDyK was PEGylated (Chen et al. 2004a). Similarly, a dimeric cyclic RGD peptide conjugated to (18)F also exhibited increased tumor retention (Chen et al. 2004c). In addition, 64Cu-labeled dimeric RGD peptides were used to image xenografts of human breast tumors with a high signal to background ratio and significant tumor retention. PEGylation of this peptide tracer reduced nonspecific binding of the tracer and allowed identification of the primary tumor as wells as metastases (Chen et al. 2005). These imaging approaches have been able to identify xenograft tumors as small as 1.5 mm in diameter. Paramagnetic polymerized liposomes conjugated to an anti-αvβ3 antibody were used to detect neovascular tumors in experimental rabbit tumors (Sipkins et al. 1998; Garanger et al. 2007). Integrin αv-targeted ultrasound microbubbles also preferentially bound neovasculature at the periphery of experimental tumors (Leong-Poi et al. 2003). Integrin targeting is useful in tumor imaging and agents that target distinct integrins may be useful in preclassifying patients for receipt of anti-integrin drugs.

Conclusions

Fibronectin and its ligands have crucial roles in many aspects of tumor development and metastasis. Many tumors upregulate both fibronectin and fibronectin binding integrins, suggesting that these may be useful targets in imaging and drug development. Integrins are an obvious choice to target as they are expressed on both tumor cells and EC and blocking them can lead to decrease in angiogenesis which will prevent tumor growth and metastasis. In addition, blocking integrin signaling on tumor cells will affect survival signals needed for the tumors to grow. In addition, inhibiting factors that upregulate fibronectin may also have therapeutic potential as lack of fibronectin will affect integrin signaling.

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Chapter 7
Vitronectin-Binding Integrins in Cancer

Eric Murphy and Dwayne Stupack

Abstract Integrins, as cell adhesion receptors that integrate signaling and mechanotransduction functions, are critical effectors of cell attachment to the extracellular matrix (ECM), migration, proliferation, and survival. The vitronectin-binding family of integrins, in particular, have been shown to play critical roles in the pathology of disease. This family of integrins contains a common αv subunit, and while each family member maintains some capacity to bind to the ECM protein vitronectin, they also bind a wide array of other ligands. These integrins have been implicated in regulating stromal cell responses to tumors, including alterations in angiogenic and inflammatory responses and have also been shown to account for tumor malignancy directly via their capacity to promote invasion and survival. Nonetheless, αv integrins are not absolutely essential for cell survival, and early clinical studies using integrin antagonists have been essentially toxicity free, making them very attractive targets for continuing development of therapeutics.

Keywords Angiogenesis • cell adhesion • cell migration • extracellular matrix • metastasis

The Basic Biology of Integrins

The integrins are cell adhesion receptors that mediate cell attachment to the extracellular matrix (ECM) or to adjacent cells. In most untransformed cells, integrins mediate cell invasion and migration and are also required for proliferation and cell survival. Integrin signaling also interacts with the programmed cell death pathways. While ligated integrins confer resistance to chemical insults or irradiation, unligated integrins can promote apoptosis. When evaluating the roles integrins play in facilitating cancer progression, it is useful to consider their role(s) in maintaining normal cell

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_7,

physiology. These "normal" functions can be exploited to promote tumor growth, and malignant tumors tend to exploit the survival and invasion properties of integrins.

Integrins Are Mechanoreceptors

In human, the integrin family includes proteins encoded by 26 different genes. There are 8 different integrin β subunits (β1–β8) and 18 different α subunits (α1–11, αIIb, αD, αE, αL, αM, αX, and αv). Each integrin is composed of a single α and a β subunit that assemble into a heterodimeric cell surface receptor of about 200–300 kDA. Each heterodimer may bind cell surface ligands, ECM proteins, or both. Integrins typically bind more than one ligand. "Bind" is an operative term since the interaction of the integrin with substrate ligands generates tensional forces within the cell (Brown et al. 2002; Schwartz and Denninghoff 1994). A pivotal point in the process is the formation of the "focal adhesion," a multiprotein complex composed of cytoskeletal scaffolding and signal-transducing proteins (Ingber 2002; Schlaepfer and Hunter 1996). Mechanical tension is applied by the engagement of the actomyosin system and provides a force-dependent capacity for the cell to align its cytosolic architecture with fibrils of the ECM. This capacity to integrate internal and external fibrillar systems was the original rationale for the name "integrins" coined by Richard Hynes (Tamkun et al. 1986). In the decade that followed, integrins became increasingly recognized for their roles in signaling and cell survival. The interaction of integrins with diverse cell signaling elements, such as receptor tyrosine kinases (RTKs), G proteins, antigen receptors (ARs), and caspases, has served to reinforce the theme of "integration" of the cell responses.

The combination of signaling and tension-generating function provides a unique mechanism for the cell to interrogate its microenvironment. In particular, the ability to exert a contractile force following ligation provides an important mechanism by which a cell may sense whether a given integrin ligand is present as a part of a crosslinked and rigid ECM (or is perhaps present on a neighboring cell) or is simply a free soluble ligand that exerts little or no opposing force (Paszek et al. 2005). The relative resistance of a ligand is an important factor in integrin signaling, as it influences the nucleation of a focal adhesion complex that requires both mechanical resistance and stable integrin interaction with ligand. The largest focal adhesions are observed on very rigid matrices, such as the "matrix" formed when ECM components are immobilized on tissue culture plastic. Cells in less rigid three-dimensional matrices have smaller focal adhesions (Larsen et al. 2006), while interactions with viruses promote short-lived signaling events (i.e., sufficient to promote viral uptake) (Stewart and Nemerow 2007). Importantly, small soluble ligands do not promote integrin signaling (Meredith et al. 1993; Stewart and Nemerow 2007). The inherent beauty of the scheme is that it permits soluble ligands to generate either abortive bursts of signaling (small particles or large ECM fragments) or complete inhibition of signaling, wherein single molecules act to "antagonize" integrins. In a remodeling microenvironment, this feedback provides an important mechanism by which a cell may test the relative structural integrity of its microenvironment.

In contrast to many other cell surface receptors, which may be highly mobile in the plane of the membrane, the combination of extracellular ligand binding outside the cell with intracellular engagement of the actin cytoskeleton means that a ligated integrin will be physically constrained.

Since most ECM components can form polymeric arrays, this immobilization is reinforced as other integrins bind extracellular ligands adjacent to the first. The overall assemblage is thought to provide a cooperative binding effect; as one integrin disengages, it is restrained by its ligated neighbors, and may re-engage ligand. Intracellularly, the array of integrin cytosolic domains is exploited by the cell as the basis of a crosslinking scaffold that forms the signaling complex. Focal adhesion complexes are observed to be larger on rigid plastic surfaces than in three-dimensional gels, as the more rigid nature opposes the cytoskeletal tension generated by the cell (Cukierman et al. 2001). The contacts appear stable, but are nonetheless slowly turned over, with new integrin-mediated contacts engaging along the contractile edge of the complex, and others disappearing at the distal end of the focal contact (Zamir et al. 2000). The overall effect is that, in cultured cells, nascent focal adhesions form in the cell periphery, and migrate medially at about 300 nm/min, and become smaller and dissociate centripetally.

This will vary somewhat from cell to cell and with different ECM composition. Different integrin heterodimers exhibit specific ligand-binding characteristics, thus the specific heterodimers involved in adhesion events and the relative tensional forces generated via linkage may "customize" the cellular response to a given extracellular milieu. In fact, although different integrins have been implicated in eliciting signals via common cellular pathways, each integrin differs somewhat in its capacity to do so (Giancotti and Ruoslahti 1999). This is due to differences in ligand affinities as well as variations in integrin cytosolic tails. Since even relatively common components of the focal adhesion such as the kinase FAK (focal adhesion kinase) (Mitra and Schlaepfer 2006), the cytoskeletal protein talin (Banno and Ginsberg 2008), and the scaffolding protein p130Cas (Klemke et al. 1998) are expressed at different levels in a given cell type, different cells may interpret the same ECM via the same integrins in different ways. For example, many hematopoietic cells express high levels of talin, but no FAK. A given cell's reaction to the local microenvironment results from the integration of these many signals, interpreted in the context of cell adhesion events and cellular programming.

The Vitronectin-Binding Integrins Are One of Several Integrin Subfamilies

The integrin α and β subunits are type I transmembrane glycoproteins that pair in the endoplasmic reticulum to form the heterodimeric holoprotein; complex glycation on both subunits follows during transport to the cell surface via the golgi. The pairing of the α and β subunits is not random; only 24 integrin pairs are known despite the existence of 18 α and 8 β subunits (and thus the potential for 144 unique

heterodimers). Thus, specific molecular properties are critical for heterodimer formation. Early studies revealed that integrin β1, β2, and β3 each associated with unique groups of α subunits (Hynes 1986), prompting speculation that the β subunits formed core subfamilies, with the specific α subunit determining the ligands bound. The model was attractive, as the known α subunits tended to be homologous and bind distinct but similar ligands. Nonetheless, it became evident with time that the converse is also true; one integrin can bind several ligands (Cheresh and Spiro 1987), and α subunits can nucleate structural "subfamilies" (Cheresh et al. 1989). Integrin αv forms the largest of these by pairing with five of the eight known β subunits. Notably, three of these β subunits pair exclusively with integrin αv (Fig. 7.1).

Integrin αv was originally cloned in Erkki Ruoslahti's laboratory in La Jolla (Suzuki et al. 1987); its genetic organization would be determined much later (Sims et al. 2000). The name α"v" was derived for its capacity to mediate attachment to vitronectin, a blood-borne serum component that is deposited with provisional ECM. Vitronectin, originally called "serum spreading factor" (or S factor), is a principle component of tissue culture media that is deposited on plastic and promotes cell attachment (Hayman et al. 1983). Other adhesion receptors, most notably the homologous fibrin/fibrinogen receptor found on platelets (integrin αIIbβ3) may also bind vitronectin. However, this chapter focuses on the αv integrin subfamily and the role of these vitronectin-binding receptors in promoting tumor progression.

αv Integrin Structure

Both αv and its associated β subunits possess relatively short (<50 amino acid) cytosolic domains, often referred to as the "cytosolic tails." Together with the single transmembrane domain, these tails account for less than 10% of the integrin; the remainder of the integrin is extracellular, including the ligand-binding region, the heterodimerization interface, structural domains, and cation-binding regions. A recombinant form of the extracellular region of integrin αvβ3 has been crystallized (Xiong et al. 2001). In good agreement with prior observations via EM, the integrin heterodimer was determined to have an ovoid-shaped head region with two stalks that extend away from it (one stalk each for the α and β subunits). The ligand-binding "head domain" region is approximately $9\times\ 6\times 4.5\ nm^3$ and comprises the specific ligand-binding region present at the interface of the α and β subunits. The integrin was crystallized in the "inactive" form, a lower energy conformation thought to represent the weaker affinity form of integrin. This structure has the "head domain" folded back on the stalks. Similar structures were seen previously in integrin activation studies (again via EM), and support the notion that the head domain swings away from the stalks and outward from the cell surface, with the stalks separating, in the "higher affinity" form(s) of the integrin (as depicted in Fig. 7.1). Nonetheless, subsequent crystallization efforts showed that even the low-affinity form of the integrin was competent to bind small soluble ligands (Xiong et al. 2002) or even fibronectin (Adair et al. 2005), though its not clear whether the latter takes place to great degree due to steric limitations imposed

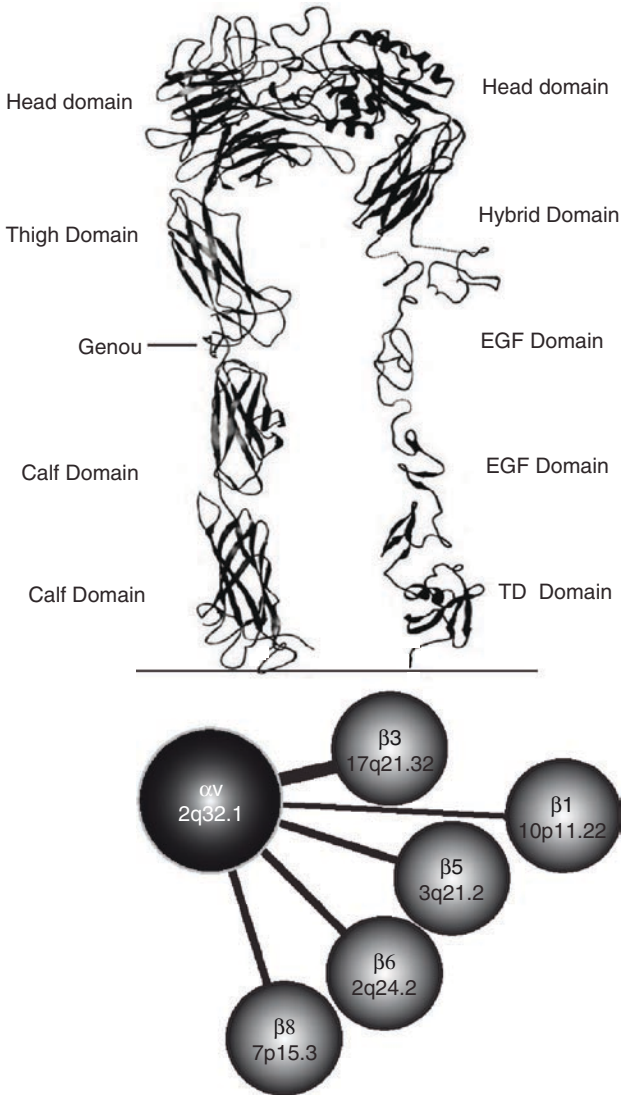

Fig. 7.1 General structure and pairing of αv integrins. A model of the active form of the extracellular regions of integrin αvβ3, extending away from the cell membrane (*line*). The α subunit is show on the *left*, and the β subunit on the *right*. The head domain of the α subunit is a β propeller – the head domain of the β subunit is a Rossman fold. The flex point of the α integrin is at the "genou" (knee). The *lower panel* shows the integrin αv binding partners (and their position in the genome). The relative "pairing preference" of αv (β3 > β5 ~ β6 ~ β8 > β1) that has been observed in cultured cell lines is indicated by the proximity of the subunits in the scheme

by the cell membrane. Still, the results were consistent with prior antibody-based studies that showed that the ligand-induced binding sites, referred to as LIBS epitopes, were present in "active" integrin αvβ3 (Frelinger et al. 1990). Similarly, engineered antibodies that bind to the active site act as surrogate reporters of αvβ3

activation (Pampori et al. 1999). Thus, the activity of an integrin such as αvβ3 is regulated on a "global" level by steric effects, ligand access to the binding site (particularly for large ligands), conformational changes permissive for binding larger ligands (which will interact more extensively with the integrin than a small soluble ligand), and cytosolic anchorage or alteration that will tend to separate the stalks of the αv and β3 subunits (thus favoring activation and force generation).

This phenomenon, called "inside-out" regulation, was initially characterized in platelets and other hematopoietic cell populations, but was found to be common to most integrins (Ginsberg et al. 1992). The ability to control integrin activation is likely important in helping to direct cell migration. Although integrin αvβ3 is more active on melanoma cells than lymphoid cell lines, only a subpopulation of the αvβ3 integrin on the melanoma appears to be activated (Stupack et al. 1999). In migrating breast cancer cells, active integrins are found selectively at the front of the cell (Rolli et al. 2003). Collectively, the data supporting the existence of these different integrin conformers is excellent; a variety of clinically relevant or predicted and engineered mutations have resulted in precise and predicted modulation of integrin activity (Takagi and Springer 2002; Zhu et al. 2007). The ability to modulate integrin function with conformation explains, to a great degree, the necessity for the relatively large size of integrins and their modular structure.

Domain Structure of a Vitronectin-Binding Integrin

The αv subunit is composed of 1,048 amino acids arrayed in three different types of domains; a single seven-bladed "β propeller" structure, an immunoglobulin-like domain, and two unique integrin "calf modules" that compose the αv integrin stalk region. A peptide loop within the second "calf" module of integrin αv is subject to proteolytic cleavage, but disulfide bonding within this module maintains the membrane anchorage of the α subunit. Of these domains, the β propeller domain interacts most extensively with the β integrin subunit, and also contains four solvent-exposed divalent cation-binding sites, which are important for integrin function. The dependence of integrins on divalent cations was established shortly after the discovery of the receptors; integrin binding to ECM ligands is directly regulated by the nature of the divalent cations available, ranging from calcium to cobalt (Smith and Cheresh 1991). Manganese, in particular, acts to promote integrin binding to most ligands, likely by helping to stabilize the extended conformation of the integrin. Such changes can be detected immunologically; activation-dependent antibody AP7 recognizes integrin αvβ3 in the presence of Mn^{2+}, but not Ca^{2+} ions (Kunicki et al. 1997).

Comprising 788 amino acids (including the signal peptide), integrin β3 is typical among the β integrins. Although smaller than the αv subunit, integrin β3 is composed of more domains, including a PSI domain (plexin, semaphorin, integrin domain), a G-protein-like Rossman fold (a β-sheet surround by a helix) that interacts with the αv subunit β propeller, a hybrid domain formed by regions on either side of the Rossman fold, several epidermal growth factor (EGF)-like structural domains,

and a membrane proximal "TD" domain. Extensive disulfide bonding within the small EGF-type domains, and within β integrins in general, stabilizes their structure. The β integrins are all predicted to have very similar structures, and not surprisingly those subunits that complex with αv (β1, β3, β5, β6, β8) share a high degree of homology at the amino acid level (Fig. 7.2). This homology is reflected in similar structures; the substitution of very short regions of integrin β3 are sufficient to alter the ligand-binding capacity and confer new epitopes on integrins β5 and β1 (Lin et al. 1997; Takagi et al. 1997).

The number of discrete protein domains found in the β integrins is important for integrin function. The modular nature permits a high degree of flexibility in integrin conformation that in turn facilitates some regulation of binding function that might

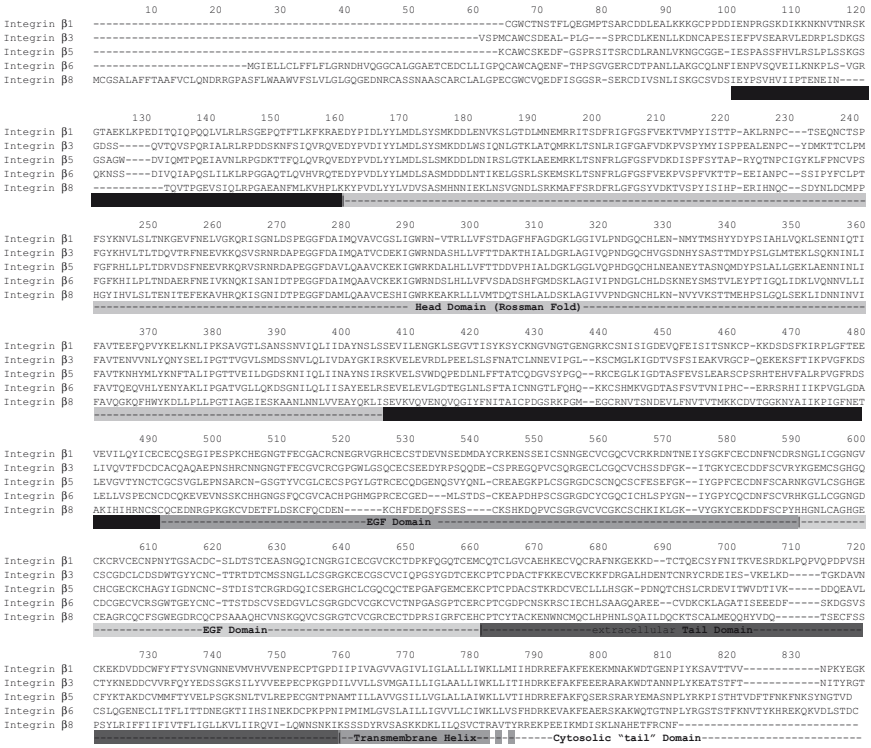

Fig. 7.2 Alignment of β subunits that pair with αv integrin. An alignment of the β1, β3, β5, β6, and β8 integrin subunits depicted using the single letter amino acid code. Mature peptides are shown for integrins β1, β3, and β5; β6 and β8 are shown with their signal peptide. Below the alignment, the domain these residues comprise is depicted by the *colored bar*. Note that the hybrid domain is composed of a PSI region (plexin-semaphorin-integrin) N-terminal to the head domain, which is seen to interchelate with the β-sheets containing "hybrid" domain in the crystal structure. The *dark gray* EGF domain differs from the *light gray* EGF domain in that it has been homology-modeled, but not confirmed in the crystal structure of the integrin ectodomain

not otherwise be achieved with structurally "simpler" modular cell adhesion molecules. Since even the "bent" conformation of the integrin can bind ligands in solution (even when the stalks are engineered to interact with each other) (Mathias et al. 1998), the capacity to move from a bent conformation to an extended conformation in a live cell, and to subsequently separate the "leg stalks," acts principally to decreases steric factors that limit ligand access to the integrin and to facilitate separation of the integrin cytosolic domains to promote cytosolic interactions. This modular nature of integrins is conserved, even among primitive animals such as sponges, highlighting the critical nature of "regulated" ECM or attachment adhesion in metazoan physiology.

Integrin αvβ3 Mediates Cell Attachment via an Arginine–Glycine–Aspartic Acid Target Sequence

The first target sequence identified to be bound by integrins (and later shown to crystallize with integrin αvβ3) was the arginine–glycine–aspartic acid (RGD) sequence described in the pioneering studies of Pierschbacher and Ruoslahti (1984) prior to the molecular characterization of integrins. The first integrin shown to bind the RGD sequence was the fibronectin receptor now known as integrin α5β1 (Pytela et al. 1985). Shortly thereafter, the fibrin receptor on platelets (integrin αIIbβ3) and a vitronectin-binding integrin on the surface of melanoma cells (integrin αvβ3) were also identified to bind the RGD sequence (Fitzgerald et al. 1987).

All of the αv integrins and about one-third of human integrins have characterized RGD ligands. Other integrins typically bind non-RGD ligands, dispelling the common misconception that all integrins bind RGD. Even among the RGD ligands, there is significant variability in presentation and in the flanking amino acids, such that the "RGD motif" varies significantly from protein to protein, appearing on loops of differing length and flexibility. Some integrins, such as α5β1, are quite restricted in the RGD ligands bound. By contrast, integrin αvβ3 exhibits the greatest range of RGD-containing ligands. Integrin αvβ3 was shown, soon after its discovery, to bind not only vitronectin but also to fibronectin and fibrin (Cheresh 1991). In the last decade, many more ligands have been found for integrin αvβ3, with most, but not all, interacting via an RGD sequence (Table 7.1).

Integrin αvβ3 Binds to Extracellular Components of Remodeling Tissues

The array of ligands bound by integrin αvβ3 would seem to suggest that it interacts with ECM nonspecifically. Careful examination reveals a central theme among these ligands; they are typically found in a tissue-remodeling environment such as

Table 7.1 Selected ligands for the αv integrins

αv Partner	Other α possible?	Common ligands
β1	α1–11	VN, FN, Virus*
β3	αIIb (Platelet receptor)	VN, FN, Fbg, Fb, CCN family proteins, TN, Virus* Disintegrins**, LM***, denatured COL I, II, IV,IV bFGF, osteopontin, bone sialoprotein, L1 CAM thrombin, thrombospondin
β5	*only* αv	VN, Fb, Virus*,
β6	*only* αv	VN, FN, TGFβ-LAP, Fibillin, Virus*
β8	*only* αv	VN, Fb, TGFβ-LAP, Virus*

The table shows the protein interactions of the β1, β3, β5, β6, and β8 integrin subunits, including other α integrin subunits the β subunit may pair with, and some of the most common ligands of the heterodimer. *Abbreviations*: CCN, Cyr61-CTGF-NOV family proteins; bFGF, basic fibrolast growth factor (via a reverse RGD motif); dCOL, (denatured collagen) types I–IV, VI; disintegrin, small soluble peptides which antagonize integrin function, such as echistatin; Fb, fibrin; Fbg, fibrinogen; LM, laminin (***requires Mn^{2+}); TGFβ-LAP, transforming growth factor-β latent-activating peptide; TN, tenascin, virus, any of the RGD-capsid viruses, including adenovirus as well as selected picornavirus, polyomavirus, and hantavirus strains

site of wound healing. As described, the blood-borne proteins vitronectin, fibronectin, and fibrin that accumulate at sites of vascular leakage or wounding are major ligands for integrin αvβ3. Consistent with a role in facilitating cell interaction with a remodeling environement, αvβ3 also binds to proteolyzed (Petitclerc et al. 2000) or denatured (Dedhar et al. 1987) collagen fragments, to oncofetal proteins such as tenascins, and to matricellular proteins such as cyr61/CCN1 (Babic et al. 1998). Integrin αvβ3 can also interact with extracellular proteases, directly in the case of MMP2 (Brooks et al. 1996), or via lateral association with transmembrane-anchored proteases such as MT-MMP, or with receptors for soluble proteases such as uPAR (Gellert et al. 2004).

The broad range of ligands recognized by integrin αvβ3, coupled with the integrin's ability to mobilize proteases, suggests some obvious mechanisms by which αvβ3 might impart significant functionality on cells invading a remodeling ECM. Nonetheless, the expression of integrin αvβ3 is a relatively uncommon event in a normal adult. Integrin αvβ3 is itself an oncofetal protein that is highly regulated and is normally found on very few cells in an adult vertebrate (Varner and Cheresh 1996). Typically, αvβ3 can be found on hematopoietic lineage cells including macrophages or osteoclasts (Crippes et al. 1996) and on "activated" (growth factor-stimulated) vascular cells (Brooks et al. 1994). The otherwise limited expression of integrin αvβ3 reflects, with few exceptions, the lack of any invasive requirement for general tissue homeostasis. In fact, one would expect the presence of αvβ3, to be a negative prognostic indicator when present in tumors. This appears to be true, as the expression of integrin αvβ3 is an independent negative prognostic indicator in many types of cancer, frequently present on malignant tumors and always present on the associated vascular cells (Cheresh 1991; Gasparini et al. 1998; Partheen et al. 2008; Sato et al. 2001).

Expression of Integrin αvβ3 on Aggressive Metastatic Tumors

Integrin αvβ3 was first shown on the surface of aggressive melanoma cell lines in vitro (Cheresh et al. 1987). The expression of integrin αvβ3 correlated very closely with the capacity of melanoma to engage in the "vertical growth phase," a progression of disease wherein the melanoma cells penetrate downward through the dermis (rather than engaging in a less invasive radial growth) and invade the lymphatics, while also metastasizing to other distant tissues (Albelda et al. 1990). An analysis of integrin αvβ3 on melanoma validated it as a negative prognostic factor in disease survival and progression (Si and Hersey 1994). Continuing analysis supported the concept that αvβ3 was both functional and causal in disease progression, not simply "associated" with more aggressive disease or poorly differentiated cell types. In fact, the simple expression of αvβ3 is sufficient to induce a highly aggressive and metastatic phenotype in otherwise nonmetastatic melanoma (Felding-Habermann et al. 1992; Jacob et al. 1995), promoting invasion to lymph nodes (Nip et al. 1992) and association with platelets, facilitating hematogenous spread (Felding-Habermann et al. 1996). The association with platelets masks and protects blood-born melanoma cells, and promotes lodging in the microcirculation. Platelets also contain numerous growth factors, such as vascular endothelial cell growth factor (VEGF) and platelet-derived growth factor (PDGF), which can condition cells in the "metastatic niche," thereby increasing the probability of a successful tumor seeding event. Integrin αvβ3 continues to be both a prognostic marker and a therapeutic target in melanoma.

Integrin αvβ3 is also associated with disease progression in glioblastoma (Gladson and Cheresh 1991). However, glioblastoma contrasts with melanoma in that the progression of the primary tumor, rather than metastases, is largely responsible for disease morbidity. Although metastasis to distant organs is observed selectively in advanced disease, glioblastoma is nonetheless highly invasive within brain tissue, and glioma cells can be found infiltrating neural tissue far from the primary tumor site (Brandes et al. 2008). At first glance, it would seem that the tumor microenvironments in skin and brain would have little in common, and the link with αvβ3 would be unclear. Nonetheless, the presence of secreted vascular permeability factors such as VEGF leads to vascular leakage in both tissues (and indeed, in any tissue). This, in turn, promotes the deposition and polymerization of blood-borne provisional matrix components (fibronectins, vitronectins, fibrinogen) within the local brain microenvironment that serve as ligands for integrin αvβ3 (Nagy et al. 2008). Thus, despite very different tumor environments, tumors will retain common features that can be exploited by the expression of αvβ3 integrin. Therefore, it is perhaps not surprising that αvβ3 is also associated with malignant disease in other types of cancers. In particular, these typically involve aggressive and metastatic carcinoma, which may be derived from a variety of epithelial tissues, including breast (Takayama et al. 2005), cervix (Gruber et al. 2005), prostate (McCabe et al. 2007), and pancreas (Kawaguchi et al. 2001).

Because of vascular leakage and the associated provisional ECM deposition, tumor cells expressing integrin αvβ3 appear to be advantaged with respect to "overcoming" normal anatomical barrier functions within tissues. Part of this is likely due to a capacity to orchestrate the actions of proteases at the front of invading cells (Brooks et al. 1998; Deryugina et al. 2004), and part is related to the ability to exploit changes in the local ECM. In both glioblastoma and melanoma, targeted antagonism of integrin αvβ3 attenuates tumor malignancy (Brooks et al. 1995; MacDonald and Ladisch 2001), supporting the idea that integrin αvβ3 contributes directly to cancer aggression (Chatterjee et al. 2000). Although aggressive and invasive tumors may exhibit greater tumor cell dissemination, actual metastatic growth will depend on the tumor type and capacity of the local niche to support tumor cell survival, and secondarily, new tumor growth (Gilbertson and Rich 2007). Thus, most cases of aggressive invasive behavior in vitro can translate to an increased incidence of distant metastases. However, in addition to its role in promoting tissue invasion, integrin αvβ3 also strongly influences cell survival decisions via its downstream signaling functions.

Mechanisms of Integrin αvβ3 Potentiation of Tumor Malignancy

Integrin αvβ3 has been well-studied with respect to the molecular mechanisms that underlie its capacity to influence cell behavior. The expression of αvβ3 alters cell shape from epithelioid to mesenchymal morphology (Filardo and Cheresh 1994; Leavesley et al. 1992) and is sufficient to promote spontaneous cell migration and invasion. The expression of integrin αvβ3 is sufficient to alter the intrinsic character of a cell, and this is accomplished not only via substrate ligation and potentiation of invasion but also via the transmission of signals into the cell itself. Several signaling pathways downstream of integrin αvβ3 have been identified that explain its potent influence on cell behavior. Principal among these are the proximal pathways initiated by FAK, the Src family of kinases (SFKs) (Mitra and Schlaepfer 2006), the phosphoinositide 3′ kinase (PI3K), and cytoskeleton-mobilizing serine/threonine kinases (DeMali et al. 2003).

Following integrin αvβ3 ligation, SFKs, and c-Src in particular, associate with the integrin cytosolic domain and become activated (Shattil 2005). Src substrates include a second non-RTK, FAK (Mitra and Schlaepfer 2006), a number of modular adaptor/cytoskeletal proteins such as Shc (Phillips et al. 2001), p130CAS (Klemke et al. 1998), and paxillin (Mitra and Schlaepfer 2006). Cytosolic phosphatases (von Wichert et al. 2003), and even the integrin itself (Ling et al. 2005) may be phosphorylated as well. However, the potency of the signal depends in part on the assembly of the signaling complex, the focal adhesion, which is responsible for anchoring actin filaments as well as serving as a scaffold for signaling molecule assembly and activation.

The ligation of clustered integrins on the cell surface promotes sustained signaling, cooperatively facilitating the assembly of a focal adhesion complex that

mediates downstream signaling to key cellular migration and survival pathways. For example, among nonadherent lymphoid cells, clusters of αvβ3 ligands are far more efficient than dispersed αvβ3 ligands at activating the kinase Syk (Stupack et al. 1999), an upstream mediator that promotes activation of the mitogen-activated protein kinases ERK1 and ERK2. Similar results appear true with respect to FAK, rather than SYK, in anchorage-dependent cells. In these cells, FAK is an important mediator of adhesion-dependent signaling via the ERK1/2 pathway. FAK is bound by the adaptor protein Grb2, which couples to son of sevenless (SoS) proteins and promotes Ras signaling (Schlaepfer et al. 1999). The canonical MAPK pathway (Ras to Raf to MEK) engages, resulting in the activation of ERK (Schlaepfer et al. 1999). Aside from the induction of proliferation, active ERK can also directly phosphorylate myosin light chain kinase, fostering cell migration (Klemke et al. 1997). FAK activation promotes migration through more direct mechanisms, including via the recruitment of the Src substrate (and large scaffolding protein) p130Cas. This expanding framework permits continuing recruitment of signaling proteins, including the adaptor protein p85, and its associated lipid kinase, PI3K. Both FAK and p130Cas bind p85α, and coprecipitate in complex with the p110 catalytic domain following integrin ligation. This event occurs relatively early after integrin activation, and can be activated by a variety of different ligands with different geometries, or even by "soluble particulate" ligands such as viruses (Li et al. 1998). Activation of the PI3K pathway is a key element in regulating cell survival, largely through downstream signals via protein kinase B (PKB/Akt) (Sedding et al. 2005), which serves to promote remodeling of the focal adhesion structure (Greenwood et al. 2000).

Integrins Govern RTK Signaling

Interestingly, these same central signaling pathways were initially identified to be activated by RTKs (Holland et al. 2005). Dimerization of specific RTKs induced by the presence of their cognate growth factor ligands, including insulin, bFGF, or EGF, induces reciprocal phosphorylation of the receptor's cytosolic domains. This, in turn, promotes the recruitment of adaptor proteins, many of which are common to focal adhesion complexes (such as Shc, Grb2, and p85) and lead to subsequent signaling via the ERK1/2 and PI3K pathways, thereby promoting cell proliferation, survival, and migration. Perhaps more intriguing, integrin-mediated adhesion has been shown to be required for signaling by many RTKs, and has been proposed as one basis for cellular anchorage dependence (Eliceiri and Cheresh 1999). It is clear that RTKs such as the insulin receptor (Lebrun et al. 1998), platelet-derived growth factor receptor (PDGFR) (Ding et al. 2003; Riemenschneider et al. 2005), and epithelial growth factor receptor (EGFR) (Marcoux and Vuori 2005) can be found within focal adhesions. In the latter case, it appears that integration of EGFR into focal adhesions may be sufficient to promote EGFR signaling in the absence of the EGF ligand, where it collaborates with other integrin signals (Marcoux and Vuori 2005).

This "collaboration in signaling" is a general phenomenon among integrins and RTKs in vitro, under situations where the integrins interact with a nonphysiological, rigid substrate. Nonetheless, this is likely to reflect the situation in vivo, where sustained RTK signaling in response to growth factors requires integrin function (Eliceiri et al. 1998).

Although RTKs and integrins interacted within the cell via common signaling elements and shared downstream targets, direct association between integrins and RTKs has been described. Since differences in integrin signaling have been attributed to their cytosolic domains (Eliceiri et al. 2002), it is perhaps surprising that integrins interact with growth factor receptors via their extracellular domains. Just as different integrin heterodimers maintain distinct ligand specificities, it appears that the specific αβ pairs may also associate preferentially with different RTKs. For example, integrin αvβ3, but not α2β1, can preferentially associate with the PDGFR and the insulin receptor, with functional consequences(Ding et al. 2003; Schneller et al. 1997). This capacity to differentially associate with cell surface receptors may explain why in some cases, integrins influence cell behavior in a cytosolic domain-dependent manner, whereas in others, the extracellular domain is key (Filardo et al. 1996). This may be due to direct interactions between the integrin and specific RTKs. For example, integrin αvβ3 associates with its extracellular domain with both the PDGF receptor and the VEGF receptor (Borges et al. 2000), whereas β1 integrins do not. Nonetheless, numerous cell membrane proteins associate with integrins, including CD47 and other tetraspanins (CD9, CD81, CD82, and CD151), uPAR, CD98, tissue transglutaminase, as well as K^+ channels (and possibly other membrane channel proteins) (Akimov et al. 2000; Petty et al. 2002).

Complexity in Signaling Is not Unique to Integrins and RTKs

The picture is somewhat analogous to antigen-mediated stimulation in lymphoid cells. The AR complex includes numerous transmembrane receptors that aggregate in a lateral plane, in the process engaging extracellular or cytosolic effectors (or both) to form an "immunological synapse" (Dustin 2008). The synapse is composed of the heterodimeric AR, additional ancillary proteins such as CD4 or CD8, SFKs such as lyn or lck, cytoskeletal proteins, and integrins. Depending on the context, signaling through the AR complex can promote cell survival and proliferation or apoptosis. RTK and integrin signaling may represent a variant on this theme, requiring coordination of several different signaling pathways for efficient signal transduction. An appreciation of the complexity of the signaling complex in lymphoid cells may provide insights as to why, under some circumstances, ligation of integrins in adherent cells could promote apoptosis rather than proliferation (i.e., why soluble ligands often induce apoptosis, whereas substrate ligands promote proliferation and survival). Indeed, while small soluble antagonists of integrins mediate apoptosis of normal vascular cells in vivo (Storgard et al. 1999), many tumors cells accumulate lesions that render them resistant to the proapoptotic effects of unligated integrins

(Stupack et al. 2001). In contrast, substrate-ligated integrin αvβ3 acts to actively counter a variety of death inducing insults, such as serum withdrawal, exposure to chemotherapeutics, or ionizing radiation in both primary and tumor cells. In this case, the recruitment of PI3K and the downstream target Akt appears critical to survival, while the contributions of the ERK pathway may be dispensable. In particular, signaling downstream of ligated integrin αvβ3 has been described to suppress the proapoptotic activity of the p53 gene (Stromblad et al. 2002), which serves to promote cell survival in response to internal stresses. Ligation also coordinately activates NFkB signaling (Scatena et al. 1998), which can result in increased expression of the inhibitors of apoptosis such as IAPs and cFLIP (Aoudjit and Vuori 2001; von Wnuck Lipinski et al. 2006) and maintain a high ratio of "good" Bcl-2 proteins to proapoptotic Bcl-2 proteins (Matter and Ruoslahti 2001; Stromblad et al. 1996). In tandem, the activation of c-Raf, by integrin αvβ3 and the basic fibroblast growth factor receptor, promotes translocation of c-Raf to the mitochondria and kinase-independent protection against mitochondrial depolarization (Alavi et al. 2003). Evasion of host surveillance, and survival under stress, has been established as important parameters for tumor progression. The ability of integrin αvβ3 to contribute to both processes provides further mechanisms by which this integrin can confer a highly aggressive phenotype on tumor cells.

Integrin αvβ5 Is an Anchorage Receptor

Integrin αvβ5, though the second αv integrin characterized, has been studied to a lesser extent than αvβ3. This is somewhat ironic, since, in contrast to αvβ3, integrin αvβ5 is expressed ubiquitously among cells in most tissues (excluding those of the hematopoietic system). Integrin αvβ5 was identified as an "alternative" vitronectin receptor, distinct from αvβ3, on the surface of carcinoma cells (Cheresh et al. 1989), and shared some, but not all, of the RGD-containing ligands bound by integrin αvβ3. These differences in ligand binding are due to differences between αvβ5 and αvβ3 at the substrate-binding interface (Lin et al. 1998). A second notable difference is in the cytosolic tail of integrin β5 that differs from integrin β3 in that it contains an additional loop of amino acids present near the terminus which may substantially change the structure of the tail. Although present on carcinoma cells and capable of mediating migration (Klemke et al. 1994; Yebra et al. 1996), integrin αvβ5 appears to play a physiological role as an anchorage receptor, mediating adhesion as part of the hemidesmosome structure (Wayner et al. 1991). Although integrin αvβ5 mediates cell attachment to vitronectin, it does not appear to promote downstream signaling to the ERK and PI3K system in the same manner that integrin αvβ3 does. For example, although integrin αvβ5 associates with FAK, this is not associated with FAK activation without other activating stimuli (Lewis et al. 1996). Rather, productive association of FAK with the β5 cytosolic domain requires independent activation of SFKs by RTKs (Eliceiri et al. 2002), suggesting that β5 is not fully competent to spontaneously mobilize SFKs (in contrast to αvβ3).

Src Activation Promotes αvβ5-Dependent Migration and Metastasis

However, in the presence of an appropriate stimulus, integrin αvβ5 readily promotes migration on vitronectin in vitro, which corresponds to metastasis in vivo (Brooks et al. 1997), which is blocked by antagonism of integrin αvβ5. This is a key observation; the induction of migration on vitronectin via αvβ5 following RTK activation, but not the capacity to migrate on collagen, laminin, or fibronectin via β1 integrins, is predictive for tumor dissemination in vivo. In colon carcinoma patients, integrin αvβ5 is thought to mediate metastases that occur early in disease (Enns et al. 2005). Functionally, the requirement for Src activation may not be a significant barrier to cell invasion, since (1) Src activation occurs readily downstream of mutations to RTKs or upregulation of signaling RTKs such as ErbB2, (2) external activation can be circumvented by activating mutations in the Src pathway itself (such as downregulation of Csk or expression of v-Src), and finally (3) Src is likely to undergo activation in vivo to some extent due to the production of endogenous growth factors.

However, it is worth noting that the activation of Src via any of these mechanisms facilitates a number of contributory changes in the cell which may promote migration independent of the recruitment of active FAK (and other focal contact signaling proteins) to αvβ5. For example, Src-dependent phenomena include the dissociation of cell–cell junctions and the internalization and degradation of cadherins (Avizienyte et al. 2002). Similarly, Src activates proteases such as calpains, which accelerate the turnover of the focal adhesion complex, similarly promoting the change from simple anchorage to migration (Avizienyte et al. 2002; Carragher et al. 2003) Thus, the shift from αvβ5 "anchorage" function to migration occurs in concert with global changes within the cell that eliminate cell–cell contact, dynamically alter the actin cytoskeleton, and promote FAK association and focal adhesion turnover activity in αvβ5-initiated focal complexes. The major difference between αvβ5 and αvβ3, and one reason that integrin αvβ3 may promote a more aggressive phenotype, appears to be that αvβ3 directly recruits Src (Arias-Salgado et al. 2003), and can accomplish most of these activities spontaneously.

Other Vitronectin-Binding Integrins and Cancer

The roles played by other αv integrins in cancer are less clear. Integrin αvβ1 appears to be expressed at lower levels than either αvβ3 or αvβ5, and tends to be expressed to a greater degree among cells lacking these integrins. This may result from inherent pairing preference of αv integrin, or may be due to cell specific pairing factors, or simply arise from the fact that β1 integrins pair with many α subunits, and thus there is greater competition for β1 binding. Functionally, αvβ1 maintains the capacity to bind vitronectin but does not appear to elicit the same signaling events that αvβ3 binding does, and in the presence of other receptors, appears to

play a lesser role in cell adhesion. For example, while αvβ1 appears to be present on the surface of a number of breast cancer cells, it seems to contribute to cell attachment in only a minority (Meyer et al. 1998). This could, however, be due to a lower affinity (as higher concentrations of ligand are required for similar adhesion). We have seen, for example, that CS1 melanoma cells expressing αvβ1 attach to vitronectin only at coating concentrations above 5 μg/ml, while expression of αvβ3 or αvβ5 in the same cells decreases this requirement by tenfold or more. The picture that emerges with αvβ1, so far, is one of an ancillary receptor, a receptor that forms, perhaps due to evolutionary homologies, and which can perform some or the functions of other αv integrins, but which does not appear to play a dominant role. Indeed, αvβ1 is not yet definitively reported to play a key role in cancer pathology.

Integrin αvβ6 is related to integrin αvβ5, and similarly contains an additional inserted region within the cytosolic tail. It is also widely expressed on a variety of epithelial cells, though not as ubiquitously as αvβ5. Like all other αv integrins, αvβ6 binds to provisional ECM components such as vitronectin and fibronectin via the RGD motif. However, unlike αvβ5, αvβ6 binding to ECM readily promotes ERK activation and cell proliferation (Dixit et al. 1996), and through these mechanisms, as well as the upregulation of ERK-target genes, promotes the malignant properties of ovarian tumors in vitro (Ahmed et al. 2002). Indeed, ovarian tumors often progress by seeding throughout the peritoneal cavity, where fibronectin and vitronectin are major ligands; other tumors found in serous effusions also demonstrate upregulation of αv integrin (Sigstad et al. 2005). Clinically, ovarian carcinomas express αv integrins, and in particular, αvβ6 appears to be upregulated in late stage, metatstatic disease (Maubant et al. 2005). Similarly, αvβ6 can promote lymph node metastasis in gastric cancer (Kawashima et al. 2003). Thus, it would appear that αvβ6 has some similar properties to integrin αvβ3 in promoting proliferation and, at least indirectly, invasion. However, this may be a context-dependent phenomenon. Although integrin αvβ6 has been shown to be elevated on some oral squamous cell tumors (Dang et al. 2004), it also appears to be decreased on others. In fact, simple expression of αvβ6 in vitro appears to be sufficient to reverse the transformation phenotype in tumors (Mogi et al. 2005), and αvβ6 plays a critical role in suppressing tumorigenesis in mice (Ludlow et al. 2005).

αv Integrins in Stromal Cell Contributions to Tumor Development

However, in this case, such "contradictory" roles for αvβ6 may be explained by interactions between tumor cells and other host cells. Tumor cells are intermixed with, and supported by, a variety of stromal elements including vascular cells, fibroblasts, and cells derived from the innate and adaptive immune systems. In the last decade or so, the role of these cells in promoting tumor growth has become increasingly recognized. In the case of integrin αvβ6, the discovery of a critical ligand, the latent activating protein (LAP) of TGFβ, by Sheppard and colleagues

(Munger et al. 1999), provided an interesting new perspective on integrin function – the capacity to regulate the function of latent or matrix-associated cytokines (Morris et al. 2003).

TGFβ1 is a pleiotropic cytokine that can promote or inhibit tumor development. TGFβ1 also interacts with somatic cells and contributes to the pathology of fibrosis independent of its signaling roles in tumor cells. TGFβ1 is secreted as an inactive precursor, which is associated with LAP. The complex is bound within the ECM, frequently covalently associated with it. Active TGFβ1 requires dissociation from the LAP, and αvβ6 binds to an RGD sequence in the LAPs of TGFβ1 (and TGFβ3). Binding is not sufficient to activate TGFβ function, but rather requires the application of mechanical force and the induction of a conformational change in the latent complex. Integrins are perfect receptors for this role; the integrins can continue to signal via interactions with the matrix-immobilized LAP, while the "mature" TGFβ1 can bind TGFβ receptors on adjacent cells and trigger signaling in this manner.

A second integrin αvβ8 also binds to LAP, via the same sites as αvβ6, yet cannot directly activate TGFβ via the mechanisms characterized for αvβ6, possibly due to differences in the cytosolic domains of β6 and β8. However, αvβ8 instead acts to secure the LAP/TGFβ complex, permitting efficient presentation to cell surface transmembrane metaloproteases, such as MT-1MMP. This, in turn, permits cleavage of LAP and TGFβ release (Mu et al. 2002). In contrast to integrin αvβ6, this mechanism of activation liberates the TGFβ from the matrix, permitting it to diffuse to neighboring cells. It may then act on proximal tumor cells, promoting an epithelial to mesynchymal transition (Yi et al. 2002) and subsequent malignant behavior such as metastasis (Galliher-Beckley and Schiemann, 2008; Subramanian et al. 2004). Thus, upregulation of integrin αvβ6 is associated with poor outcome in colon carcinoma (Bates et al. 2005). Integrins αvβ1 and αvβ5 appear to be able to bind to LAP weakly, though it is not clear if they activate TGFβ function in vivo. In contrast, the presence of integrin αvβ3 suppresses TGFβ signaling (Reynolds et al. 2005), suggesting that αvβ3 either does not activate LAP, or that the presence of the β3 subunit competes with β6 and β8 for the pool of αv integrin. Under normal physiological circumstances, this may represent a feedback mechanism since TGFβ signaling can upregulate integrin αvβ3 (Pechkovsky et al. 2008).

It is tempting to assign "cancer-promoting" activity to TGFβ, yet its important to consider that its capacity to promote tumor malignancy is limited to established tumors that have already accumulated multiple genetic lesions. In fact, TGFβ can potently suppress immune responses, which may be relevant to carcinogenesis. The presence of TGF can act to curb ongoing inflammatory responses, thus suppressing tumorigenesis, by preventing the accumulation of oncogenic lesions in precancerous cells (Ludlow et al. 2005).

While integrins such as αvβ5 or αvβ1 are expressed ubiquitously on many cells, they are also expressed in an inducible fashion on monocytes (Lafrenie et al. 2002). Integrin αvβ3 can be induced in mature osteoclasts (Zambonin Zallone et al. 1989) or macrophage and on vascular cells (pericytes and endothelial cells) (Brooks et al. 1994) that have been stimulated to execute the angiogenic program. Integrin αvβ8 is expressed on leukocytes and, as discussed earlier, plays a critical role in control-

ling chronic inflammatory responses (Travis et al. 2007). These examples of the inducible αv integrin expression and the consistent conservation of the αv integrin family among vertebrate would generally lead one to propose an essential role for these receptors. Yet, αv knockout mice develop normally with 20% or so of the pups born alive (Hynes and Hodivala-Dilke 1999). The principle problems with these mice are a lack of patent cerebral vasculature, and these mice typically succumb rapidly to the associated complications. However, the results still provide an indication that αv integrins are not absolutely essential to all developmental processes. The results strongly support a role for αv integrins as critical in regulating cellular responses to insults and in the restoration of architecture for normal physiological function. A major component of this type of tissue remodeling is angiogenesis.

Tumor Angiogenesis

The work of Dr. Judah Folkman drove an appreciation of the role of angiogenesis, or new blood vessel growth, as a critical factor promoting tumor growth and progression. Angiogenesis is induced by cytokines such as VEGF or bFGF, which can be secreted by tumor cells or stromal cells. In particular, the αvβ3 and αvβ5 integrins are expressed on vascular cells, and have been implicated as critical players during angiogenesis, although playing slightly different roles.

The role of integrin αvβ5 has been tightly linked to the actions of VEGF, which was initially described as vascular permeability factor due to its capacity to induce vascular leak (Senger et al. 1986). Vascular leak occurs as endothelial cells lining the vessels disassemble contacts with each other, providing access to the underlying tissues for blood-borne elements, including latent serum ECM components, coagulation factors/proteases, and even, in some cases, cells and platelets (Weis et al. 2007). Thus, vascular permeability permits a rapid response and capacity to remodel the local ECM microenvironment. Mice deficient in integrin αvβ5 are also blocked in their VEGF-induced vascular leak response (Eliceiri et al. 2002). Mechanistically, this is due to SFK-induced cooperation between VEGF and αvβ5, and is triggered by VEGF binding to its respective RTKs (VEGFR1 and VEGFR2) on the of endothelial cell surface. The activation of these receptors and their immediate downstream targets leads to activation of the Src and Yes kinases that are critical for mobilization of αvβ5 from an "anchorage" receptor to an "invasion" receptor in endothelial cells – an observation that mirrors the situation in tumor cells. Mechanistically, the studies also demonstrated the subsequent lateral association of αvβ5 and Src near VE-cadherin sites that permits Src-mediated VE-cadherin phosphorylation and disruption of cell–cell junctions.

By contrast, integrin αvβ3 is not present on resting endothelial cells and is not involved in the early angiogenic response. Integrin αvβ3 is upregulated at the transcriptional level in response to growth factor-induced "reprogramming" of the vascular endothelial cells mediated by several members of the Hox family of homeobox genes (Boudreau et al. 1997). As in tumors, the expression of αvβ3 on endothelial cells

optimizes their capacity to invade and survive in a remodeling ECM. For example via αvβ3, sustained bFGF signaling to the ERK/MAPK pathway and/or PI3K/Akt pathways can be observed (Eliceiri et al. 1998). Treatment with "antagonists" of this integrin promoted apoptosis in vitro and in vivo and inhibited angiogenesis and tumor growth.

Since mice lacking integrin αv or β3 (Hodivala-Dilke et al. 1999) develop blood vessels, it would appear that either other integrins can compensate for its role in invasion or the role of integrin αvβ3 was more complex than simply facilitating tissue interactions. Clues as to αvβ3 function were provided in both the broad nature of its ligands: ECM components and proteolyzed fragments of these proteins. Interestingly, αvβ3 is also utilized as a receptor by many RGD-bearing viruses and venoms, suggesting that bioactive molecules targeting this integrin exhibit functional (and pathological) consequences. This likely relates to the role of integrins in transmitting signals as mechanoreceptors; integrins can act as biosensors, interrogating their milieu, and conferring information back to the cell that can help to control cell fate decisions (life, death, maturation). For example, the angiogenic milieu surrounding a tumor contains provisional ECM that is constantly replenished from the blood or via local production. This is necessary, as the actions of proteases such as plasmin, uPA, MMPs, or extracellular cathepsins can rapidly degrade the ECM, establishing equilibrium between soluble ECM fragments and substrate ECM. The broad ligand specificity of integrin αvβ3 permits it to act broadly as a biosensor in this environment, binding soluble ligands (antagonized integrin) or immobilized ligands, and effecting alterations in cell behavior. While ligated integrin αvβ3 promotes survival, unligated or "antagonized" integrin αvβ3 induces apoptosis.

The binding of soluble ligands, in particular, has been associated with the induction of apoptosis downstream via at least two mechanisms; the activation of caspase 8 (Stupack et al. 2001), the suppression of signaling via the ERK (Eliceiri and Cheresh 1999), or Akt pathways (Maeshima et al. 2002). These events appear distinct since mutations in the tail of integrin β3 that disrupt signaling can nonetheless trigger apoptosis (Zhao et al. 2005). Apoptosis induced by unligated integrin occurs in a caspase 8-dependent manner initially, but may proceed via subsequent caspase 9 activation when caspase 8 is absent or its activation is blocked. Such a negative feedback mechanism would be expected to be absent among mice deficient in αvβ3, and in fact these mice exhibit increased pathological vascularization during tumor development (Reynolds et al. 2002). Conversely, antagonizing integrin αvβ3-mediated invasion in the presence of inhibitors of endothelial cell apoptosis does not prevent angiogenesis (Stupack 2007). Thus, other integrins are sufficient for invasion, but the presence of αvβ3 acts to regulate the process.

Therapeutic Strategies Involving Vitronectin Receptors

Given the role of integrins in both the tumor cells and the supporting stromal elements in cancer, and the limited expression of certain integrins (αvβ3), it was recognized from the earliest studies that strategies which target this family of

adhesion receptors could have merit. Angiogenesis represents one critical component of tumor growth that has been targeted aggressively with integrin antagonists (Brooks et al. 1994; Folkman 1995; Friedlander et al. 1996), in part due to the strong correlation that was established between the percent of αvβ3 positive vessels within the tumor and disease progression (Gasparini et al. 1998). A diverse array of strategies have been aimed at either inhibiting integrin αv function directly or utilizing these receptors for targeted drug delivery to the angiogenic endothelium present in tumors (Temming et al. 2005). Efforts in the early to mid 1990s focused on developing antagonists of integrin function by designing peptides based on the RGD sequence that would compete for binding to respective ligands in the ECM (Heckmann and Kessler 2007). Such studies have yielded small molecule peptidomimetics that have been used as targeting agents and antagonists. Related studies have also utilized integrin function-blocking monoclonal antibodies, perhaps the best studies of these is LM609, which antagonizes integrin αvβ3 (Cheresh 1987).

Integrin αvβ3-Targeted Antibodies

Abegrin (also called Etaracizumab, MEDI-522 or Vitaxin), is a humanized function-blocking antibody that was developed based on the mouse monoclonal (LM609) antibody directed against αvβ3. Abegrin recognizes a conformational epitope formed by the αv and β3 subunits (Gutheil et al. 2000), and has been proven to inhibit angiogenesis and tumor growth in preclinical studies. In addition to its effects on tumors directly, Abegrin may target tumor cell αvβ3, and Abegrin is currently being tested in multiple clinical trials including late stage malignant melanoma (where it appears to prolong survival), androgen-independent prostate cancer, and renal cell carcinoma, in addition to trials in rheumatoid arthritis and psoriasis. Abegrin is somewhat unique as an anti-vitronectin receptor strategy, as it is the only approach that is absolutely specific for integrin αvβ3; no other αv integrins are recognized. Other antibodies that have been clinically tested, such as YM337 (also known as 7E3), recognize both the platelet integrin αIIbβ3 and αvβ3. Although tumor cells can accumulate genetic lesions that promote survival and/or signaling even when integrin αvβ3 is blocked, Vitaxin binds with sufficient affinity that it may still block the ligand-binding function of the tumor integrin, while having full effects on untransformed stromal elements. In addition to angiogenesis and tumors, Vitaxin has been used to target osteoclasts-associated with bone destruction arising from bone metastasis (as is the case during carcinoma, myeloma, or melanoma, e.g.). While antibodies hold clear promise for targeting and therapy, they are nevertheless associated with the increased expense common to the development of complex, biological-based therapies. Auxiliary strategies could clearly be useful if integrin-targeted therapies are to become inexpensive and widely available.

RGD Peptides and RGD Mimetics

The identification of the RGD motif by Pierschbacher and Ruoslahti forms the basis for a second mechanism of integrin antagonism. Linear RGD peptides were initially used to antagonize αv integrins, supplemented by attempts to develop flanking sequences which would enhance integrin-binding activity, though relative specificities were low. A large advance occurred when the domains of natural RGD-containing ligands such as vitronectin and fibronectin were crystallized, leading to the acknowledgement that RGD peptides functioned in a context dependent manner, and prompted attempts to restrict the conformational space of the amino acid sequence via cyclization. This enhanced the affinity of the small peptides for integrins, while also permitting selectivity for a particular αv receptor (Aumailley et al. 1991; Heckmann and Kessler 2007), since RGD ligands spanned the interface of the integrin α and β subunits (Fig. 7.3). The effect of cyclization mimicked the secondary and tertiary structures of natural protein ligands, creating a defined orientation of the RGD motif. By contrast, the linear RGD peptides initially used low specificity due to the inherent flexibility of the RGD sequence, but were more adaptable to different integrin receptors (Gurrath et al. 1992).

Varying the chirality of amino acids within the cyclized RGD peptide permitted further tailoring of secondary interactions to enhance integrin specificity (Gurrath et al. 1992). On the basis of structure–activity relationships found by studies with a peptide library and the D-amino acid scans in the cyclized peptides, cRGDfV, where f is D-Phe, demonstrated selective αvβ3 antagonism, while exhibiting very low affinity for the related platelet receptor-αIIBβ3 (Pfaff et al., 1994). This peptide demonstrated efficacy in a rheumatoid arthritis rabbit model by diminishing arthritis severity and inducing apoptosis among the angiogenic endothelial cells (Storgard et al. 1999) and likely by inhibiting osteoclast function. A second enhancement was discovered by doing an *N*-methyl scan of the amide bonds in the cRGDfV peptide producing cRGDf (N-MeV) (Dechantsreiter et al. 1999), known as Cilengitide (EMD 121974), which achieved the highest affinity for αvβ3 (and also exhibited activity against αvβ5) among the *N*-methylated peptides. Nabors et al. (2007) reported the results of a Phase I clinical trial in recurrent malignant glioma using this cyclic peptide. An objective response was reported in 5 of 51 patients, with another 16 of 51 patients described as stable for a median duration of 5 months. No clear pattern of toxicity emerged in this study with only mild joint and bone pain, thrombosis, thrombocytopenia, and anorexia reported. Similar promising results have been obtained in pediatric studies (MacDonald et al. 2008). The essentially side-effect free nature of these agents has prompted suggestions that they may work universally as adjuncts to other therapies (Hariharan et al. 2007; Idbaih et al. 2008; Reardon et al. 2008; Stupp et al. 2007).

A second, discrete strategy to discover selective integrin antagonists utilized phage display libraries containing an RGD core, and random flanking regions. The approach was used to pan for novel peptide sequences that could bind selectively to either α5β1 (Koivunen et al. 1994) or αv integrins. In the studies on

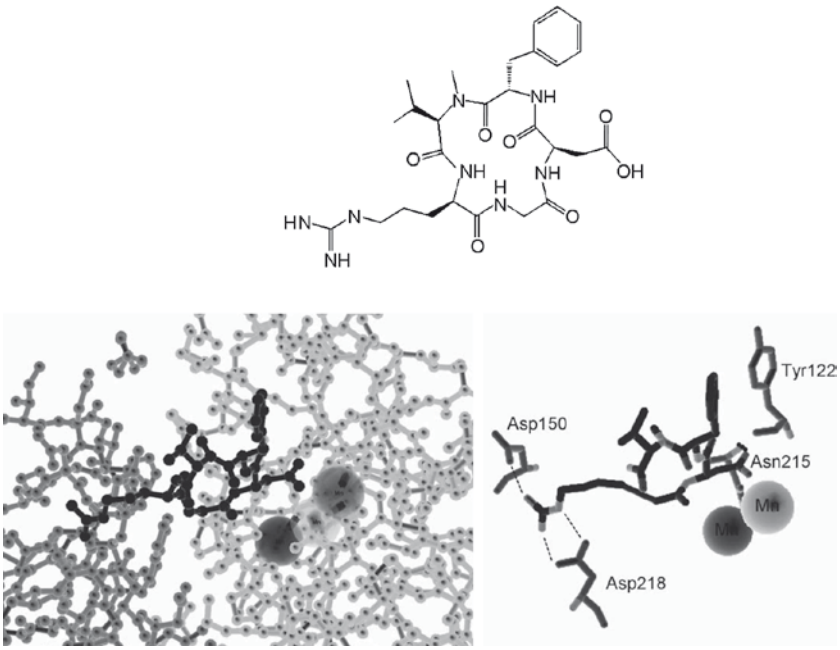

Fig. 7.3 A cyclic arginine–glycine–aspartic acid (RGD) peptide bound to integrin αvβ3. The figure shows Cilengitide (*above*) within the ligand binding region of integrin αvβ3, including proximal amino acids from both the α subunit (*dark gray* amino acid residues) and β subunit (*light gray* amino acid residues) (*lower left*). The cRGDf(N-Me)V peptide, Cilengitide (shown in black), is positioned spanning the αv-β3 interface (*left panel*). The significant molecular interactions are highlighted (*right panel*). In particular, aspartic acid residues 218 and 150 in the αv subunit form a bidentate salt bridge with the guanidinium moiety of the RGD peptide, whereas the carboxylate from Asp of the RGD coordinates Mn^{2+} binding at MIDAS as well as hydrogen bonding with the backbone amide of Asparagine 215 of β3. An additional π–π interaction occurs between the aromatic rings of the ligand D-phenylalanine and tyrosine 122

αvβ3, the peptide ACDCRGDCFCG (RGD4C) was identified as a potent inhibitor of αvβ3/αvβ5-mediated adhesion to vitronectin, and was 200-fold more effective than a linear RGD sequence (Pasqualini et al. 1995). Of the potential disulphide-bonding patterns, the C1–C4, C2–C3 disulphide bond arrangement lead to the most significant activity against the αv integrins (Assa-Munt et al. 2001). An additional peptide termed RGD10 was discovered by panning an RGD peptide phage library and this sequence (GARYCRGDCFDG) formed only one disulphide bond (Holig et al. 2004). Similar to the cRGDfV and related variants, the RGD4C and RGD10 improved selectivity over linear RGD peptides and improved affinity for the target integrins.

On the basis of the studies completed with cyclic RGD peptides, a pharmacophore model has been generated to guide the design of small-molecule RGD peptidomimetics designed to improve selectivity for binding to the various integrin α/β heterodimers as well as improvements in oral bioavailability (Takahashi et al. 2007). On the basis of the cyclized peptides, the peptidomimetics needed to incorporate a basic (guanidinium or other) and an acidic moiety as well as an optimal spacer distance between the pharmacophores to direct the appropriate specificity for the integrin target. The cocrystal structure of the extracellular segment of αvβ3 with cilengitide bound was solved providing an opportunity for molecular modeling and rational design of RGD peptidomimetics (Xiong et al. 2002). This ligand-induced conformation clearly identified the key residues in both the αv and β3 subunits, which are important for ligand binding. In this interaction, the α subunit contributes the acidic residues Asp150 and Asp218 forming a salt bridge with the Arg of the cyclic peptide. On the opposite side in the β subunit, the Asp of the cyclic peptide coordinates a metal ion located in MIDAS and further hydrogen bonds to the amide backbone at both Tyr122 and Asn215. On the basis of this molecular understanding, distances between the basic moiety (guanidine, pyridine, etc.) and the acidic moiety were found to correlate well with data generated from peptide libraries (Muller et al. 1994). Analoging of the guanidinium group and the linker distances lead to advances in both affinity and selectivity of the RGD mimetics which were important since targeting αvβ3 while minimizing the inhibition of the platelet receptor αIIBβ3 was desirable.

αv-Targeting of Therapeutics

Although selective antagonists of αvβ3 exhibit some efficacy in clinical trials currently, the observation that αvβ3 was preferentially expressed on endothelial cells in the tumor neovasculature represented an exciting opportunity for imaging and targeted drug delivery. Integrin αvβ3 present on the activated neovessels associated with tumors represents a theoretically ideal receptor for targeted drug delivery for the following reasons: (1) the angiogenic endothelial cells associated with the tumor vasculature are directly accessible and do not require the delivery systems to penetrate the tumor, (2) the αv integrins are known to act as internalization receptors for several viruses including adenovirus, via RGD sequences on the viral capsid (Wang et al. 2005; Wickham et al. 1993), (3) endothelial cells expressing the integrin are genetically stable, thus loss of integrin expression or the acquisition of drug resistance observed with tumors are not observed, and (4) tumor growth is dependent upon angiogenesis to maintain a proper supply of oxygen and nutrients to the growing tumor. Multivalent presentation of the peptide or peptidomimetic on proteins, polymers, or nanoparticles (NPs) greatly enhances affinity (Boturyn et al. 2004; Eliceiri et al. 2002; Kok et al. 2002; Montet et al. 2006) but also promotes integrin clustering and efficient endocytosis similar to viruses that utilize integrins

for cellular uptake. These features of multivalent targeting define the necessity of RGD peptide/peptidomimetics with greater selectivity rather than high affinity for the integrin in the monovalent format. These properties led to a vast amount of work utilizing cyclic RGD peptides and RGD peptidomimetics for both in vivo imaging and drug delivery.

Therapeutic studies utilizing the αv integrin targeting peptide, RGD4C, demonstrated that this peptide, when directly conjugated to doxorubicin, effectively targeted doxorubicin to the tumor neovasculature and enhanced efficacy in human breast cancer xenografts in mice (Arap et al. 1998). Importantly, this demonstrated the advantage of targeted chemotherapy and greatly reduced the side effects associated with doxorubicin therapy. Since this early study, cyclic RGD peptides have been conjugated to prodrugs of doxorubicin and paclitaxel and these studies have demonstrated an advantage for targeting αvβ3 (Chen et al. 2005; de Groot et al. 2002; Haubner et al. 2001). To date, these prodrug conjugates have not advanced deeper for reasons which are not known.

In another study, an RGD peptidomimetic was conjugated to a nanoparticle for suicide gene delivery to angiogenic blood vessels and this approach led to an induction of apoptosis in the tumor endothelium that subsequently produced tumor regression in murine models (Hood et al. 2002). Previous studies with the same αvβ3-targeted NPs containing gadolinium demonstrated targeting to the tumor vasculature and revealed "hot spots" of angiogenesis within the tumor (Sipkins et al. 1998). In addition to this work, polymeric systems such as RGD-PEG-PEI (polyethylenimine) have been utilized for siRNA delivery and successful therapy has been achieved with siVEGFR2 in models of angiogenesis and neuroblastoma (Kim et al. 2004; Schiffelers et al. 2004). Additionally, cRGDfK-functionalized liposomes with encapsulated doxorubicin inhibited tumor growth in a subcutaneous CT26 colon carcinoma model (Schiffelers et al. 2003). Recently, cRGDfK-functionalized liposomes containing doxorubicin have been shown to suppress metastasis in two orthotopic murine tumor models: pancreatic carcinoma and renal cell carcinoma (Murphy et al. 2008). In this study, targeting αvβ3 enabled a 15-fold reduction in the amount of doxorubicin that was required to suppress metastasis. Interestingly, only modest effects were observed in reducing primary tumor growth while the metastatic sites were greatly impacted. This suggested that the enhanced permeability and retention effect, which is relied on in a large majority of nanoparticle studies, did not play a major role in these orthotopic tumor models. The studies define αvβ3 as a target for treating various cancers and suppressing the metastatic potential of primary tumors.

Outlook

Despite more than two decades of work, the vitronectin-binding integrins still present mystery. While not absolutely essential for development, they remain critical regulators of disease physiology and pathology. The best understood receptor, αvβ3, presents a major challenge in tumor biology in that it confers a highly malignant

and aggressive phenotype on both tumor cells and tumor-associated stromal elements. The role of more recently characterized integrins, such as αvβ8, has only recently begun to be appreciated, but the capacity to influence inflammation is clearly a significant influence on tumor progression. While these reflections seem grim, there are also therapeutic opportunities that can be directed against these specialized integrins. Approaches that impact the intrinsic biological activities of integrins directly offer the most obvious approach, but the capacity to use these integrins as markers of pathological activity could also serve to target general therapies. In this respect, the advent of nanotechnology, and in particular small targetable therapeutic platforms, offers exciting new options to exploit. Thus, there is optimism that the third decade of investigating the integrins offers the very real promise of αv-directed therapies in the clinic.

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Chapter 8
The Function of SPARC in Tumor Cell Biology: SPARC as a Modulator of Cell-Extracellular Matrix Interaction

Rolf A. Brekken and Amy D. Bradshaw

Abstract Although alterations in the level of SPARC (secreted protein, acidic and rich in cysteine; also known as osteonectin) expression have been associated with a large number of studies on tumor tissue from various anatomical locations, the mechanisms by which SPARC influences tumor progression are not well defined. The capacity of SPARC to affect cellular proliferation, adhesion, migration, and invasion in a cell and tissue-specific manner further complicates the analysis of the function of SPARC in tumor biology. In this chapter, an effort is made to bring together results generated from a number of different studies that highlight functional consequences of SPARC expression. Emphasis has been placed on cell-Extracellular matrix interactions, in particular collagen-binding and collagen receptor activity. Likely, it is the contextual nature of divergent functions associated with SPARC, based in unique tissue microenvironments, that give rise to what are seen as seemingly contradictory effects of SPARC expression in different tumors.

Keywords Osteonectin/BM40 • collagen • metastasis • counter-adhesion • migration • tumor stroma

Introduction

Matricellular proteins are defined as extracellular matrix (ECM)-associated proteins that do not contribute structurally to the ECM, such as the classical ECM proteins, collagens and laminins, but instead modulate cell interaction with the

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_8,

ECM (Bornstein and Sage 2002). SPARC (secreted protein, acidic and rich in cysteine; also known as osteonectin), is a prototypic matricellular protein composed of three modular domains (Brekken and Sage 2001). That SPARC is conserved in a wide variety of evolutionarily diverse organisms (e.g., *Caenorhabditis elegans*, *Drosophila melanogaster*, brine shrimp, zebra fish, chicken, mice, and humans), suggests a basic function of this matricellular protein in multicellular biology (Bassuk et al. 1993; Schwarzbauer and Spencer 1993; Bradshaw and Sage 2001; Tanaka et al. 2001; Martinek et al. 2002; Rotllant et al. 2008).

Although the expression of SPARC is associated with many different types of tumors, the function of SPARC in tumorigenesis and metastasis is not clearly defined. A number of excellent reviews summarizing SPARC expression in specific types of tumors and its association with either enhanced or diminished tumor progression are available (Framson and Sage 2004; Clark and Sage 2008; Podhajcer et al. 2008). SPARC activity has proven to be contextual, and thus, seemingly contradictory functions of SPARC in either promoting or inhibiting different types of cancer have emerged. In general, SPARC expression correlates with invasion and progression of gliomas and melanomas (Rempel et al. 1999; Schultz et al. 2002; Prada et al. 2007; Shi et al. 2007; Haber et al. 2008; Yunker et al. 2008). In contrast, many epithelial cancers (e.g., lung, colon, prostate, pancreatic, and endometrial) hypermethylate the SPARC promoter, thus, reducing the amount of SPARC produced by the tumor cells (Sato et al. 2003; Suzuki et al. 2005; Wang et al. 2005b; Sova et al. 2006; Rodriguez-Jimenez et al. 2007; Yang et al. 2007). Targeted promoter demethylation by the nonsteroidal anti-inflammatory drug NS398 in human lung cancer cells restored SPARC expression and reversed the inhibition of cell invasion mediated by SPARC (Pan et al. 2008). However, given the wide-range of activities attributed to SPARC, global targeting of SPARC function has the potential to introduce detrimental off-target effects. A clear description of the molecular mechanisms of SPARC action is needed to understand its divergent effects on human cancers and thus develop effective strategies to manipulate SPARC activity that might be useful in the treatment of cancer growth and metastasis.

This chapter focuses on activities associated with SPARC and proposed cellular mechanisms by which SPARC mediates these activities with primary focus on cell-ECM interaction. We seek to provide some insight into the disparate influences of SPARC and the potential of this matricellular protein to guide either tumor and/or stromal cell interaction with ECM and thereby impact tumor progression and dissemination.

SPARC Structure/Function

The SPARC gene encodes a protein with a predicted molecular weight of 32 kD (Mason et al. 1986). Tissue-specific glycosylation of mammalian SPARC decreases the mobility of this glycoprotein which frequently migrates at ~40–43 kD under reducing SDS-PAGE conditions (Hughes et al. 1987; Kaufmann et al. 2004). The

N-terminal region of SPARC contains a low-affinity, high-capacity Ca^{2+}-binding domain (Maurer et al. 1992; Brekken and Sage 2001). The central portion of the protein includes a region with homology to follistatin that includes a Cu^{2+}-binding site, whereas the C-terminal domain (E-C domain) contains two high-affinity Ca^{2+}-binding EF hands (Hohenester et al. 1996, 1997; Sasaki et al. 1998). The E-C domain of SPARC contains the cell-binding domain as well as the collagen-binding region and is the most conserved among SPARC homologs expressed in *C. elegans*, *Drosophila*, and mammals (Sasaki et al. 1998). The capacity of SPARC to bind fibrillar collagens such as types I, III, and V in addition to type IV, is dependent upon the triple helical conformation of collagen and suggests that SPARC might influence ECM composition in both connective tissue (rich in fibrillar collagens I, III, and V) and basal membranes (where collagen IV is a prominent component) (Mayer et al. 1991; Bradshaw and Sage 2001).

The affinity of SPARC binding to collagen is in the 10^{-7} M range but can differ according to the cellular source of SPARC due to differential post-translational modification (Sasaki et al. 1997). The collagen binding sites of SPARC to collagen types I, II, and III have been mapped by rotary shadowing and to collagen I by atomic force microscopy (Wang et al. 2005a; Giudici et al. 2008). Rotary shadowing, used by Giudici et al. (2008), mapped the major SPARC binding site on procollagen I to a location approximately 180 nm from the C-terminus of types I, II, and III. A lesser site located near the mammalian collagenase cleavage site in types I and II was mapped to a region 60–100 nm from the C-terminus. In contrast, the prominent site mapped by Wang et al. (Wang et al. 2005a) using atomic force microscopy was 87.5–125 nm from the C-terminus of procollagen I with a lesser site located 237.5–262.5 nm from the C-terminus (Giudici et al. 2008). The discrepancy in the two studies might arise from the sources of recombinant SPARC (rSPARC) used to perform the binding assays. rSPARC produced by mammalian cells was used to perform rotary shadowing analysis whereas rSPARC produced in insect cells was used in the atomic force microscopy studies. Differential glycosylation performed by insect versus mammalian cells is one possible explanation for differences in SPARC binding to collagen in the two studies. Similarly, the source of procollagen I differed as Wang et al. used a recombinant homotrimer of procollagen I (3 subunits of collagen α1(I)) whereas Guidici et al. performed rotary shadowing with heterotrimeric procollagen I [2 collagen α(1)I and 1 collagen α(2) I] produced by human dermal fibroblasts.

Guidici et al. went on to show that a synthetic triple-helical peptide from collagen III, GPOGPSGPRGQOGVMGFOGPKGNDGAO (O, 4-hydroxyproline) bound to SPARC with an affinity comparable to that of recombinant procollagen III (Giudici et al. 2008). Interestingly, the region of collagen shown to be bound by SPARC using rotary shadowing overlapped previously mapped binding domains of the collagen receptor DDR2, in addition to von Willebrand Factor (Agarwal et al. 2002; Konitsiotis et al. 2008). Alternately, the sites mapped by atomic force microscopy overlap a subset of those proposed to bind the collagen specific integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$ (Xu et al. 2000; Zhang et al. 2003). Hence, SPARC bound to collagens might limit cell surface receptor interaction, either mediated by DDR2 and/or integrins, with fibrillar collagens in the pericellular milieu.

Digestion of SPARC by several matrix metalloproteinases (MMP) increased the affinity of SPARC for collagen types (Sasaki et al. 1997). Removal of helix αC in the E-C domain increased SPARC affinity for collagens 7–20 fold (Sasaki et al. 1998). In addition, differential glycosylation of SPARC affected collagen-binding affinity. SPARC appears to have a single N-glycosylation site at Asn99 that is highly conserved (Brekken and Sage 2001). SPARC purified from platelets migrated more slowly in SDS-PAGE analysis than SPARC from bone due to increased complexity of the platelet oligosaccharide modification versus that of bone-derived SPARC (Hughes et al. 1987; Kelm and Mann 1991). The difference in glycosylation influenced SPARC binding to collagen as bone SPARC bound with higher affinity to collagen I, III, and V than did SPARC from platelets. Removal of oligosaccharides from both forms of SPARC increased collagen binding to collagen V and abrogated the differences in collagen binding (Kelm and Mann 1991). Therefore, the binding of SPARC to collagen in the extracellular environment is subject to modulation by cell type-specific post-translational modification and by MMP activity.

The expression of SPARC is robust during development and differentiation of most mammalian tissues (Bradshaw and Sage 2001). However, the expression of SPARC declines in most organs as organisms mature. Bones, gut epithelia, and other tissues with high ECM turnover, retain SPARC expression into adulthood (Bradshaw and Sage 2001). Increased SPARC production is associated with adult tissue remodeling events such as wound healing and those that involve fibrotic deposition of collagen such as in liver cirrhosis and in individuals with scleroderma (Reed et al. 1993; Frizell et al. 1995; Zhou et al. 2003). As stated previously, SPARC expression in transformed cells or in stromal cells adjacent to tumors is dependent upon the type of tumor and its tissue of origin (Framson and Sage 2004).

The capacity of SPARC to bind to a number of different types of collagen, including fibrillar collagens I, III, V, and to basal membrane collagen IV, and its high expression in tissues undergoing active remodeling implicates SPARC in the process of ECM assembly and turnover.

ECM Assembly

Evidence that SPARC contributes to the formation and/or stability of a functional basal lamina is suggested from studies in *C. elegans* and *Drosophila* in which disruption of SPARC expression gave rise to lethal mutations (Fitzgerald and Schwarzbauer 1998; Martinek et al. 2002, 2008). Whereas fibrillar collagens homologous to mammalian collagen types I-III are not expressed in worms and flies, collagen IV, a primary constituent of basal membranes, is present throughout each organism. In *C. elegans*, exogenously expressed SPARC-GFP was localized to basal lamina in several tissues (Fitzgerald and Schwarzbauer 1998). In *Drosophila*, localization of SPARC protein closely followed that of collagen IV (Martinek et al. 2002).

In flies with mutated expression of collagen IV, SPARC protein was significantly decreased in basal laminae of certain internal organs. Abrogation of SPARC expression in *Drosophila* gave rise to disrupted basal laminae similar to that exhibited by collagen IV mutants (Martinek et al. 2008). The function of SPARC in invertebrates appears to be essential for patent basal laminae formation and is implicated in either production and/or assembly of collagen IV into extracellular structures.

Although abrogation of SPARC expression in mice does not give rise to embryonic lethality, SPARC-null mice display a range of phenotypes, the basis of which also appear to reside in disruption of ECM organization (Bradshaw and Sage 2001). The existence of a family of SPARC-related proteins, including hevin, SMOC-1 and 2, and testican, might provide some compensation for the absence of SPARC in mice (Soderling et al. 1997; Roll et al. 2006; Liu et al. 2008). One of the first phenotypes described in SPARC-null mice was premature cataractogenesis. In two independently generated SPARC-null mice, cataract formation in mice of 4 weeks and younger was observed (Gilmour et al. 1998; Norose et al. 1998). Yan et al., went on to show that the basement membrane surrounding the lens epithelial cells exhibited disorganized collagen IV and laminin compared with those of WT mice (Yan et al. 2002). Whereas the plasma membrane of wild type lens epithelial cells formed a sharp demarcation between cells and ECM, the plasma membrane produced by SPARC-null lens epithelial cells was invaginated with integrin β_1-positive protrusions extending into the disorganized basement membrane. In the absence of SPARC, lens epithelial cells were not able to deposit and correctly assemble a patent basement membrane so that fluid balance across this ECM was not maintained (Yan et al. 2003). Yan et al. hypothesized that the increased porosity of the SPARC-null ECM, demonstrated by toluidene blue penetrance, gave rise to cataract formation in the SPARC-null mice. SPARC-null lens epithelial cells also demonstrated changes in adhesion and integrin expression versus wild type cells (Weaver et al. 2006).

In addition to aberrations in lens basement membrane, SPARC-null mice also exhibited deficiencies in connective tissue. Reduced levels of collagen I were reported in skin, adipose, heart, and bones of SPARC-null mice (Bradshaw et al. 2003a, b; Delany et al. 2003). The collagen fibrils in the skin of the null mice displayed significant decreases in diameter and a uniformity of size in comparison to those of wild type mice. The decrease in collagen content has been linked to reduced tensile strength of the skin and to accelerated closure of full-thickness wounds (Bradshaw et al. 2003b). Improved healing of dermal wounds was attributed to an increase in skin contractility brought about by decreases in the collagenous ECM generated in the absence of SPARC (Bradshaw et al. 2002). As collagen gels of lesser collagen concentration were contracted by fibroblasts more quickly than those of higher collagen concentration, an extrapolation was made that the dermis of SPARC-null mice, with lesser amounts of collagen than wild type, was more susceptible to contraction by cells in the wound.

Foreign materials, when implanted into mice, are encapsulated by resident cells to “wall off” the exogenous material. A similar event has been observed in some solid tumors. Formation of a collagen capsule is accompanied by increased expression of SPARC. In SPARC-null mice, a decrease in the dimensions of the collagen capsule

synthesized in response to such an implanted foreign material was evident versus that formed in WT mice (Puolakkainen et al. 2003). The collagen surrounding the foreign material in SPARC-null animals exhibited more immature fibers that were smaller and more uniform in diameter than those that were seen in samples from WT mice. Collagen fibrils formed by adult dermal fibroblasts in response to an implant retained the phenotypic changes noted in collagen fibrils formed during development in SPARC-null skin (Puolakkainen et al. 2003). Hence, SPARC most likely serves a basic function in the regulation of collagen fibril assembly at least by dermal fibroblasts.

A decrease in collagen deposition was also reported in bleomycin-induced injury in the lungs of SPARC-null mice and, in an animal model of diabetic nephropathy, diminished fibrosis in the kidney of SPARC-null mice treated with streptozocin was observed (Strandjord et al. 1999; Taneda et al. 2003). An increase in collagen production and SPARC expression was associated with both bleomycin-induced injury and in diabetic nephropathy; hence, the absence of SPARC was shown to have significant effects on collagen deposition in response to injury in adult lungs and kidney (Pichler et al. 1996).

SPARC production in some types of tumors was associated with changes in collagen I deposition as well. Lewis lung carcinoma cells injected subcutaneously into SPARC-null mice formed substantially larger tumors in comparison to wild type mice, with a reduction in the collagenous capsule surrounding the tumors in the SPARC-null mice (Brekken et al. 2003). Although the carcinoma cells expressed SPARC in vitro and in vivo, the host response to tumor progression was clearly influenced by the absence of SPARC expression by stromal cells. In addition, a decrease in the infiltration of macrophages was observed in tumors from SPARC-null mice in comparison to wild type mice (Brekken et al. 2003).

We observed that murine pancreatic cancer cells (Pan02, aka Panc02) injected into SPARC-null mice formed larger tumors versus those injected into wild type animals (Puolakkainen et al. 2004). The tumors from SPARC-null mice had decreases in associated ECM (Fig. 8.1) and decreases in macrophage recruitment and invasion, results similar to those found upon injection of Lewis Lung carcinoma cells into SPARC-null and wild type mice (Brekken et al. 2003). Furthermore, when Pan02 cells were implanted orthotopically into the pancreas of SPARC-null and wild type animals, the number of metastatic events was also increased in SPARC-null mice (Arnold et al. 2008). Interestingly, when Pan02 cells engineered to over-express matrix metalloproteinase (MMP)-9 were injected, growth of tumors continued to be enhanced in SPARC-null animals; however, the metastatic burden was decreased in both SPARC-null and wild type mice. Microvessel density was diminished in tumors formed in SPARC-null versus WT mice whereas forced expression of MMP-9 by tumor cells reversed the angiogenic decrease in SPARC-null mice (Arnold et al. 2008). These results suggest a complex interaction between MMP-9 and SPARC, which is a substrate for MMP cleavage. This interaction between a protease prominent in the tumor microenvironment (MMP-9) and an extracellular adaptor protein (SPARC) impacts many of the hallmarks of cancer including invasion and angiogenesis.

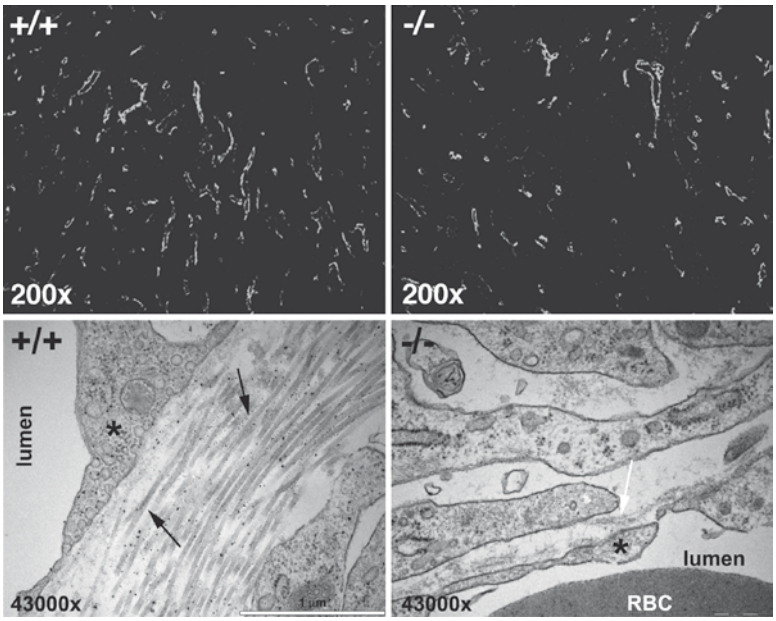

Fig. 8.1 Decreased ECM Deposition in Pan02 tumors grown in SPARC-null animals. *Upper panels*: Pan02 tumors grown in pancreas of WT (+/+) or SPARC-null (–/–) mice were harvested, snap frozen, and analyzed for collagen IV by immunohistochemistry. *Lower panels*: TEM analysis of orthotopic Pan02 tumors in WT (+/+) and SPARC-null (–/–) animals. Endothelial cells (*), red blood cells (RBC), and lumens of blood vessels are labeled. Note the reduction of ECM and collagen deposition under endothelium in tumors from SPARC –/– mice (*white arrows*) but prominent deposition of collagen fibrils in WT mice (*black arrows*). Total magnification and scale bar (1 μm) are shown

Glioblastomas are heterogeneous tumors that can exhibit diverse cellular regions including those involved in proliferation, angiogenesis, apoptosis, and invasion. SPARC is expressed highly in gliomas and promotes invasion of this cell type while inhibiting overall tumor growth. Yunker et al. demonstrated that overexpression of SPARC in transplanted glioma cells gave rise to increased collagen deposition associated with the SPARC-expressing tumors versus control (Yunker et al. 2008). The author postulated that SPARC expression reduced tumor growth through an increase in ECM deposition as well as a reduction in VEGF-induced angiogenesis. Here, increased SPARC expression by tumor cells was sufficient to drive increased collagen production and incorporation into the ECM and clearly influenced tumor progression.

Hence, there is convincing evidence from a variety of studies that SPARC is a critical factor in the synthesis, deposition, and/or stabilization of collagen I in the ECM of connective tissue and might contribute to inhibition of some tumors through increased production and deposition of a stromal or tumor ECM rich in collagen I.

Cellular Mechanisms of SPARC in ECM Assembly and Cell Signaling

The molecular basis of SPARC to influence collagen I deposition and fibrillogenesis has been investigated in primary fibroblasts from SPARC-null mice. In Rentz et al., SPARC-null dermal fibroblasts were shown to exhibit increased cell-associated procollagen I in comparison to wild type cells (Rentz et al. 2007). Procollagen I is processed to mature collagen I by removal of the C and N-terminal propeptides. Typically, procollagen I associated with fibroblast cell layers exists in four forms: procollagen I with N and C terminal propeptides, pC collagen I (C-propeptide attached, N-propeptide removed), pN collagen I (N-propeptide attached, C-propeptide removed), and collagen I (both propeptides removed). In the absence of SPARC, an increase in the proportion of total collagen I in the cell layer was present as enzymatically cleaved collagen I, with propeptides removed. As the propeptides of procollagen I are generally considered to be inhibitory to collagen fibril incorporation, these results suggested that SPARC influenced procollagen processing and enhanced production of fibril-forming collagen I. However, SPARC-null fibroblasts were inefficient in the incorporation of collagen I into a detergent-insoluble ECM. We proposed that SPARC bound to collagen I might diminish cell surface receptor interaction and promote assembly of collagen I into insoluble ECM. Without SPARC, increased collagen engagement by receptors might enhance collagen turnover either through phagocytosis and/or pericellular degradation pathways (McCulloch 2004; Lee et al. 2006).

One class of collagen receptors is the integrin family of ECM receptors (White et al. 2004). Interestingly, a function of SPARC in the regulation of integrin linked kinase (ILK), a down-stream component of the integrin signal transduction pathway, is emerging. Primary lung fibroblasts from SPARC-null mice exhibit reduced fibronectin-induced ILK activation. Associated with the decrease in ILK activity in cells lacking SPARC, a diminished capacity to generate stress-fibers on fibronectin - a critical step in fibronectin assembly - was observed (Barker et al. 2005). Expression of exogenous SPARC in SPARC-null cells restored the capacity of the lung fibroblasts to form fibronectin-induced stress fibers and fibronectin-dependent activation of ILK. As fibronectin is required for collagen I ECM assembly in vitro, inefficient fibronectin assembly in vivo, predicted from the absence of SPARC, might impair collagen I ECM deposition (Velling et al. 2002).

The capacity of SPARC to enhance ILK activation in lens epithelial cells promoted cell survival in vitro. Weaver et al. further showed that SPARC bound to ILK through an integrin β_1 complex (Weaver et al. 2008). The copper-binding region of SPARC located in the modular domain with follistatin homology was implicated in the interaction of SPARC with β_1 integrin and ILK.

In glioma cells, two studies examining SPARC and ILK activity have been reported. In one case, inhibition of SPARC expression by short interfering RNA (siRNA) reduced ILK activity coincident with reduced Akt and Focal Adhesion

Kinase (FAK) activation (Shi et al. 2007). Golembieski et al. reported that in glioma cells expressing SPARC tagged with green fluorescent protein (GFP), FAK and Akt activity were not changed in response to fibronectin versus control cells whereas total levels of ILK were increased in SPARC-expressing cells (Golembieski et al. 2008). In the latter study, heat shock protein (HSP) 27 was shown to be a major downstream effector of SPARC activity. HSP27 is a protein implicated in actin polymerization, cell contraction, and migration and, as such, is postulated to have potent effects on cell behavior.

In addition to the function of SPARC in the regulation of ECM-cell interaction, an active role of SPARC in the regulation of collagen fibrillogenesis is suggested from in vitro and in vivo results. In vitro, SPARC was shown to inhibit collagen fibrillogenesis using recombinant SPARC and purified collagen (Giudici et al. 2008). Addition of SPARC following initiation of fibrillogenesis had little effect on collagen fibril formation whereas near complete inhibition of fibril assembly occurred when SPARC was added during the fibril nucleation phase. Collagen fibrils in SPARC-null dermis, as mentioned above, displayed a distinct morphology versus those of WT and suggested that SPARC participates in collagen fibril assembly in vivo (Bradshaw et al. 2003b).

As extracellular SPARC is difficult to detect in tissues, presumably SPARC is not retained in collagen fibrils incorporated into insoluble ECM. The spatial and temporal regulation of procollagen processing is believed to be essential for collagen deposition. One possibility is that SPARC bound to procollagen I serves to reduce collagen binding to receptors in the pericellular environment following procollagen secretion while inhibiting fibril nucleation events. A number of collagen-binding proteoglycans including decorin, lumican, fibromodulin, and dermatopontin, influence collagen fibril diameter as well as incorporation of collagens type III and V into collagen I fibrils (Danielson et al. 1997; Ezura et al. 2000; Takeda et al. 2002). SPARC might promote appropriate processing of propeptides and perhaps association with other proteins incorporated into fibrils so that assembly of collagen I into tissue-specific ECMs is accomplished. In such a scenario, SPARC could be considered a type of extracellular chaperone for collagen that is released following initiation of fibrillogenesis.

Alternatively, a function for SPARC as an intracellular chaperone for collagen, similar to perhaps HSP47, has been proposed (Tasab et al. 2000; Martinek et al. 2007). Whereas HSP47 is required in the endoplasmic reticulum to assemble procollagen molecules, Martinek et al. put forth evidence supporting a function of SPARC to facilitate post-endoplasmic reticulum events in procollagen maturation that influence collagen fibrillogenesis (Martinek et al. 2008). Along these lines, SPARC exhibited classic chaperone activity in thermal aggregation assays carried out in vitro (Emerson et al. 2006). A distinct possibility is that SPARC has both intra and extracellular activities that influence collagen ECM assembly.

With regard to cell-ECM interaction, it is noteworthy that SPARC was shown to be a substrate of transglutaminase (Hohenadl et al. 1995). Transglutaminase cross-links ECM components and has been implicated in fibronectin assembly via interaction with integrin receptors at the plasma membrane (Telci et al. 2008).

The expression of SPARC is therefore predicted to influence transglutaminase-dependent events in the pericellular environment.

Cell Motility

Unlike many other matricellular proteins, SPARC does not contain a classical cell attachment, integrin-binding, RGD sequence. In fact, purified SPARC protein induces rounding when added to a number of different types of cell cultures, most notably endothelial cells (Lane and Sage 1990). Consequently, SPARC has been designated a counter-adhesive protein. Expression of SPARC has been suggested therefore to enhance migration of certain cell types that must disengage from existing ECM ties to initiate movement. In prostrate cancer cells, SPARC supported migration of metastatic cells to bone (De et al. 2003). The increase in migration generated in response to SPARC was dependent upon activation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$, RGD-binding integrins, and SPARC therefore did not mediate the migration directly. Furthermore, the SPARC-induced migration was supported by an autocrine vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway on the prostrate cancer cells.

HSP27, as described above, has been implicated in SPARC-mediated effects in glioma. As HSP27 mediates various cellular activities shown to be affected by SPARC expression such as motility and stress fiber formation, HSP27 as a downstream target of SPARC activity is plausible. SPARC expression by melanoma cells was associated with aggressive invasion whereas inhibition of SPARC expression diminished tumorgenicity of melanoma cells. Proteomic analysis of proteins affected by inhibition of SPARC expression in melanoma cells revealed an increase in HSP27 in cells with diminished SPARC expression (Sosa et al. 2007). N-Cadherin, a cell adhesion molecule, and clusterin, in contrast, were decreased in response to decreased SPARC expression in the melanoma cells.

Robert et al. reported that over-expression of SPARC in normal melanocytes resulted in a phenotypic shift to a fibroblast-like morphology (Robert et al. 2006). A decrease in two cell adhesion molecules, E-cadherin and P-cadherin expression, was associated with the mesenchymal transition induced by over-expression of SPARC. As loss of E-cadherin contributes to melanoma cell growth and invasion, SPARC might represent an important regulator of E-cadherin expression in melanoma cells.

Growth Factors and Cytokines

The capacity of SPARC to modulate the activity of several growth factors including basic fibroblast growth factor (bFGF), VEGF, platelet derived growth factor (PDGF), and transforming growth factor (TGF)-β1, has been established (Hasselaar and Sage 1992; Raines et al. 1992; Kupprion et al. 1998; Francki et al. 2004).

In the case of bFGF, PDGF, and VEGF, SPARC inhibited the action of these growth factors. SPARC was shown to bind directly to PDGF to diminish PDGF receptor activation whereas in the case of bFGF, SPARC was inhibitory for bFGF-induced migration of endothelial cells but did not bind directly to bFGF (Hasselaar and Sage 1992; Raines et al. 1992).

Similar to PDGF, SPARC was shown to bind directly to VEGF and diminished VEGF interaction with receptors on microvascular endothelial cells. As SPARC had previously been shown to decrease proliferation of endothelial cells in response to mitogenic stimuli, the function of SPARC as a negative regulator of angiogenesis was proposed (Funk and Sage 1991). In an in vivo model of angiogenesis, sponges implanted into the sub-dermal space of SPARC-null mice demonstrated an increased fibrovascular invasion in comparison to that of WT mice (Bradshaw et al. 2001). An increase in VEGF expression was noted in SPARC-null sponges and by SPARC-null dermal fibroblasts versus expression levels in WT mice and levels of VEGF produced by WT cells. The capacity of SPARC to reduce VEGF activity provided additional evidence to support an anti-angiogenic function of SPARC (Nozaki et al. 2006).

In glioblastomas, increased expression of SPARC decreased VEGF expression in part due to reduced levels of mRNA encoding VEGF 165 (Yunker et al. 2008). Likewise, purified SPARC protein inhibited angiogenesis in neuroblastoma xenografts inoculated to athymic nude mice (Chlenski et al. 2004). Chlenski et al. mapped the domain of SPARC responsible for inhibition of bFGF-induced migration of endothelial cells using a matrigel plug containing neuroblastoma cells delivered to athymic nude mice. Cysteine-linked peptides associated with distinct regions of SPARC were used to isolate anti-angiogenic activity. Peptide FS-E (representing amino acids 55–76) within the follistatin domain of SPARC was found to confer significant and specific inhibition of microvessel density (Chlenski et al. 2004), although within the follistatin domain, FS-E represents a distinct site from that found to be responsible for ILK activation mapped by Weaver et al. (Weaver et al. 2008).

In a mouse model of ovarian cancer, host-derived SPARC was shown to be an important contributor to cancer dissemination and lethality (Said and Motamed 2005). SPARC-null mice injected with syngeneic ID8 ovarian cancer cells developed greater peritoneal nodule dissemination and increased lethality in comparison to WT mice (Said and Motamed 2005). An increase in levels of VEGF was detected in SPARC-null ascitic fluid and was proposed to contribute to the increased invasion of the ID8 cells. SPARC was also shown to diminish basal and VEGF-induced activation of integrins in ID8 cells. Said et al. demonstrated that SPARC substantially reduced integrin activation and clustering, two critical aspects of integrin receptors required for cell movement and signal transduction events in ovarian cancer cells (Said et al. 2007).

SPARC activity has also been implicated in the regulation of TGF-β1. Mesangial cells isolated from SPARC-null mice were found to synthesize decreased amounts of collagen I and TGF-β1 in vitro (Francki et al. 1999). Addition of rSPARC restored collagen I and TGF-β1 expression to that approximating the level produced

by WT cells. Francki et al. showed that SPARC appeared to influence TGF-β1 activity through an interaction with the TGF-β1/receptor II (TGFβRII) complex that was dependent upon TGF-β1 bound to receptor II (Francki et al. 2004). Schiemann et al. also found SPARC to influence TGF-β1 signaling pathways in epithelial cells (Schiemann et al. 2003).

Adenocarcinoma of the pancreas is a highly desmoplastic disease and is also frequently associated with mutations that effect the TGFβ pathway (Truty and Urrutia 2007). For instance, deletion of SMAD4 or mutation of TGFβRII in tumor cells occurs in greater than 50% of cases of pancreatic adenocarcinoma. We found recently that there is a significant increase in the level of active TGFβ1 in pancreatic tumors (Pan02) grown in SPARC-null mice, which corresponded to a more aggressive phenotype of these tumors in the absence of host-derived SPARC. The change in phenotype of the Pan02 tumors, which was SMAD4 null, might reflect an increase in TGF-β1 driven epithelial to mesenchymal transition or exacerbate known immune-suppressive effects of TGF-β1 in the tumor.

Immune System

SPARC activity has been shown to contribute significantly to inflammatory mediators particularly in animal models of tumor growth and invasion. A familiar theme with SPARC activity in cancer progression also held true with regard to immune response; the source of SPARC expression either by host stromal cells or by transformed tumor cells seemed to be an important contributor to the outcome (Prada et al. 2007; Haber et al. 2008).

For example, melanoma cells with suppressed SPARC expression injected into nude mice resulted in increased polymorphonuclear leukocyte (PMN) recruitment and abrogated tumor growth in comparison to tumors generated from melanoma cells with high SPARC expression (Alvarez et al. 2005). In vitro, melanoma cells with reduced SPARC expression induced PMN migration and antimelanoma cytotoxic activity whereas addition of rSPARC counteracted these effects. Seemingly, SPARC expression by melanoma cells decreases PMN recruitment, a first-line of defense in the immune surveillance against cancer, so that an inhibition of SPARC expression in melanoma cells enhanced the capacity of PMNs to combat tumor growth.

Conversely, SPARC expression by leukocytes might be an important factor in the recruitment of leukocytes from the vasculature. SPARC was identified as a counter ligand for the cell adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells (Kelly et al. 2007). SPARC expressed by leukocytes interacted with VCAM-1 to initiate actin rearrangement in endothelial cells which led to the generation of intercellular gaps that allowed leukocyte transmigration through the endothelial monolayers, a process referred to as diapedesis. SPARC-null mice were shown to exhibit abnormalities in leukocyte recruitment to inflamed peritoneum (Kelly et al. 2007). Whereas SPARC expression by melanoma

cells was inhibitory to PMN recruitment, SPARC expression by leukocytes was a critical step in inflammatory cell recruitment. These results highlight the complexity and potential pitfalls of targeting SPARC activity in cancer treatment.

SPARC-null mice have now been shown to exhibit aberrant splenic morphology - a demonstration that SPARC is a critical factor in the development of a competent immune system at least in mice. The spleens of SPARC-null mice were larger and had increased amounts of white pulp, hyperproliferative B cells in germinal centers, and marginal zones that were decreased compared with those of WT mice (Rempel et al. 2007). SPARC-null mice failed to generate an immune response after administration of lipopolysaccharide to the footpad whereas WT mice treated identically exhibited significant swelling. Although Rempel et al. stated that an increase in infections was observed in mouse colonies under their care - particularly in older mice - in >10 years of maintaining a SPARC-null colony, no differences in infection rates or progression to infection of superficial wounds between SPARC-null and WT mice have been noted at other sites. In addition, significant differences in life span of SPARC-null versus WT mice have not been reported.

In agreement with results that demonstrated an impaired immune response in SPARC-null mice, tumors induced with either Lewis lung carcinoma cells or pancreatic Pan02 adenocarcinoma cells demonstrated reduced macrophage recruitment in SPARC-null versus WT mice (Brekken et al. 2003; Puolakkainen et al. 2004). Therefore, SPARC has been proposed as an important mediator of macrophage recruitment. In the event that SPARC actively recruits macrophages, a process for regulating extracellular SPARC by macrophages might serve as an effective feedback control mechanism. Stabilin-1 is a scavenger receptor expressed on alternatively activated macrophages and sinusoidal endothelial cells known to internalize and degrade acetylated low density lipid. Stabilin-1 was also shown to be a receptor for SPARC (Kzhyshkowska et al. 2006). Upon endocytosis of SPARC through stabilin-1, SPARC was targeted for lysosomal degradation. Hence, expression of stabilin-1 by macrophages enabled these inflammatory cells to clear SPARC from the extracellular milieu and thus reduced SPARC concentration and perhaps further macrophage recruitment. That SPARC is important for immune cell function is supported in part by two studies from Sangaletti et al. (Sangaletti et al. 2003; Sangaletti et al. 2005). In the first report, the authors demonstrated that SPARC produced by infiltrating leukocytes was instrumental in appropriate deposition of collagen IV in tumors from mammary carcinoma (Sangaletti et al. 2003). The second study found that dendritic cell migration and T-cell priming was enhanced in the absence of host SPARC (Sangaletti et al. 2005).

Conclusions

Significant changes in levels of mRNA encoding SPARC or SPARC protein are frequently revealed in studies that analyze expression profiles of tumors and transformed cell lines versus non-cancerous tissue and cells. SPARC has been shown to

act as a modulator of cell adhesion, proliferation, survival, growth factor activity, and ECM assembly. As integrin receptors have also been implicated in each of the cellular processes mentioned above, the concept that SPARC regulates cell-ECM interaction through modulation of integrin binding is provocative. Integrin signaling is complex and contextual. For example, in endothelial cells, trans-inhibition of RGD binding integrins was observed upon engagement of collagen-binding integrins (Orr et al. 2006). Hence, one type of integrin receptor has the capacity to regulate function and down-stream signaling pathways of other types of integrin receptors within the same cell. In view of the fact that SPARC is generally classified as a counter-adhesive protein and integrins are best known as mediators of adhesion, one might anticipate that SPARC bound to a β1 integrin complex decreases integrin activity. However, a scenario in which SPARC influences a specific subset of integrin receptors might invoke a layer of complexity to cell adhesion/ECM assembly pathways that are yet to be fully characterized.

Convincing results generated from SPARC-null mice and tumor studies have established SPARC as a significant participant in collagen deposition and assembly in the ECM. As tumor biologists have long appreciated that different types of tumors possess different ECM signatures, perhaps that SPARC has diverse roles in different tumors is not surprising. Future experiments that define the molecular mechanisms and binding partners of SPARC in tumors will contribute enormously to future strategies to exploit the promise of manipulating host response to control tumor progression and invasion.

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Chapter 9
Proteoglycans and Cancer

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Abstract Proteoglycans are ubiquitous molecules composed of glycosaminoglycan chains attached covalently to core proteins. Proteoglycans perform a myriad of functions and participate in regulating tumor cell growth, survival, adhesion, metastasis and angiogenesis. These functions are largely mediated through interactions between their charged glycosaminoglycan chains and effector proteins such as growth factors, cytokines and chemokines. In addition, emerging data is revealing that the core proteins of proteoglycans can also form complexes with other proteins such as integrins and regulate their signaling. Because proteoglycans are at the crossroads of many signaling events, they are currently being extensively investigated for their potential as therapeutic targets for cancer. This review focuses on the expression, structure and function of proteoglycans in cancer and provides an overview of the field as well as specific examples of how these diverse molecules regulate tumor behavior.

Keywords Proteoglycan • glycosaminoglycan • cancer • growth factors • therapy

Introduction

Proteoglycans represent a wide range of molecules composed of core proteins having covalently attached unbranched glycosaminoglycan chains. Proteoglycans are abundant, and virtually all cells express multiple types of proteoglycans. Their functions are attributed largely to binding of their glycosaminoglycan chains to various regulatory proteins (e.g., growth factors, chemokines), but emerging evidence clearly identifies core protein domains which also serve important functions.

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_9,

Owing to their regulated expression, their broad range of functional activities and the fact that they are found within cells, at the cell surface and throughout the extracellular matrix, these molecules are uniquely endowed with the capacity to regulate cell behaviors ranging from organogenesis to apoptosis. The purpose of this review is to provide a broad overview of roles of the various proteoglycans in cancer. These complex molecules play important roles in all steps of cancer progression including viral infection, growth, alterations of cell adhesion, motility and spreading, cell migration, tumor stroma formation, angiogenesis, and metastasis. Proteoglycans are classified by the type of glycosaminoglycan chains that they bear (heparan sulfate, chondroitin sulfate, or keratan sulfate). Additionally, there is hyaluronic acid (HA), a glycosaminoglycan that lacks a core protein but whose expression is widespread and abundant and important in regulating cancer. In this review, we have selected several examples from each major proteoglycan type for the discussion of their roles in tumor biology. For a more comprehensive overview of each proteoglycan type, we refer the reader to the numerous reviews cited within the text. Overall, what has become clear is that the functional affects of proteoglycans on cancer can range from stimulatory to inhibitory, dependent on both core protein and glycosaminoglycan type and structure, the type and stage of cancer and the localization of the tumor. Although the biology of proteoglycans in cancer is complex, the understanding of their structure and function that has evolved over the last two decades has opened the door for new therapeutic opportunities now being tested.

Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) contain one or multiple heparan sulfate (HS) chains composed of alternating *N*-acetylated or *N*-sulfated glucosamine and uronic acid (iduronic acid or glucuronic acid). These chains can be sulfated at numerous sites to give rise to the rich structural and functional diversity that characterizes HS (Esko and Selleck 2002). The core proteins of HSPGs to a large extent dictate tissue distribution and localization (i.e., cell surface or extracellular matrix (ECM)) and modification of core proteins by enzymatic cleavage at the cell surface can transform a cell surface HSPG into a ECM-bound or soluble molecule with important functional consequences (Bernfield et al. 1999). HSPGs have a broad range of effects in cancer largely because of their ability to bind to and regulate many effector molecules such as heparin-binding growth factors, chemokines, and angiogenic factors.

The *syndecans* are a four-member family of transmembrane proteoglycans that bear predominantly heparan sulfate chains (Beauvais and Rapraeger 2004; Bernfield et al. 1992; Couchman 2003). The core protein consists of a short intracellular domain, a highly conserved transmembrane domain, and an ectodomain that is divergent in amino acid sequence among the four family members (Bernfield et al. 1992). The syndecans are expressed virtually ubiquitously throughout embryonic

development and into adulthood, though patterns of expression differ between the individual syndecans. Often, their expression and HS structure are altered in response to certain pathophysiological stimuli, and this in turn can influence disease progression including tumor onset, progression and metastasis (Beauvais and Rapraeger 2004; Fears and Woods 2006; Sanderson 2001). The syndecans function both on the cell surface and when the intact syndecan ectodomain is shed into the extracellular milieu. On the cell surface, the core protein and HS chains of syndecans play a number of roles in promoting cell–cell and cell–ECM interactions and in regulating cell survival, adhesion, and migration (Beauvais and Rapraeger 2004; Couchman 2003; Couchman et al. 2001; Sanderson 2001). Furthermore, syndecans, via their heparan sulfate chains, bind an array of molecules, thereby, facilitating downstream signaling events (Perrimon and Bernfield 2001; Tkachenko et al. 2005). Over the past decade, a number of studies have been performed, analyzing the role of individual members of the syndecan family in regulating the behavior of tumors. Syndecan-1 is highly expressed at the basolateral membrane of simple epithelial cells, where it is believed to promote cell adhesion to the ECM and regulate cell shape by interacting with the actin cytoskeleton (Bernfield et al. 1992). Often, transformed epithelial cells lose expression of syndecan-1 along with E-cadherin which together appear to be key steps in regulating epithelial to mesenchymal transition (Kato et al. 1995; Sun et al. 1998). Hence, downregulation or loss of syndecan-1 in most epithelial tumors, such as squamous cell and esophageal carcinomas, cervical, lung, and laryngeal cancers, is associated with accelerated tumor progression and reduced patient survival (Inki et al. 1994a, b; Klatka 2002; Mikami et al. 2001; Nackaerts et al. 1997; Pulkkinen et al. 1997). Conversely, increased syndecan-1 expression has been observed in breast, ovarian, pancreatic, and endometrial tumors (Barbareschi et al. 2003; Conejo et al. 2000; Davies et al. 2004; Hasengaowa et al. 2005), as well as some hematologic malignancies (multiple myeloma, some leukemias, and in Hodgkin's disease (Bayer-Garner et al. 2001; Carbone et al. 1998; Chilosi et al. 1999; Jilani et al. 2008; Ridley et al. 1993; Sebestyen et al. 1999, 1997; Seftalioglu et al. 2003; Seidel et al. 2000)), where its overexpression is associated with an increased tumor invasiveness and poor prognosis. For example, in myeloma, expression of syndecan-1 regulates cell growth, adhesion, and migration (Sanderson and Borset 2002; Sanderson et al. 2004). Myeloma cell lines expressing low levels of syndecan-1 grow poorly in vivo, while their counterparts expressing elevated levels of syndecan-1 grow very aggressively and form large, highly vascular tumors (Yang et al. 2007). Although much of the function of syndecan-1 in cancer is attributed to its heparan sulfate chains, in breast cancer syndecan-1 is a key regulator of $\alpha_v\beta_3$ integrin signaling activity, via an interaction between the syndecan-1 core protein and the integrin (Beauvais et al. 2004). These divergent roles of syndecan-1 imply tissue- and tumor-type specificity of this HSPG.

Interestingly, expression of syndecan-1 by tumor stromal cells may also be important in regulating tumor behavior. For example, in breast cancer, syndecan-1 is found at relatively high levels in the stroma of some patients and one study indicates that this correlates with poor prognosis (Stanley et al. 1999; Tsanou et al. 2004). In support of this notion, in vivo studies have revealed that syndecan-1 shed

by stromal cells can promote breast tumor growth and angiogenesis (Maeda et al. 2006; Su et al. 2007). A functional role for shed syndecan-1 is not limited to that produced by stromal cells. In myeloma, tumor cells expressing high levels of soluble syndecan-1 exhibit accelerated invasion in vitro and form large, aggressive tumors in vivo. These tumors are highly vascular and show increased metastatic propensity (Yang et al. 2002). Moreover, immunohistochemical studies show that high levels of shed syndecan-1 can accumulate in the bone marrow of myeloma patients where it may sequester growth factors that help promote tumor growth and perhaps even tumor relapse following therapeutic intervention (Bayer-Garner et al. 2001).

In addition to its role in regulating tumor growth and metastasis, in some tumors syndecan-1 may also be critical for initial steps leading to tumorigenesis. The best evidence for this lies in studies using syndecan-1 null mice. Wnt transgenic mice exhibit a high level of spontaneous breast tumor formation. However, when these mice are crossed with syndecan-1 null mice, tumor formation is dramatically reduced (Alexander et al. 2000). Further studies with this model and with 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumorigenesis indicate that syndecan-1 may be required for the activation of tumor precursor cells drawn from the stem/progenitor cell compartment (Liu et al. 2004; McDermott et al. 2007).

Like syndecan-1, syndecan-2 also plays an important role in mediating cell shape and adhesion. Possessing a cytoplasmic domain that interacts with the ezrin, radixin, and moesin (ERM) protein family, syndecan-2 regulates organization of the actin cytoskeleton and activation of focal adhesion kinases (Granes et al. 2000). These interactions create a physical connection between the cytoskeleton and the ECM, thereby regulating migratory characteristics of differentiated cells. Co-dimerization of syndecan-2 with $\alpha_5\beta_1$ integrin in Lewis lung carcinoma cells induces actin stress fiber formation (Munesue et al. 2002). A highly metastatic variant of Lewis lung carcinoma cells exhibits low syndecan-2 expression, and recent studies revealed that syndecan-2 acts to suppress activation of matrix metalloproteinase-2, thus suggesting that syndecan-2 inhibits metastasis of these cells (Munesue et al. 2007). Similarly, osteosarcoma cells that overexpress syndecan-2 are reduced in their migration and also exhibit enhanced apoptosis in vitro as compared to those cells expressing low levels of syndecan-2 (Modrowski et al. 2005; Orosco et al. 2007). In contrast, expression of syndecan-2 in colon cancer promotes cell adhesion, spreading and proliferation indicating that it is important in colon cancer survival and perhaps initial tumorigenesis and metastasis (Contreras et al. 2001; Han et al. 2004; Park et al. 2002). Together, these findings indicate that syndecan-2 can play important roles in the behavior of tumors dependent on the tumor cell type, as well as microenvironmental factors.

In regard to the tumor microenvironment, syndecan-2 is highly expressed on endothelial cells in a murine glioma model and its inhibition leads to reduced tubulogenesis in vitro, consistent with its role in cell adhesion and migration (Fears et al. 2006). In this same model, addition of exogenous matrix metalloproteinases or pro-angiogenic factors (e.g., VEGF, FGF2) leads to increased syndecan-2 shedding and promotion of endothelial cell migration on matrigel. Thus, like

syndecan-1, in some tumors syndecan-2 may play an important role in the positive regulation of tumor angiogenesis to help promote tumor growth and progression.

Syndecan-4 is expressed endogenously by breast cancer cells, where it mediates their adhesion to and spreading on the ECM (Beauvais and Rapraeger 2003). Similar to syndecan-1, syndecan-4 forms FGF-2/syndecan-4/FGFR-1 complexes, promoting pro-angiogenic signaling events through the protein kinase C_{α} (PKC_{α}) pathway (Mundhenke et al. 2002). Also, enhanced expression of syndecan-4 has antimigratory effects on both ARH-77 lymphoid and CHO-K1 cells in vitro (Liu et al. 1998; Longley et al. 1999). Within the tumor microenvironment, glioblastoma and breast carcinoma stromal cells express high levels of tenascin-C, an adhesion-modulatory ECM protein that disrupts syndecan-4/fibronectin interactions, thereby decreasing focal adhesions present on the tumor cells (Huang et al. 2001; Woods and Couchman 1988). This disruption of tumor cell adhesion leads to increased tumor cell proliferation and may contribute to poor survival of patients bearing these tumors. Thus, regulation of cell adhesion via syndecan-4 is critical in controlling both the migration and proliferation of some tumor types.

Glypicans are HSPGs characterized by their interaction with the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Like syndecans, glypicans are expressed in cell-, tissue-, and development-specific patterns, interact with a multitude of extracellular matrix proteins, including chemokines, growth factors/morphogens and their receptors, and regulate cell-signaling events during morphogenesis, adult physiology, and carcinogenesis (Filmus et al. 2008; Hacker et al. 2005; Lin 2004). Six members of the glypican gene family have been identified in mammals. Members of the glypican family have core proteins of ~60–70 kDa and these proteins exhibit a pattern of 14 conserved cysteine residues. All glypican C-terminal regions have attachment sites for heparan sulfate chains, as well as the signal sequence for the GPI anchor. Although much of glypican function is mediated through heparan sulfate chains, emerging evidence has demonstrated their core proteins also serve important functions independent of the HS chains. For example, mutant forms of glypicans lacking heparan sulfate chains can stimulate apoptosis and support Wnt signaling (Capurro et al. 2005; De Cat et al. 2003; Gonzalez et al. 1998).

Glypican-1 is reportedly upregulated in pancreatic and breast cancers and is also frequently overexpressed in human gliomas where it enhances FGF2 signaling (Kleeff et al. 1998; Matsuda et al. 2001; Su et al. 2006). Downregulation of glypican-1 in the human pancreatic cancer cell line PANC-1 prolonged doubling times and decreased anchorage-independent growth in vitro and tumor growth, angiogenesis, and metastasis in vivo (Aikawa et al. 2008; Kleeff et al. 1999). Similarly, glypican-5 increases tumor proliferation in rhabdomyosarcomas by potentiating the action of FGF2, hepatocyte growth factor (HGF), and Wnt1A (Matsuda et al. 2001).

Among the human malignancies, glypican-3 has been studied most extensively (Capurro et al. 2005; Filmus 2001; Filmus et al. 2008; Motomura et al. 2006; Nakatsura et al. 2004a; Nakatsura and Nishimura 2005; Wichert et al. 2004). The expression and function of glypican-3 is apparently related to tumor type and

cell of origin and thus can aid in the diagnosis and prognosis of specific forms of cancer. For example, glypican-3 is prevalent in lung squamous cell carcinoma but rarely found in lung adenomas (Aviel-Ronen et al. 2008; Kim et al. 2003). In patients with ovarian carcinomas, serous and endometroid carcinomas rarely showed positivity for glypican-3, whereas more than 60% of ovarian clear-cell carcinomas expressed this marker (Stadlmann et al. 2007). In cancer cell lines that originated from glypican-3-positive adult tissues, such as breast (Xiang et al. 2001), lung (Kim et al. 2003) ovary (Lin et al. 1999), or mesothelium (Murthy et al. 2000), its expression is often downregulated during tumor progression. On the other hand, in neoplasms that originated from tissues that express glypican-3 only in the embryo, its expression reappears during malignant transformation as an oncofetal protein (Ota et al. 2006; Yamauchi et al. 2005; Zhu et al. 2001). For example, glypican-3 is expressed in embryonic liver, absent in adult liver but is overexpressed in hepatocellular carcinomas (HCC) (Jakubovic and Jothy 2007; Nakatsura and Nishimura 2005; Zhu et al. 2001). In addition, soluble glypican-3 can also be detected in the serum of 40–53% of HCC patients but is not present in the serum of healthy individuals (Capurro et al. 2003; Nakatsura et al. 2003). Functionally, glypican-3 has been shown to promote Wnt signaling and also to promote HCC oncogenesis via the IGF signaling pathway (Cheng et al. 2008).

Initial studies have revealed that in animal models glypican-3 is an effective immunotherapeutic target in the treatment of glypican-3-overexpressing tumors. For example, in HCC transgenic mice, preclinical studies have shown that antigenic peptide (144–152 of glypican-3) induces peptide-reactive cytotoxic T lymphocytes that can reduce tumor mass without inducing autoimmunity (Komori et al. 2006). In a mouse model of colorectal carcinoma, a protective effect was achieved by the transfer of glypican-3 peptide-pulsed dendritic cells or glypican-3-reactive cytotoxic T lymphocytes (Nakatsura et al. 2004b). Glypican-3 was also shown to be an effective target antigen for antimelanoma immunotherapy (Motomura et al. 2006). In a different approach, phosphoinositide-specific phospholipase-C (PI-PLC), which cleaves the GPI anchor of glypican-1, inhibited the mitogenic responses of PANC-1 and COLO-357 pancreatic cancer cells to FGF-2 and heparin binding EGF, two growth factors that are commonly overexpressed in pancreatic cancer (Kleeff et al. 1998).

Perlecan is the predominant HSPG found in the basement membrane and also present within the ECM. Perlecan is an unusual HSPG in that it has a large core protein (~470 kDa) and up to four heparan sulfate chains (Dolan et al. 1997; Friedrich et al. 1999). It plays important structural roles, particularly in the basement membrane, but also interacts with an array of factors such as FGF2, VEGF and PDGF through both core protein and HS interactions (Iozzo 2005). In addition, perlecan can act as a reservoir for heparin-binding growth factors where they await release by the action of extracellular enzymes such as heparanase (Whitelock et al. 1996). Regarding cancer, perlecan has been shown to regulate hedgehog signaling, a pathway important in prostate cancer growth and metastasis (Datta et al. 2006a, b; Park et al. 2003). Perlecan may also play a more general and widespread role in cancer by regulating angiogenesis. It acts as a scaffold for blood vessel formation

and knockdown of perlecan blocks angiogenesis and tumor growth in vivo of both colon carcinoma and melanoma cells (Sharma et al. 1998). Interestingly, endorepellin, a proteolytic fragment of perlecan derived from domain V of the core protein, inhibits endothelial cell migration, collagen-induced capillary morphogenesis and blood vessel growth in matrigel (Iozzo 2005). When recombinant endorepellin was administered to mice bearing squamous cell carcinoma or Lewis lung carcinoma, angiogenesis was inhibited resulting in diminished tumor growth (Bix et al. 2006). Thus, perlecan is strategically placed within the ECM to either promote or inhibit tumor growth depending on the available growth factors and on its degree of modification by enzymes.

Chondroitin Sulfate Proteoglycans

Proteoglycans containing chondroitin sulfate (CS) chains are ubiquitous components of extracellular matrices and cell surfaces, and play important roles in regulating tumor behavior (Wegrowski and Maquart 2006). CS is a glycosaminoglycan composed of repeating disaccharides of glucuronic acid (GlcA) and *N*-acetyl galactosamine (GalNAc) with sulfation at various sites. Chondroitin sulfate interacts with a wide variety of key molecules, such as growth factors, cytokines, chemokines, adhesion molecules, and lipoproteins, and these interactions regulate biological processes including signaling, cell differentiation cell–cell or cell–matrix interactions and morphogenesis. The ability of CS to regulate these processes is dependent on the presence of distinct sulfation motifs (Wegrowski and Maquart 2006). Several CS proteoglycans have been identified within the ECM including aggrecan, the major PG of cartilage, *versican* the common PG of noncartilaginous connective tissues and *brevican* and *neurocan* found mainly in brain. *Decorin* and *biglycan*, which belong to another class of proteoglycans, the small leucine-rich proteoglycans (SLRPs), are usually substituted by CS chains (Schaefer and Iozzo 2008). Several cytokines such as CSF-1 and M-CSF-1 have been bound on occasion to have CS chains and are thus considered part-time proteoglycans. Several other neural CSPGs like *phosphacan*/RPTPbeta/PTPzeta, *appican*, amyloid precursor like protein-2 and *neuroglycan* have been isolated from brain. Proteoglycans like perlecan, *bamacan* and syndecan can also bear CS chains.

Tumor stroma and tumor fibrotic tissue contain abnormally high concentrations of CSPGs, especially decorin and versican. The expression of decorin, the most abundant SLRP, is enhanced in pancreatic cancer, colorectal carcinoma, colon adenocarcinoma, basal cells carcinoma, melanoma and osteosarcoma (Hunzelmann et al. 1995; Ladanyi et al. 2001; Skandalis et al. 2006; Theocharis 2002; Tsara et al. 2002; Zafiropoulos and Tzanakakis 2008). Numerous benign tumors with strong fibrosing reaction are also characterized by enhanced decorin expression and deposition, especially in central, fibrosing zones (Berto et al. 2003). In contrast, in lung adenocarcinoma, hepatocellular carcinoma, breast carcinoma and ovarian carcinoma, decorin expression is low. This may be due to intracellular degradation of

newly synthesized molecules (Nash et al. 2002; Wegrowski and Maquart 2004). In some instances, decorin acts as a powerful tumor cell growth and migration inhibitor by both modulating tumor stroma deposition and cell signaling pathways (Csordas et al. 2000; Santra et al. 2000). This can occur via binding of the decorin core protein to growth factors and neutralizing their activity, thereby retarding tumor growth. Decorin also binds to ECM components including fibronectin, thrombospondin and several types of collagen and regulates the formation of collagen fibrillar network (Iozzo 1999). Decorin can also modulate tumor angiogenesis although its role is somewhat controversial. Decorin can be either pro-angiogenic or anti-angiogenic. The anti-angiogenic property of decorin is mediated by interfering with thrombospondin-1 and by suppressing the endogenous production of VEGF by tumor cells (Iozzo 1998). Much less is known about the expression of other SLRP family members, lumican (a keratan sulfate discussed below) and biglycan, but studies have shown that they participate in the arrest of cancer cell growth by upregulating p27 (a cell cycle inhibitor protein) and by downregulating cyclin expression (Vuillermoz et al. 2004; Weber et al. 2001).

Versican is the most abundant CSPG expressed in the stroma of most human cancers (Isogai et al. 1996; Wegrowski and Maquart 2006). The deposition of versican in the tumor stroma, particularly in the hyaluronic (HA) rich region, supports cancer cell growth, proliferation, migration and differentiation, all processes vital for tumor development (Theocharis 2002). Versican binds to ECM, principally HA and tenascin, leading to structural aggregation of tumor matrix and modulation of cellular attachment and motility (Suwiwat et al. 2004). The growth stimulatory effect of versican is thought to result from its destabilizing effect on focal cell contact, which then represses cell adhesion and promotes tumor growth. On the basis of the spliced domains of CS (CSα or CSβ), four isoforms of versican are present in different tissues: namely V0, V1, V2, and V3, with different biological consequences in tumorigenesis (Sheng et al. 2005). For example, the V1 isoform enhances cell proliferation, modulates cell cycle progression and protects cells from apoptosis, while the V2 isoform inhibits cell proliferation and does not contribute to apoptotic resistance of cells. In tumor formation, the extracellular environment becomes favorable for cell proliferation and survival by the increased expression of V1 isoform, but in contrast, the V2 isoform suppresses cell proliferation, as in the case of maintenance of mature tissue (Sheng et al. 2005). Overexpression of the *N*-terminal globular domain (G1) of versican augmented anchorage-independent cell growth in vitro and enhanced tumor cell growth in nude mice (Cattaruzza et al. 2004).

Many of the biological properties of matrix PGs reside in the specific structural characteristics of their GAG chains (ten Dam et al. 2007). Fine structural alterations of decorin and versican in tumor stroma directly influence the biology of cancer cells, contributing to cell growth and migration. In colon adenocarcinoma, decorin and versican accumulation is accompanied by alteration in the type of GAG bound on core proteins (Theocharis 2002). In normal colon, dermatan sulfate (DS) is the major type of GAG present on both versican and decorin, while in colon adenocar-

cinoma DS is replaced by CS chains. This alteration is also associated with the sulfation pattern of the GAG chain of both the PGs. While the 4-sulfated CS disaccharide units are predominant in normal colon, the tumor-associated versican and decorin contains more 6-sulfated and nonsulfated CS disaccharide units (Theocharis 2002; Theocharis et al. 2006). Laryngeal squamous cell carcinoma is also characterized by increased catabolism of the cartilage PG aggrecan, followed by a significant increase in matrix PGs, versican and decorin. But, in contrast to cancer development in gastrointestinal tract the CS chains in laryngeal tumor contain more 4-sulfated disaccharide units with a significant reduction of 6-sulfated units, when compared to normal tissue (Skandalis et al. 2006).

The cell surface CSPGs include *CD44* (certain isoforms), *betaglycan* (both of which can also contain heparan sulfate), and melanoma cell surface PG. In addition, syndecan-1, although predominantly bearing HS, has some CS as well. The minor or restricted full time CSPGs of non-neural origin include *endocan* and *thrombomodulin*, although thrombomodulin may be substituted with HS chains. Cell surface CSPGs are known to induce cell motility, collagen-dependent cell migration, cell invasion and anchorage-independent growth. CD44 is a transmembrane glycoprotein, and certain isoforms of CD44 are highly glycosylated and can be expressed as a CS or HS cell surface PG in a cell type specific fashion (Henke et al. 1996). CD44–hyaluronan interactions promote CD44-mediated cell movement, including metastasis (Lesley et al. 1993). Melanoma cell surface CD44-related CSPG also acts in concert with α2β1 integrin to mediate tumor cell migration and invasion on type IV collagen (Iida et al. 1996; Knutson et al. 1996). The melanoma CSPG is uniformly and abundantly expressed in most human melanoma lesions and has been implicated in tumor invasion (Faassen et al. 1992; Iida et al. 2001). Melanoma CSPG expression is an ominous prognostic factor in acral letiginous melanoma and in nonmelanoma tumors such as infantile acute myeloid leukemia. It is also expressed in different nervous tissue tumors (glioma, astrocytoma, sarcoma, or neuroblastoma), basal cell and breast carcinoma. Chondroitin sulfate modification of the core protein of melanoma CSPG has been linked to its ability to bind the heparin-binding domain of fibronectin. In association with the transmembrane matrix metalloproteinase MT3-MMP, melanoma CSPG facilitates invasion of melanoma cells into type I collagen gels as well as degradation of denatured type I collagen (Yang et al. 2004). Melanoma CSPG also acts as a coreceptor for α4ß1 integrin to modulate cell adhesion, spreading, and metastasis (Yang et al. 2004). Because of its expression in a large percentage of melanoma lesions and its restricted distribution in normal tissues, the high molecular weight-melanoma-associated antigen (HMW-MAA), has potential as an immunotherapeutic target for melanoma treatment (Campoli et al. 2004).

Betaglycan, a cell surface PG that bears both heparan sulfate and chondroitin sulfate chains is the most abundantly expressed TGF-ß receptor and binds all three TGF-ß isoforms with high affinity. The TGF-beta growth factor is a crucial molecule in tumor development and stromal reaction and the local concentration of this growth factor on betaglycan initiates cellular events that are advantageous

to cancer progression, including promoting migration, invasion, epithelial to mesenchymal transition, and angiogenesis (Bierie and Moses 2006). The role for betaglycan in cancer is less clear. Increased expression of betaglycan is found in higher grade lymphomas, while reduced expression of betaglycan is found associated with advanced stage neuroblastomas and ovarian carcinomas (Hempel et al. 2007; Iolascon et al. 2000; Woszczyk et al. 2004). Thrombomodulin, another cell-bound CSPG is expressed on the surface of many tumor cells and is predicted to be involved in the hemostatic shift that occurs locally around malignant tumors (Hanly et al. 2005). Thrombomodulin is expressed in several tumors of neural (brain) and epithelial origin including squamous cell carcinoma (oral, esophageal, and pulmonary) and adenocarcinoma (breast and colorectal), where a consistent finding is the correlation of loss of expression with advanced stage and poor prognosis. Reduction of thrombomodulin expression plays an important role in facilitating the metastatic process of several cancer types, but exactly how thrombomodulin inhibits metastatic spread remains to be delineated. The cell-to-cell adhesion properties of thrombomodulin are thought to underpin its role in limiting metastatic behavior. Also, tumor cells lacking thrombomodulin have low anticoagulant activity, facilitating adhesion to the endothelium in target tissues and thus promoting metastatic spread (Hanly and Winter 2007). A recently identified novel endothelial cell-specific PG, termed endocan, is also involved in mediating tumor growth. Endocan contains a single chain of DS and in contrast to most PGs, endocan does not localize to the subendothelial basement membrane but rather circulates freely in the blood. Endocan promotes tumor growth by interaction with growth factors through the glycan and with the effector cells of the immune response through the polypeptide (Scherpereel et al. 2003). An upregulation of the expression of different CSPGs is also observed in nervous tissue tumors (bervican in glioma and phosphacan in glioblastoma), with some being strong inhibitors of nerve regeneration and axonal growth (Lorente et al. 2005). In general, the large CSPGs of the ECM and cell membranes possess protumoral activities whereas the small CSPGs of the SLRP family possess antitumoral activities.

Keratan Sulfate Proteoglycans

Keratan sulfate proteoglycans (KSPGs) are made up of a core protein with attached keratan sulfate chains composed of a linear polymer molecule having repeating galactose and *N*-acetylglucosamine disaccharide units with sulfation at the C6 position of either one or both of the monosaccharide units (Greiling 1994). Though proteoglycans bearing KS chains have been identified in various cells and tissues, a majority of the currently identified KSPGs are localized predominantly in cornea, cartilage, and neural tissues (Funderburgh 2000). Different KSPGs show distinct tissue distribution, for example, *fibromodulin* is detected primarily in skin and cartilage, whereas *lumican* and *keratocan* are present in high levels in cornea compared

to other tissues (Funderburgh 2000). Importance of KS and KSPG in physiological functions like corneal hydration (Bettelheim and Plessy 1975), as a structural component of bone and cartilage (Roughley and Lee 1994), neurite outgrowth (Jones and Tuszynski 2002), and in wound healing (Fullwood et al. 1996) have been well documented.

The expression and distribution of KS and many KSPGs are altered in many diseased states including cancer. For example, keratan sulfates were found to be expressed in high levels in malignant astrocytic tumors and not in low-grade astrocytic tumors, suggesting a possible association with the malignancy of these tumors (Kato et al. 2008). In another study, keratan sulfate modification of CD44H, the cell surface receptor for HA (Thomas et al. 1992), significantly modulated adhesion of colon carcinoma cells to HA in vitro (Takahashi et al. 1996).

Lumican, a major KSPG of the corneal stroma, is expressed in many tissues such as skin, muscle, and cartilage (Ying et al. 1997). Lumican, a member of the SLRPs, exhibits altered expression in cancers of the breast (Leygue et al. 1998), colon (Lu et al. 2002), skin (Sifaki et al. 2006) and pancreas (Ping Lu et al. 2002). The higher levels of lumican expression have been correlated with poor prognosis and nodal metastasis in advanced colorectal cancer (Seya et al. 2006). Interestingly, lumican has been shown to decrease colony formation and tumorigenicity of *K-ras* or *v-src* oncogene transformed rat fibroblast F204 cells (Yoshioka et al. 2000). The in vitro antitumor effects of lumican expression have been shown to be mediated via p21/Waf-1 (Li et al. 2004). In the same study, the authors demonstrated that lumican is a target for the action of metalloproteinases such as MT1-MMP. Similarly, expression of lumican in melanoma cells also inhibits their tumor-forming ability in vivo (Vuillermoz et al. 2004). Collectively, the above observations emphasize a possible role for lumican in negatively regulating the progression of malignancy in vivo.

Fibromodulin, a well-studied KSPG, is found in the extracellular matrix and plays an important role in the maintenance and assembly of extracellular matrix protein collagen (Ameye and Young 2002). By controlling collagen matrix structure and assembly, fibromodulin plays a critical role in the proper strength and function of tendons and ligaments (Ezura et al. 2000; Gill et al. 2002). Additionally, a role for fibromodulin in endochondral and intramembranous bone formation has also been reported (Gori et al. 2001). Expression of fibromodulin RNA has been reported in lung cancer (Garber et al. 2001), prostate carcinomas (Welsh et al. 2001), and in benign uterine tumors such as leiomyomas (Levens et al. 2005). Using experimental carcinoma models, a role for fibromodulin in the maintenance of stromal matrix structure and fluid balance of tumor stroma has been established (Oldberg et al. 2007). Interestingly, fibromodulin has been shown to bind, and thus localize the availability and activity of TGF-beta (Fukushima et al. 1993; Hildebrand et al. 1994), an important signaling molecule in diseased states like cancer (Derynck et al. 2001). This particular and largely unexplored function of fibromodulin could be of very important consequence during the progression of cancer, as TGF-β is a well-established modulator of tumor progression and growth (as mentioned above).

Hyaluronic Acid

Hyaluronan (hyaluronic acid, hyaluronate, HA) is a large, unbranched glycosaminoglycan abundant within the extracellular matrix. It is composed of 2,000–25,000 repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine [-β(1,4)-GlcUA-β(1,3)-GlcNAc-]$_n$, and having total molecular masses of 10^6–10^7 kDa. HA can be found in high and low molecular weight forms. The high molecular weight form can associate with proteoglycans and function in maintaining the extracellular matrix thereby regulating a multitude of cell behaviors such as proliferation, motility and cell–cell interactions. The low molecular weight form is derived from high molecular weight HA following catalysis and is typically found in malignancies which possess many different biological functions known to promote disease progression (Stern et al. 2006). Unlike other GAGs, which are synthesized in the rough endoplasmic reticulum and Golgi and typically covalently linked to a core protein to form a proteoglycan, HA is present at the inner face of the plasma membrane as a free linear polymer lacking any association with a core protein (Tammi et al. 2008; Weigel et al. 1997). HA is synthesized by three transmembrane glycosyltransferase isoenzymes know as hyaluronic acid synthases (HAS1, HAS2 and HAS3). The nascent HA chains are synthesized directly on to the cell surface where they are retained by prolonged attachment to the synthase or by interaction with its receptors (CD44, RHAMM), or it can be released and assembled into pericellular and extracellular matrices (Toole et al. 2005). In cancer, HA is known to accumulate within the tumor and surrounding matrix and has been shown to play a role in angiogenesis, epithelial-mesenchymal transition (EMT), multi-drug resistance, and, in general, to promote the malignant phenotype.

It is well documented that high levels of HA are associated with many human malignancies (Tammi et al. 2008; Toole 2004). HA is found not only in malignant cells, but also in the surrounding peritumor stroma. The increased accumulation of HA seen in the malignant state can be attributed to direct production by the tumor or indirectly via tumor-stimulated stromal cell production. The interactions that lead to increased HA production have been studied for some time and were found to be caused by increased activity of the synthases (Knudson et al. 1984; Leonard et al. 1978). Increased expression of the three HAS isoforms has been documented both in vitro and in vivo. Using rat 3Y1 fibroblasts, oncogenic transformation with v-HA-ras only showed increased expression of HAS2 and demonstrated less malignancy. On the other hand, highly malignant v-Src transformed cells stimulated both HAS1 and HAS2 expression, suggesting that HAS isoforms may be involved in various stages of malignancy (Itano et al. 2004). Overexpression of HAS2 and HAS3 resulted in excess production of HA and enhanced tumorigenicity; while suppression of either of these two genes markedly decreased HA production and tumorigenic ability in a multitude of cell lines (Kosaki et al. 1999; Liu et al. 2001). In a mouse model of mammary carcinoma, mutants displaying low levels of HA synthesis and metastatic capability became more metastatic when HAS1 expression was

induced (Itano et al. 1999). Although increased synthetic activity of the HAS proteins correlates with tumorigenesis in several models, the ability of HA to promote cancer may be dependent upon tumor type. For example, overexpression of HAS2 in glioma cells, which lack hyaluronidase activity, resulted in decreased tumorigenesis (Enegd et al. 2002). In contrast, high levels of HA in the tumor/stroma are associated with poor survival and malignancy in many cancers, including breast, prostate, ovarian, and non-small-cell lung adenocarcinomas (Anttila et al. 2000; Auvinen et al. 2000; Lipponen et al. 2001; Pirinen et al. 2001). Additionally, HA can be detected in the urine, serum and saliva of patients with bladder carcinoma, breast cancer, and head and neck cancer, respectively, thus potentially serving as a biomarker of disease status (Delpech et al. 1990; Franzmann et al. 2003; Lokeshwar et al. 2002).

Degradation products of HA can promote angiogenesis while high molecular weight forms of HA inhibit angiogenesis (Deed et al. 1997; Sattar et al. 1994; Slevin et al. 1998; West et al. 1985), thus implying that metabolism of HA may be an important regulatory point controlling tumor growth and metastasis. Some potential mechanisms of HA-induced angiogenesis include, but are not limited to: synergy between HA oligosaccharides and the pro-angiogenic growth factors VEGF and FGF2 stimulating endothelial cell proliferation, migration and capillary formation in vitro (Deed et al. 1997) and interaction with the HA receptors CD44 and RHAMM on the surface of endothelial cells (Slevin et al. 2007). In addition, it was recently proposed that HA-rich matrices may serve as a "soil" for endothelial progenitor cells, recruiting them from the bone marrow and retaining them within the tumor microenvironment (Itano and Kimata 2008).

Epithelial to mesenchymal transition (EMT) is a critical step during embryogenesis and in the conversion of carcinoma cells to the metastatic state. In both of these settings, HA has proven to be a critical component in driving this phenomenon (7). Using an adenoviral vector to deliver HAS2, forced expression of HA in MDCK canine kidney and MCF-10A human mammary epithelial cells resulted in the acquisition of several mesenchymal characteristics, including loss of E-cadherin at intercellular boundaries, a hallmark of EMT, and increased expression of vimentin and dispersion of cytokeratin (Toole et al. 2005; Zoltan-Jones et al. 2003). Additionally, hyperproduction of HA in a HAS2 conditional transgenic mouse model of mammary carcinoma resulted in decreased E-cadherin expression along with nuclear translocation of β-catenin, an EMT-inducing agent (Koyama et al. 2007). These data suggest that increased HA production, as seen in many types of malignancy, may be sufficient to induce EMT.

The acquisition of drug resistance by tumor cells is a major problem in the clinical setting. The ability of tumor cells to become resistant to chemotherapy can occur in a multitude of ways; ranging from decreased uptake of the drug and increased drug efflux to cell adhesion-mediated drug resistance (Baumgartner et al. 1998). An early indication that HA may influence drug sensitivity was identified when HA, administered with various chemotherapeutic agents, enhanced the efficacy of these drugs (Misra et al. 2005). It was believed that HA enhanced

sensitivity to the drugs by increasing the transport of the drugs to the tumor. In contrast, an interesting mechanism whereby HA promotes drug resistance is engagement with its receptor CD44 resulting in increased expression of P-glycoprotein (Ohashi et al. 2007), MRP2 (Gilg et al. 2008), and BCRP (Biswas et al. 1995), all of which function to remove drug from the cell. EMMPRIN, a cell surface glycoprotein and member of the Ig-like superfamily is highly expressed in many types of malignant tumors and high levels of this molecule on the surface of tumor cells correlates with multidrug resistance (Marieb et al. 2004; Yang et al. 2003). Interestingly, EMMPRIN is known to stimulate the production of HA and was later identified to induce drug resistance in an HA-dependent manner (Misra et al. 2003; Toole and Slomiany 2008). Lastly, in recent studies, a very low molecular weight form of HA (6–18 sugar units) has been shown to induce apoptosis in animal models without affecting normal cells (Ghatak et al. 2002; Gilg et al. 2008; Toole et al. 2008). The inhibitory effect of small HA oligomers on tumors likely occurs because of interference with the normal HA function within the tumor microenvironment.

Summary

Proteoglycans represent a broad range of molecules with widespread and diverse expression. Thus, as would be expected, they play an array of functions within tumors and have the ability to promote or inhibit the initiation and progression of cancer dependent on the structure, function, and localization of the proteoglycan and on the tumor type and stage. The diversity inherent in proteoglycans thus provides the potential for multiple layers regulation of tumor behavior. Although not discussed in this review, there are additional points of regulation of proteoglycan function that can be accomplished by enzymatic remodeling of proteoglycans after their synthesis, some of which can occur extracellularly (e.g., extracellular sulfatases and heparanase (Ilan et al. 2006; Sanderson et al. 2005)). Also, other regulatory functions of proteoglycans may be accomplished via transportation of proteoglycans or glycosaminoglycans into the nucleus where they regulate transcriptional activity of tumor cells (Hsia et al. 2003; Ishihara and Conrad 1989). Taken together, the expansion of our knowledge of the functional control of cancer by proteoglycans has opened the door to multiple novel therapeutic approaches now being explored both in the laboratory and in the clinic. As our understanding of the complex relationships between proteoglycans and cancers evolves, the field will undoubtedly progress toward perfecting proteoglycan/glycosaminoglycan-based therapies for cancer and other diseases.

Acknowledgments Support to the authors includes grants from the National Institutes of Health CA135075 and CA055819 (to RDS), Institutional Training Fellowship T32 AR047512 (to JR), the Veterans Administration (to RDS), and the Multiple Myeloma Research Foundation (to YY). We apologize to authors whose work was not cited because of space limitations.

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Chapter 10
Tetraspanins in Cancer

Andries Zijlstra

Abstract The Tetraspanins comprise a distinct family of small transmembrane proteins capable of molecular organization of its non-tetraspanin partners. The 33 family members are characterized by a recurrent structural theme and conserved cysteines. While catalytic domains and ligands have not been identified, tetraspanins appear to function as molecular organizers by recruiting non-tetraspanin partners into organized membrane structures known as tetraspanin-enriched microdomains (TERM). These interactions allow tetraspanins to regulate adhesion, cytoskeletal interactions, molecular signaling, and protein trafficking. Tetraspanins are involved in a multitude of biological processes ranging from synaptic contacts at neuromuscular junctions to epithelial integrity and T-cell activation. In recent years, their role in several malignancies has become particularly apparent. Some tetraspanins appear capable of limiting cancer progression while others promote tumor growth, invasion, and metastasis. This chapter focuses on reviewing the current understanding of tetraspanins in tumorigenesis.

Keywords Tetraspanin • Cancer • Migration Tetraspanin Enriched Microdomain (TERM) • TSPAN • TM4SF • Scaffold

What are Tetraspanins

Members of the Tetraspanin family are identified by their similarity in structure and sequence homology. A small extracellular loop (SEL) and large extracellular loop (LEL) are created by the membrane insertion of 4 transmembrane domains while relatively small cytoplasmic tails are retained at the N and C terminus (Fig. 10.1). Close packing of the transmembrane domains is thought to give tetraspanins, a rod-like structure which protrudes from the membrane by approximately 4 nm. Conserved sequences include hydrophilic residues in the transmembrane domains, a CCG motif, critically conserved cysteines in the LEL and membrane proximal

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_10,

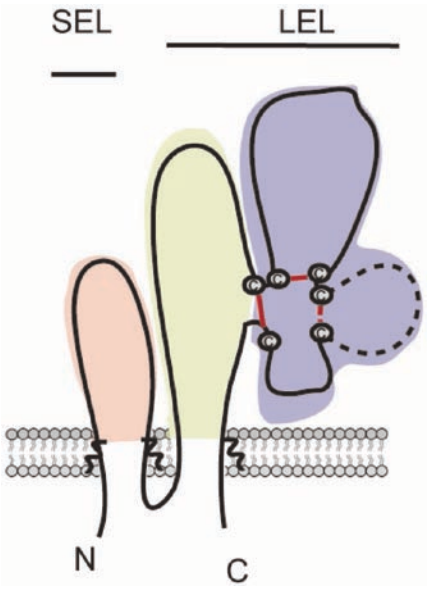

Fig. 10.1 Tetraspanin structure. Each of the 33 family members is characterized by four transmembrane domains that create a distinct small extracellular loop (SEL) and large extracellular loop (LEL). Conserved sequences are particularly abundant in the transmembrane region which are palmitoylated. The LEL is further folded into subdomains by disulfide bridging among conserved cysteines

palmytilation sites. The LEL consists of a constant region which may be responsible for tetraspanin dimerization and a variable region responsible for non-tetraspanin interactions. The vertebrate tetraspanin family has grown to 33 members (Table 10.1). The uroplakins (UPK1a and 1b Sun et al. 1996) and the retinal dystrophy syndrome genes (Peripherin and ROM1 van Soest et al. 1999) were among the first to be identified and characterized. Subsequently, many of the initial members were identified as leukocyte differentiation antigens using monoclonal antibodies (CD9, CD37, CD53, CD63, CD81/TAPA-1, CD82, and CD151). New members have been identified as tumor-associated antigens including CO-029 and SAS (Jankowski et al. 1994; Szala et al. 1990). The identification of a conserved sequence homology (Wright and Tomlinson 1994; Maecker et al. 1997; Rubinstein and Boucheix 1999) led several laboratories to the "in silico" discovery of additional members within cDNA and genomic databases including Tspan-1–6 (Todd et al. 1998), NET-1–7 "New EST tetraspanin" (Serru et al. 2000) and Tspan16/TM4-B (Puls et al. 1999).

Tetraspanins and the Tetraspanin Enriched Microdomain

Tetraspanins can interact with themselves as well as a wide variety of non-tetraspanin proteins including integrins, Ig superfamily members, proteases, and signaling molecules (Fig. 10.2). Oligomerzation among members of the family forms the

Table 10.1 The tetraspanins

Tspan	Symbol	Other Aliases	Protein ID UniProt	Gene ID NCBI	Chromosomal location	RefSeq	
						DNA	Prot
1	TSPAN1	NET-1	O60635	10103	1p34.1	NM_005727.2	NP_005718.2
2	TSPAN2		O60636	10100	1p13.2	NM_005725.3	NP_005716.2
3	TSPAN3	TM4SF8	O60637	10099	15q24.3	NM_005724.4	NP_005715.1
4	TSPAN4	NAG2	O14817	7106	11p15.5	NM_001025237.1	NP_001020408.1
5	TSPAN5	NET-4, TM4SF9	P62079	10098	4q23	NM_005723.2	NP_005714.2
6	TSPAN6	TM4SF6	O43657	7105	Xq22	NM_003270.2	NP_003261.1
7	TSPAN7	A15,TALLA-1, TM4SF2	P41732	7102	Xp11.4	NM_004615.3	NP_004606.2
8	TSPAN8	CO-029, D6.1, TM4SF3	P19075	7103	12q14.1-q21.1	NM_004616.2	NP_004607.1
9	TSPAN9	NET-5	O75954	10867	12p13.33-32	NM_006675.3	NP_006666.1
10	TSPAN10	OCSP	Q9H1Z9	83882	17q25.3	NM_031945.3	NP_114151.3
11	TSPAN11		A1L157	441631	12p11.21	NM_001080509.2	NP_001073978.1
12	TSPAN12	NET-2, TM4SF12	O95859	23554	7q31.31	NM_012338.3	NP_036470.1
13	TSPAN13	NET-6, TM4SF13	O95857	27075	7p21.1	NM_014399.3	NP_055214.1
14	TSPAN14	TM4SF14	Q8NG11	81619	10q23.1	NM_030927.2	NP_112189.2
15	TSPAN15	NET-7, TM4SF15	O95858	23555	10q21.3	NM_012339.3	NP_036471.1
16	TSPAN16	TM4SF16, TM4-B	Q9UKR8	26526	19p13.2	NM_012466.2	NP_036598.1
17	TSPAN17	TM4SF17	Q96FV3	26262	5q35.3	NM_012171.1	NP_036303.1
18	TSPAN18		Q96SJ8	90139	11p11.2	NM_001031730.1	NP_001026900.1
19	TSPAN19		P0C672	144448	12q21.31	NM_001100917.1	NP_001094387.1
20	UPK1B[a]	UPIB	O75841	7348	3q13.3-q21	NM_006952.3	NP_008883.2
21	UPK1A[a]	UPIA	O00322	11045	19q13.13	NM_007000.2	NP_008931.1
22	PRPH2[b]	RDS, RP7	P23942	5961	6p21.2-p12.3	NM_000322.4	NP_000313.2
23	ROM1[c]	ROM, ROSP1	Q03395	6094	11q13	NM_000327.2	NP_000318.1
24	CD151	PETA-3, RAPH, SFA1	P48509	977	11p15.5	NM_004357.4	NP_004348.2
25	CD53	MOX44, TSPAN25	P19397	963	1p13	NM_001040033.1	NP_001035122.1

(continued)

Table 10.1 (continued)

Tspan	Symbol	Other Aliases	Protein ID UniProt	Gene ID NCBI	Chromosomal location	RefSeq	
						DNA	Prot
26	CD37		P11049	951	19q13.3	NM_001774.2	NP_001765.1
27	CD82	KAI1, SAR2, ST6	P27701	3732	11p11.2	NM_002231.3	NP_002222.1
28	CD81	S5.7, TAPA1	P60033	975	11p15.5	NM_004356.3	NP_004347.1
29	CD9	MRP-1, DRAP-27	P21926	928	12p13.3	NM_001769.2	NP_001760.1
30	CD63	LAMP-3, ME491	P08962	967	12q12-q13	NM_001780.4	NP_001771.1
31	TSPAN31	SAS	Q12999	6302	12q13.3	NM_005981.3	NP_005972.1
32	TSPAN32	PHMX, TSSC6	Q96QS1	10077	11p15.5	NM_139022.2	NP_620591.3
33	TSPAN33	PEN	Q86UF1	340348	7q32.1	NM_178562.2	NP_848657.1

[a]Uroplakin, TM4SF (tetraspanin superfamily)
[b]Peripherin 2
[c]Retinal outer segment membrane protein 1
Links: Each tetraspanin symbol is linked to its respective gene card. The protein and gene IDs are linked to The Universal Protein Resource (UniProt) and NCBI "gene," respectively. Tetraspanins that functionally contribute to cancer are highlighted in GREY while those highlighted in BLACK are only associated with tumor progression.

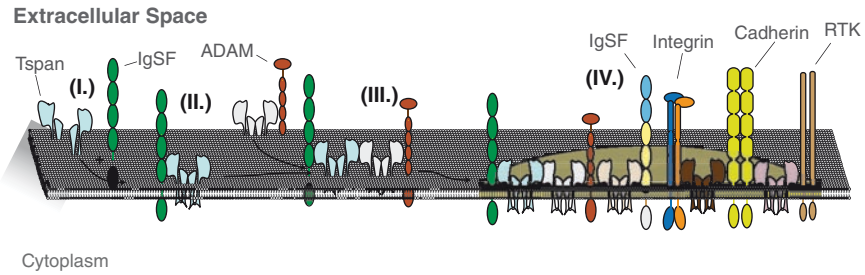

Fig. 10.2 TERM assembly. While catalytic domains and ligands have not been identified, tetraspanins appear to function as molecular organizers by recruiting non-tetraspanin partners into organized membrane structures known as tetraspanin enriched microdomains (TERM). Direct Tspan-Tspan interactions (I) and primary interactions with select partners (II) create the core complex which forms secondary interactions with other Tspan-partner complexes (III). Multimerization and heterotypic interactions recruit Tspan complexes into a unique membrane compartment referred to as "Tetraspanin-Enriched Microdomain" (TERM, IV). Through their interacting partners, tetraspanins can regulate adhesion, cytoskeletal interactions, molecular signaling, and protein trafficking. The dynamics of protein clustering in TERM is thought to be regulated by the affinity of the interactions between the various components of TERM as well as palmitoylation of the Tspans. This stability is identified biochemically by detergent solubility

scaffold of a macromolecular complex known as the tetraspanin-enriched microdomain (TEM). Various non-tetraspanin transmembrane and cytosolic proteins can interact with the LEL, cytoplasmic tails, or transmembrane regions and become incorporated into the TEM. The importance of the protein interactions of the LEL is apparent in both PRPH2 and TALLA-1 where the pathology is associated with point mutations located in the LEL (Karamatic Crew et al. 2008; Karamatic Crew et al. 2004). Primary complexes formed through direct protein–protein interactions between the tetraspanins themselves and with non-tetraspanin proteins form the core of a ~0.2 μm membrane complex. These interactions can be identified by crosslinking and their stability during Triton X-100 solubilization. However, secondary interactions, which are only stable under milder detergent conditions, can also recruit tetraspanin partners to the TEM. In fact, the vast majority of the 100+ identified partners, including critical signaling molecules such as PKC, engage in such secondary interactions. This lateral organization of signaling molecules, adhesion and cytokine receptors into macromolecular structures enables tetraspanins to influence biological activity by altering affinity/avidity, availability, and cellular localization (Fig. 10.2).

Genealogical Appearance of Tetraspanins

While tetraspanins were only identified in 1990 (Oren et al. 1990), phylogenetic studies have identified a tetraspanin gene origin that predates its prominent appearance in vertebrate biology. The family coevolved with the formation of

increasingly complex cell–cell interactions in multicellular organisms (Fig. 10.3) and expanded primarily through gene duplication. Tetraspanins have been identified in entamoeba, mytecozoa, fungi, and sponges suggesting that tetraspanins arrived with the evolution of multicellularity (Garcia-España et al. 2008; Huang et al. 2005a). Equally intriguing is the multiplicity of members: fungi (Rhizopus; 3), nematode (Caenorhabditis; 20), fly (Drosophila; 35), sea urchin (Puls et al. 1999), zebrafish (Yamada et al. 2008), chicken (Berditchevski et al. 2001), mouse (Hadjiargyrou and Patterson 1995), and human (Kazarov et al. 2002). Moreover, the gene structure of tetraspanins is conserved and suggests a common ancestor (Garcia-España et al. 2008; Huang et al. 2005a). Of the 33 mammalian family members, CD9, CD63, CD81, CD82, CD151 are expressed almost ubiquitously

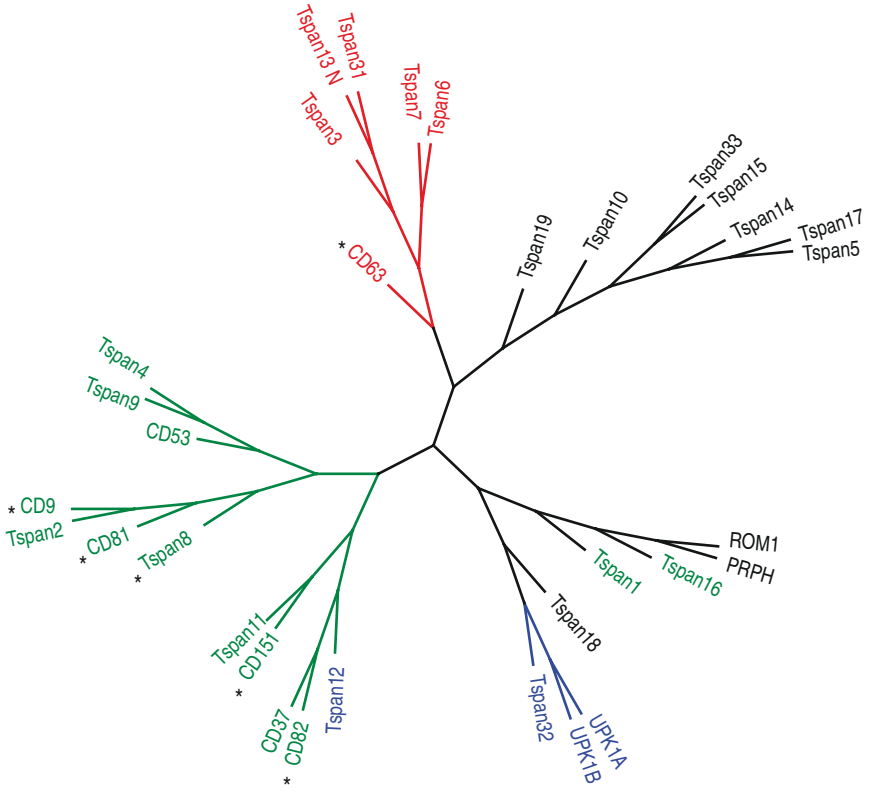

Fig. 10.3 Evolutionary tree of the tetraspanin superfamily drawn as an unrooted tree. Phylogenetic analysis of the 33 human tetraspanins was performed using Clustawl alignment and maximum likelihood phylogenetic analysis Guindon and Gascuel (2003) with PHYLM (Sun et al. 1996) in the software "Geneious." The superfamily can be subdivided into four major monophyletic subfamilies (the CD family (*green*), CD63 family (*red*), uroplakin family (*blue*), and RDS family (*black*) van Soest et al. 1999). The six tetraspanins that functionally contribute to cancer are indicated by asterisk (*)

indicating their participation in common cellular processes shared among all cell types. Other members exhibit cell-specific expression such as UPK1a/b in bladder epithelium and ROM1/PRPH2 in photo receptors. The discovery of several tetraspanins as tumor-associated antigens led to further inquiry into the role of tetraspanins in cancer. To date, of the 33 mammalian family members six have been shown to participate in neoplastic disease while the expression of four additional members correlates with disease progression. Phylogenetic analysis had classified four major monophyletic subfamilies: the CD family, CD63 family, uroplakin family, and RDS family (Garcia-España et al. 2008). It is interesting to note that all the tumor-associated tetraspanins, except for CD63, are relatively closely related members of the CD subfamily (Fig. 10.3). It is currently unknown if these members share a common mechanism or biological activity that explains their participation in tumor biology. The association of many members with neoplastic transformation may reflect the broad participation of tetraspanins in cell and matrix adhesion. Cancer progression involves the disruption of cellular adhesions and tissue architecture. This may explain the participation of so many family members in a variety of neoplastic diseases.

Molecular Functioning of Tetraspanins

The molecular and biological function of tetraspanins has been investigated using antibodies, genetic ablation, mutation, and overexpression. Most of these efforts have been directed at identifying the biological function and the molecular domain responsible for this activity.

Tetraspanin Characterization, Genetics

The genetic evidence, in the form of pathological mutations and gene ablation, illustrates diverse pathologies arising primarily from disrupted protein interactions. While, to date, no tetraspanin has been found to be required for survival, the mutation and/or loss of tetraspanins in humans can lead to distinct pathologies. Mutation of PRPH2 leads to several retinal diseases (Kohl et al. 1998) while targeted deletion in RDS leads to disrupted photoreceptor morphogenesis (Sanyal et al. 1980). Inactivation of the human tetraspanin Tspan7 (TALLA-1) by a chromosomal translocation (X;2), or point mutations (P172H), is associated with mental retardation (Zemni et al. 2000). Moreover, the tissue-specific expression of TALLA-1 (nervous system) and PRPH2 (photoreceptors) explains why the pathology is limited to those tissues. The situation is different for more globally expressed tetraspanins such as CD9, CD81, and CD151. A hereditary frameshift mutation in CD151 leads to the

expression of a truncated tetraspanin unable to bind to its integrin partner. Patients carrying the truncated tetraspanin exhibit nephritis, sensorineural deafness, pretibial epidermolysis bullosa and thalassemia minor (Karamatic Crew et al. 2004). These joined pathologies of the kidney, skin, blood, and neurosensory organs reflect a diversity of functions performed by this tetraspanin. Genetic ablation of CD151 in mice has reproduced only one of these pathologies: nephritis (Baleato et al. 2008; Sachs et al. 2006). Likewise, the targeted disruption of other globally expressed tetraspanins (CD9 and CD81) resulted in very specific pathologies. The deletion of CD81 in mice resulted in enhanced T cell proliferation (Miyazaki et al. 1997) and impaired B cell functions (Maecker et al. 1997; Miyazaki et al. 1997; Tsitsikov et al. 1997; Deng et al. 2000). CD9 null mice produce oocytes that are deficient in sperm egg fusion (Miyado et al. 2000; Le Naour et al. 2000). The lack of global failure suggests that other family members might compensate in many circumstances. In fact, the absence of embryonic lethality for individual tetraspanin null animals infers functional redundancy among the family members. This hypothesis is supported by phylogenetic analysis of LEL evolution and genetic studies of CD9 and CD81 which appear to complement each other in several functions.

Tetraspanin Characterization, Mutational Analysis

Genetic modification of conserved sequences has shed insight into the molecular requirements for tetraspanin function. Mutation of palmitoylation sites proximal to the transmembrane domains alters the lateral interactions of tetraspanins with its partners and disrupts the function of the TERM (Yang et al. 2004; Zhou et al. 2004; Berditchevski et al. 2002). The importance of the palmitoylation is affirmed by the ablation of the palmitoylating enzyme DHHC2 (Asp-His-His-Cys 2) which is the sole protein acyltransferase (PAT) capable of palmitoylating tetraspanins (Sharma et al. 2008). Loss of DHHC2 results in diminished palmitoylation of CD9 and CD151. The tetraspanins subsequently exhibit greater interaction with their respective partners and diminished lysosomal degradation. While the palmitoylation can influence the stability of the tetraspanin interactions, the specificity of that interaction resides in the LEL. If the LEL of CD151 is exchanged with the LEL from Tspan4 (NAG2) or CD9, the CD151-specific partners ($\alpha 3\beta 1$) can no longer bind and the contribution of CD151 to integrin function is lost (Berditchevski et al. 2001; Berditchevski and Odintsova 1999; Zhang et al. 2002). This specific interaction of CD151 and integrins occurs through the QRD sequences resident within the LEL (Kazarov et al. 2002).

Tetraspanin Characterization, Tetraspanin Antibodies

While some tetraspanins have been identified as the source of a genetic pathology (ROM1, TALLA-1, PRPH2), most members were identified as the antigens targeted

by antibodies raised against hematopoietic cells (CD9, CD37, CD81, CD82, CD53, CD151, and Tspan32). Interestingly, anti-tetraspanin antibodies have resulted in more prominent phenotypes than their counterpart gene ablation. Anti-CD9, CD81, and CD151 promote strong cell-matrix or cell–cell adhesion (Oren et al. 1990; Hadjiargyrou and Patterson 1995; Masellis-Smith and Shaw 1994). Antibodies to CD37 and CD81 can inhibit B cell proliferation while anti-CD82 can stimulate T cells (Oren et al. 1990; van Spriel et al. 2004). Antibody-linked activities appear to require the bivalent nature of the antibody suggesting that clustering of the tetraspanin is involved (Zijlstra et al. 2008). Initial experiments in hematopoietic cells suggested that anti-CD9 activity required a non-specific cooperation with the Fc receptor. However, subsequent studies with immobilized antibodies and antibody fragments have confirmed that clustering of CD9 conveys tetraspanin-mediated platelet activation (Griffith et al. 1991). Thus anti-tetraspanin antibodies appear capable of blocking or promoting tetraspanin activity depending upon the exact epitope recognized by the antibody. Indeed, additional evidence of such interactions is found in the inability of some anti-CD151 antibodies to bind CD151 bound to integrins. Detailed biochemical analysis shows that epitopes on the LEL are blocked when the tetraspanin interacts with its partner (Geary et al. 2001; Yamada et al. 2008). Conversely, anti-CD151 antibodies against the integrin-binding region are capable of blocking the tetraspanin interaction with its partner molecule and thereby block the endogenous function of the tetraspanin (Nishiuchi et al. 2005). The ability to control the activity of a tetraspanin suggest that therapies based on tetraspanin targeting antibodies may provide a means to regulate tetraspanin-related pathologies including cancer (Hemler 2008).

While 30% of the 33 mammalian members have been associated with cancer and malignant progression (Table 10.2), no cancer-related genetic alterations of tetraspanins have been identified. In contrast to non-cancerous pathologies, the alteration in tetraspanins appears to be limited to the epigenetic regulation of gene expression. CD151 and CO-029 (Tspan8) appear to be tumor-promoting tetrasapanins which promote migration, invasion and metastasis. CD151 is broadly upregulated in malignancies including cancers of breast, prostate, colon, lung and head and neck. Conversely, CO-029/Tspan8 has been associated almost exclusively with colorectal and hepatic cancers. The tetraspanins CD9, CD63, CD81, and CD82 appear to be suppressors of malignant progression and metastasis. Although variation is seen among organs sites, these tetraspanins are generally lost during cancer progression and, when up regulated, and inhibit malignant progression. See Table 10.2 for a comprehensive summary.

Taken together, current functional studies indicate that the biological activity of tetraspanins lies within their ability of association with function-specific partners, and subsequently organize themselves and their partners into macromolecular structures (Fig. 10.2). In this manner, the tetraspanins become molecular organizers that facilitate the interaction between a large cohort of molecular effectors and exert regulation of a large number of biological activities.

Table 10.2 Tumor-associated tetraspanins

Tspan	Symbol	Expression pattern:	Solid tissue	Hematopoietic cancer	Direct partner	Functional contribution?	Reference
1	TSPAN1 (NET-1)	Unknown	Unknown	Gastric, hepatocellular, and cervical	Unknown	Correlation	Chen et al. (2008), Chen et al. (2007), Shen et al. (2008)
7	TSPAN7 (TALLA-1)	Brain, skeletal muscle	Spleen	Neuroblastoma	Unknown	Correlation	Ito et al. (2003), Takagi et al. (1995)
8	TSPAN8 (CO-029)	Incomplete	Lymphoid progenitors	Colorectal, esophageal, hepatocellular	CO-029, Claudin 7, EpCAM, CD44	Promote cancer progression	Szala et al. (1990), Kanetaka et al. (2001), Kuhn et al. (2007b), Kanetaka et al. (2003), Claas et al. (1998), Gesierich et al. (2006)
13	TSPAN13 (NET-6)	Unknown	Incomplete, osteoclasts	Breast carcinoma	Unknown	Correlation	Huang et al. (2005b), Huang et al. (2007)
24	CD151 (PETA-3)	Broad	Platelets, megacaryocytes and monocytes	Various	CD151. α3β1, α6β1, α6β4, α7β1, MMP14	Promote cancer progression	Zijlstra et al. (2008), Kohno et al. (2002), Tokuhara et al. (2001), Ang et al. (2004)
27	CD82 (KAI1)	Broad	Lymphoid and myeloid cells, platelets and granulocytes	Various	CD82. EWI-2, α3β1, α4β1, α5β1, α6β1	Suppress cancer progression	Sridhar and Miranti (2006), Miranti (2009), Huang et al. (1998), Bienstock and Barrett (2001), Jackson et al. (2005), Liu and Zhang (2006)

28	CD81 (TAPA-1)	Broad	B cells, germinal center	Hepatocellular carcinoma	CD81. EWI-2, EWI-F, CD19. α4β1, GPR56	Suppress cancer progression	Oren et al. (1990), Mazzocca et al. (2008), Schöniger-Hekele et al. (2005), Koenig-Hoffmann et al. (2005), Xu et al. (2006)
29	CD9 (MRP-1)	Broad	Platelets, activated lymphoid and myeloid cells	Various	CD9. EWI-2, EWI-F, Claudin-1, HB-EGF	Suppress cancer progression	Huang et al. (1998), Miyake et al. (1996), Si and Hersey (1993)
30	CD63 (LAMP-3)	Broad	Platelets and monocytes	Melanoma, lung	Syntenin-1	Suppress cancer progression	Kwon et al. (2007), Radford et al. (1995), Radford et al. (1997), Funakoshi et al. (2003)
31	TSPAN31 (SAS)	Unknown	Unknown	Sarcoma	Unknown	Correlation	Jankowski et al. (1994)

[a]Peripherin 2, [b]retinal outer segment membrane protein 1, [c]Uroplakin, TM4SF (tetraspanin superfamily),
[d]Various tumors are known to be afflicted including melanoma, breast, prostate, and colorectal carcinoma
Links: Each tetraspanin symbol is linked to its respective gene card. Tetraspanins that functionally contribute to cancer are highlighted in GREY while those highlighted in BLACK are only associated with tumor progression.

Tetraspanin Activity: Signaling, Adhesion, Cytoskeletal Anchoring, and Protein Trafficking

The interactions between tetraspanins and their numerous partners have been detailed in several reviews (Maecker et al. 1997; Hemler 2008; Hemler 2005; Zöller 2009; Levy and Shoham 2005). Tetraspanins have been shown to interact with each other (Charrin et al. 2003), integrins (Berditchevski and Odintsova 1999; Berditchevski 2001), other tetraspans (Claudin and L6 family members) (Lekishvili et al. 2008; Kuhn et al. 2007a), cytokine receptors (Klosek et al. 2005; Sridhar and Miranti 2006), Immunoglobulin superfamily members (Le Naour et al. 2006; Le Naour and Zoller 2008; Yang et al. 2006; Sala-Valdés et al. 2006; Ley and Zhang 2008), cytosolic signaling molecules (André et al. 2006; Zhang et al. 2001a), gangliosides (Todeschini et al. 2007), and proteases (adams and MMPs) (André et al. 2006; Yanez-Mo et al. 2008; Lafleur et al. 2009; Arduise et al. 2008). The interactions between tetraspanins and their partners vary in affinity and can be classified according to their ability to maintain their association under mild (Brij98) or stringent (TX-100) detergent conditions (Fig. 10.4). Chemical crosslinking and fluorescence energy transfer (FRET) have been used to confirm some of these interactions. Ultimately, however, the ability to regulate the function of its partners is the defining parameter in its interaction. Considering that the detailed interactions of only a few tetraspanins have been investigated, we can anticipate that many more partners will be identified in the near future. Moreover, the macromolecular complexes created by TERMs contain numerous partners that may not be linked directly to a tetraspanin. The biological activity of individual TERM components is likely to be influenced by the incorporation of other partners. While this makes the interpretation of tetraspanin function more difficult, it explains the broad impact of tetraspanins and the functional redundancy seen among their multiple members. While the functional consequences of tetraspanin interactions may sometimes be difficult to interpret, from the current repertoire of tetraspanin partners, we can establish the classes of interactions based on their biological activity: signaling, adhesion, cytoskeletal anchoring, and protein trafficking (Fig. 10.5).

Protein Trafficking

Protein trafficking has been best studied for CD63, also known as the endocytic marker Lamp3. Early studies identified CD63 as a marker for late endocytic/lysosomal organelles where it is often found on the internal membranes of late endocytic organelles. Sequence analysis identifies a G-Y-E-V-M sequence responsible of lysosomal targeting. However, this is not sufficient as inclusion of this sequence in a different tetraspanin (CD9) does not target the tetraspanin to the lysosome (Ryu et al. 2000). CD63 trafficking is linked to the clathrin-dependent pathways although the utilization of those pathways is cell type-specific (Rous et al. 2002). The tyrosine-based sorting sequence (Y-X-X-F) is present in 12 other tetraspanins

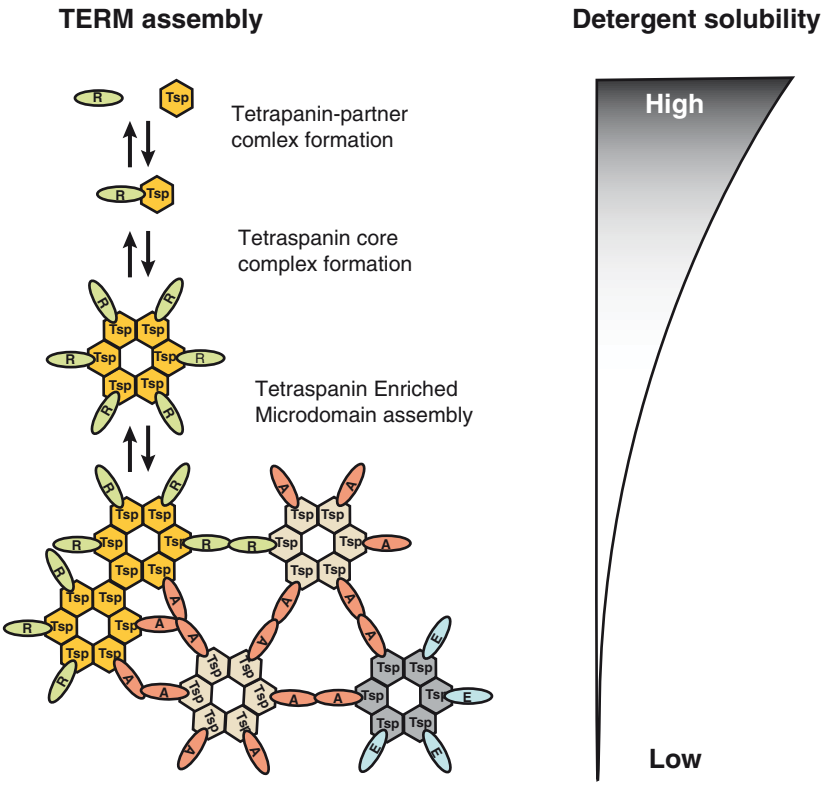

Fig. 10.4 TERM organization and detergent solubility. A simplified interpretation of TERM organization assumes that the primary interaction consists of a tetraspanin in complex with a non-tetraspanin partner. These complexes are organized into a tetraspanin core which can be assembled into a macromolecular complex enriched in tetraspanins (TERM). As "assembly" of the TERM progresses, the individual components become increasingly more difficult to extract with mild detergents

(Berditchevski and Odintsova 2007). While not functionally validated in all tetraspanins, protein trafficking is frequently associated with tetraspanin function. Altering the availability of cytokine receptors and adhesion molecules through differential trafficking could significantly alter the tumor–host interactions. CD151 is capable of regulating the trafficking of its associated integrins, as is evident from gene ablation in mice and in siRNA experiments. The loss of CD151 does not change the surface expression of integrins but reduces the rate of internalization of its partner ($\alpha 3\beta 1$). Specific mutation of the YXXϕ endocytosis/sorting motif ("YRSL" to "ARSA") diminished internalization of CD151 associated integrins (Nishiuchi et al. 2005; Winterwood et al. 2006). This cell-surface retention of the CD151-associated integrins diminished motility of tumor cells on fibronectin and laminin (Liu et al. 2007). Tetraspanins are responsible for trafficking proteins to several endocytic organelles including lysosomes, multivesicular bodies (MVP) and cytoplasmic granules (Berditchevski and Odintsova 2007). MVP are particularly enriched in

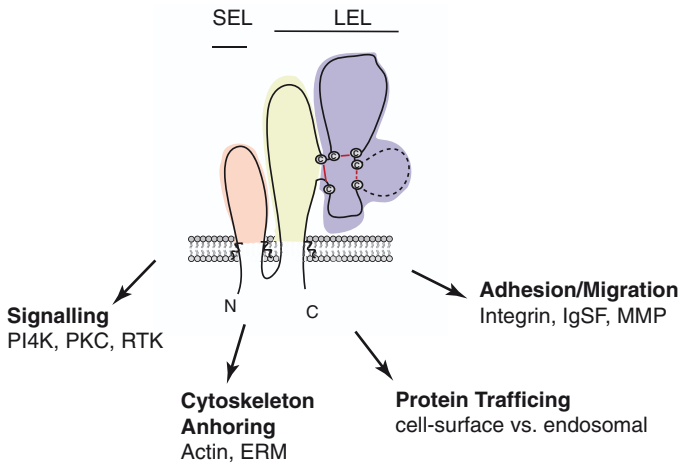

Fig. 10.5 Tetraspanin activity. Tetraspanins are capable of numerous interactions which make the functional consequences of tetraspanin–partner interactions may sometimes be difficult to interpret. However, on the basis of experimental data and clinical evidence, four classes of interactions can be established based on their biological activity: signaling, adhesion, cytoskeletal anchoring, and protein trafficking

tetraspanins (Escola et al. 1998). These unique structures form the source or exosomes (Zöller 2009) which are abundantly released from neoplasia (Schorey and Bhatnagar 2008; Andre et al. 2002). Exosomes are the most recently identified bioactive vesicles. Unlike intracellular vesicles, these structures are capable of trafficking cytoplasmic and membrane components extracellularly within the local tumor microenvironment as well as systemically via the circulation (Schorey and Bhatnagar 2008; Caby et al. 2005; Janowska-Wieczorek et al. 2005). While the exact function of tetraspanins in exosomes is poorly understood, these vesicles are though to engage in intercellular communication important in the immune response (Schorey and Bhatnagar 2008) and tumor progression (Zöller 2006). The enrichment of tetraspanins in exosomes and endocytic vesicles underscores the ability of tetraspanins to distribute asymmetrically within the spacial context of the cell body. CD151 exhibits a basolateral distribution in epithelial cells and is particularly enriched near the basil lamina whereas CD9 is uniformly distributed on all epidermal cells (Sincock et al. 1997). Such differential localization is undoubtedly linked to the associated partners and their respective functions.

Cytoskeletal Anchoring

Some of the asymmetric distribution of tetraspanins is linked to the spatial organization of the partner molecules rather than differential sorting within cytoplasmic vesicles. Several tetraspanins involved in cancer associated with a novel Ig sub-

family named EWI proteins for their characteristic Glu-Trp-Ile (EWI) extracellular motif (Sun et al. 1996; van Soest et al. 1999; Jankowski et al. 1994; Szala et al. 1990; Wright and Tomlinson 1994). This subfamily includes four members: EWI-2, EWI-3, EWI-F/CD9P-1, and EWI-101. EWI-2 and EWI-F bind strongly to CD9 and CD81. Through cytoplasmic binding of ezrin-radixin-moesin (ERM) proteins, they are capable of linking to the actin cytoskeleton. In fact, EWI-2 expression redistributes CD9 on the surface of breast cancer cells and glioblastoma, a phenomenon extremely prevalent in neoplasias (Yang et al. 2006; Kolesnikova et al. 2009). EWI-2 links directly to the cytoplasmic region of the tetraspanin CD81 and loss of EWI-2 augments cell migration and cellular polarity. The importance of this interaction is underscored by the ability of EWI-2 to limit glioblastoma growth (Kolesnikova et al. 2009). Hence, EWI proteins, through their direct interactions with ERM proteins, connect tetraspanins and their microdomains to the actin cytoskeleton (Sala-Valdés et al. 2006). While EWI–ERM connections to the cytoskeleton are the most direct anchoring mechanisms, CD82 and CD151 have also been linked to E-cadherin which links to cortical actin via beta-catenin (Chattopadhyay et al. 2003; Abe et al. 2008; Shigeta et al. 2003).

Signaling

While tetraspanins lack internal catalytic domains or enzymatic activity, they can influence molecular signaling pathways via cytoplasmic kinases (PKC and PI4K) and transmembrane receptors (EGFR and cMET). Several tetraspanins, including CD9, CD53, CD81, CD82, and CD151, associate directly with activated PKC and link this activity to specific β1 integrins (Zhang et al. 2001b). CD151-associated PKC has been shown to influence cell–cell adhesion (Shigeta et al. 2003) while CD82 is required for the suppression of EGFR signaling by PKC (Wang et al. 2007). PI4Kinase type II is associated with CD9, CD63, CD81, CD151, and Tspan7 (TALLA-1) (Yauch and Hemler 2000). PI4K activity is associated with CD63 during integrin activation in platelets while CD81-linked activity is critical for motility and metastasis of hepatocellular carcinoma (Mazzocca et al. 2008). Although tetraspanins do no exhibit strong interaction with growth factor receptors, they nevertheless have been reported to influence their activity. CD82 can regulate EGFR compartmentalization and ligand-induced dimerization (Odintsova et al. 2000; Odintsova et al. 2003) while CD9 can modulate EGFR activity (Murayama et al. 2008). CD82 has also been reported to suppress tumor cell invasion by inhibiting integrin cross-talk with c-Met receptors and Src kinases (Sridhar and Miranti 2006; Miranti 2009). CD82 regulation of c-Met receptor signaling is augmented by the ganglioside GM2/3 complex. Furthermore, CD82-ganglioside interactions inhibit tumor cell motility through the CD82-cMet pathway (Todeschini et al. 2007; Regina Todeschini and Hakomori 2008; Todeschini et al. 2008). A more indirect regulation of cell signaling can be achieved by CD63 which connects with the cytoplasmic scaffolding protein Syntenin-1 via a

PDZ binding domain. Syntenin-1 possesses a second PDZ domain and can interact with class B Ephrins, pro-TGFalpha, protein tyrosine phosphatases, syndecans, and various other transmembrane receptors. The tetraspanins also have several indirect connections to the MAPKinase and Rho GTPase pathways. CD81 expression can activate ERK1/2 and promote proliferation while anti-CD9 antibodies can suppress ERK1/2 activity and inhibit proliferation (Carloni et al. 2004; Murayama et al. 2004). CD151 has a broad tumor-promoting ability and is associated with invasion and metastasis and affects cell adhesion and motility (Zijlstra et al. 2008; Kohno et al. 2002; Lazo 2007). Motility and related changes in actin dynamics are regulated by the Rho-family GTPases (Rho, Rac, Ccd42). While no direct association between tetraspanins and GTPases has been found, CD151 did enhance Rac and Cdc42 activation when overexpressed in epidermoid carcinoma cells (Shigeta et al. 2003). CD151 has also been reported to negatively affect the Ras-ERK/MAPK1/2 pathway (Sawada et al. 2003). The exact mechanism by which tetraspanins can influence GTPase activity is currently unknown.

While any single tetraspanin can influence distinct partners, in most instances multiple family members are cooperating in a macromolecular complex that contains several tetraspanins and associated partner molecules. The temporal, spatial, and contextual organization of these macromolecular complexes (TERMs) is likely to be equally, if not more important than the singular action of one tetraspanin (Sharma et al. 2008; Hemler 2008; Hemler 2005). Signaling within TERM could be enhanced by tetraspanin clustering and recruitment. While this is frequently accomplished experimentally using tetraspanin antibodies, TERM stabilization also seems to be influenced by cholesterol (Silvie et al. 2006), tetraspanin palmitoylation (Sharma et al. 2008), and membrane anchoring by tetraspanin partners that have extracellular or cytoplasmic anchors (EWI proteins and integrins, Fig. 10.2, Table 10.2). Ultimately, it is the integration of tetraspanin availability, the molecular specificity of its partner molecule, the stability of their interaction, and the spatial organization of this complex within the cell that will control the biological activity of tetraspanins.

Adhesion and Migration

Tetraspanins have been prominent participants in tumor cell adhesion, migration, invasion, and metastasis (Zöller 2009). Indirect evidence of the participation of tetraspanins in motility is derived from the correlation of tetraspanin gene expression with malignant dissemination and the association of tetraspanins with adhesion receptors. Direct evidence of tetraspanin-mediated control of tumor cell motility is obtained from the observed changes in adhesion, migration, invasion, and metastasis in response to tetraspanin ablation and expression or the ability of anti-tetraspanin antibodies to inhibit or promote motility. The tetraspanins CD9, CD63, CD81, CD82, and CD151 have all been linked to adhesive phenotypes.

CD9 was initially identified as the target of an antibody capable of inhibiting tumor cell migration and invasion and named "motility-related protein (MRP-1) (Miyake et al. 1991). CD9 expression was able to suppress tumor cell invasion and metastasis (Higashiyama et al. 1995a; Ikeyama et al. 1993) and reduced CD9 expression correlated with disease progression and poor prognosis in cancers of breast, lung, and head and neck but not osteosarcoma (Uchida et al. 1999; Huang et al. 1998; Miyake et al. 1996; Miyake et al. 1995; Kubista et al. 2004). The molecular mechanism by which CD9 can control migration has been studied extensively but has not been conclusively determined. CD9 interacts with several proteins that can influence adhesion including integrins (Zöller 2009; Berditchevski 2001; Hood and Cheresh 2002), actin anchoring EWI proteins (Sala-Valdés et al. 2006), protein kinase C (Zhang et al. 2001a), Claudin-1 (Kovalenko et al. 2007) and heparin-binding epidermal growth factor (Higashiyama et al. 1995b). The direct correlation between CD9 expression and positive prognosis (Uchida et al. 1999; Miyake et al. 1996; Zheng et al. 2005; Hori et al. 2004; Mhawech et al. 2004; Hashida et al. 2003; Mhawech et al. 2003; Miyamoto et al. 2001; Hirano et al. 1999) and improved survival strongly indicates that CD9-mediated adhesion and motility is central to solid tumor invasion and metastasis.

CD81, in contrast to other tumor associated tetraspanins, was identified as the target of an anti-proliferative antibody (Oren et al. 1990). Only recently has its ability to regulate migration and invasion become apparent. Together with CD9 and CD151, CD81 can regulate MMP14 activity (Lafleur et al. 2009). GPR56 (Xu and Hynes 2007), EWI proteins (Sala-Valdés et al. 2006), facilitate ERK/MAPkinase signaling (Mazzocca et al. 2008; Carloni et al. 2004), and integrin adhesion strengthening (Feigelson et al. 2003; VanCompernolle et al. 2001).

CD151 was identified as a tumor-associated antigen (PETA-3). CD151 expression is negatively correlated to disease progression in various cancers and several laboratories have demonstrated that the loss or mutation of CD151 diminishes integrin activity. Anti-CD151 antibody disrupt motility (Zhang et al. 2002; Zijlstra et al. 2008; Zhang et al. 2001b; Sterk et al. 2002). Recently, we found that anti-CD151 mAb 1A5 can promote adhesion and thereby prevent migration and metastasis (Zijlstra et al. 2008). Genetic disruption of CD151 in both mice and humans results in disruption of basement membrane interactions (Baleato et al. 2008; Sachs et al. 2006). Interestingly, CD151 is not required for proper embryonic development even though migration in various tissues is central to organ formation. Nevertheless, diminished adhesive functions in CD151−/− mice are apparent in the platelets (Wright et al. 2004) podocytes (Sachs et al. 2006), epithelial cells and endothelial cells (Takeda et al. 2007). Like CD9, several mechanisms have been proposed for the regulation of tumor cell motility, including the ability to enhance integrin affinity (adhesion strengthening) (Lammerding et al. 2003) and trafficking (Berditchevski and Odintsova 2007), the activation of PKC and PI4K, signaling through Rho-family GTPases, and association with receptor tyrosine kinases (cMET). Unfortunately, it remains unclear which of these mechanisms quantitatively contributes to tumor cell dissemination.

CD63 was originally identified as a melanoma-associated antigen (ME491) (Hotta et al. 1989) and was demonstrated to reduce the H-Ras mediated transformation (Hotta et al. 1991). CD63 expression correlates inversely to melanoma metastasis (Kondoh et al. 1993). Decreased expression, indeed, characterized disease progression in melanoma (Kondoh et al. 1993) and adenocarcinoma of the lung (Kwon et al. 2007), but has not been functionally correlated to invasion and dissemination of other cancers. CD63 can suppress the melanoma proliferation and metastasis (Radford et al. 1995; Li et al. 2003) a phenotype attributed to the regulation of integrin-mediated motility in melanoma cells (Radford et al. 1995; Radford et al. 1997; Radford et al. 1996). CD63 is the endocytic marker Lamp3 and cycles between the secretory and endocytic vesicles (Kobayashi et al. 2000). It is therefore uniquely capable of controlling protein trafficking of its membrane partners including other tetraspanins and integrins (Radford et al. 1996; Mantegazza et al. 2004; Berditchevski et al. 1996).

CO-029 (Tspan8), like CD151 and CD63, was identified as a tumor-associated antigen; however, unlike the other tumor-associated tetraspanins, CO-029 has a distinctly restricted expression and is found preferentially expressed on colorectal and hepatocellular carcinomas (Szala et al. 1990; Kanetaka et al. 2001). Although little is known about this tetraspanin, it colocalizes with CD151 and associates with CD44, Claudin-1, and EpCAM (Kuhn et al. 2007b). Its expression correlates with increased migration and metastasis of colorectal and hepatocellular carcinomas (Kanetaka et al. 2001; Gesierich et al. 2005; Kanetaka et al. 2003). Thus, CD151 and CO-029 promote the mobility of tumor cells while the CD9, CD81, CD82, and CD63 appear to limit the dissemination of tumor cells.

CD82 (KAI1) is best known for its metastasis-suppressing activity identified using chromosomal transfer of 11p11.2 to rat AT6.1 prostate cancer cells (Dong et al. 1995). It has subsequently been linked to the suppression of metastatic behavior in a wide variety of cancers including cancers of the colon, lung, cervix, bladder, skin, and head (Yang et al. 2008; Miyazaki et al. 2005; Chen et al. 2004; Liu et al. 2001; Shinohara et al. 2001; Tokuhara et al. 2001; Jackson et al. 2000; Miyazaki et al. 2000; Lombardi et al. 1999; Guo et al. 1998; Hinoda et al. 1998; Takaoka et al. 1998a; Takaoka et al. 1998b; Yang et al. 1997; Guo et al. 1996). Histological analysis illustrates a strong correlation between the loss of CD82 expression and the gain of migratory capacity in tumor cells. This clinical observation is supported by the loss of migration, invasion, and metastasis ability by tumor cells in which CD82 is over expressed. Numerous investigations into the molecular mechanism of CD82 have identified several different mechanisms by which CD82 can control tumor cell motility. Like CD9, CD63, CD81, and CD151, CD82 can also associate with integrins (Berditchevski and Odintsova 1999) and possibly alter their activity, their maturation (Jee et al. 2007). CD82 is capable of numerous lateral interactions, the most notable of which are its association with EWI-F, EGF receptor, integrin $\alpha 3\beta 1$, the gangliosides GM2/3, VANGL1 (KITENIN), and DARC (reviewed in detail elsewhere, Zöller 2009; Miranti 2009). Functionally, CD82 appears primarily to be engaged to control extracellular interactions by regulating the activity and availability of cytokine receptors (EGF, cMET), integrins, leading to impaired migration and invasion.

Summary

As a family, the tetraspanins participate extensively in tumor biology. Among the six tetraspanins that influence malignant progression, CD151 and CO-029 promote tumor progression and metastasis while CD9, CD63, CD81, and CD82 are tumor and metastasis suppressors. Four additional members are differentially expressed in cancer and future investigation into their mode of action will shed more light on their mechanistic contribution to cancer. The specific function of tetraspanins within tumor biology depends upon the molecular activity of their partner molecules. Through the discovery of numerous tetraspanin partners, several tetraspanin-controlled molecular mechanisms that influence tumor progression have been identified. These mechanisms can be classified into four major categories: Signaling, Adhesion, Cytoskeletal anchoring, and Protein Trafficking. Considering their extensive involvement in cancer, those tetraspanin activities provide promising targets for future therapeutic intervention.

Acknowledgments This work was supported, in part, by -5K01 CA120711-02 (NIH/NCI).

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Chapter 11
The Role of Integrin-Linked Kinase in Cancer Development and Progression

Paul C. McDonald and Shoukat Dedhar

Abstract Integrin-Linked Kinase (ILK) is a multifunctional intracellular effector of Cell-Extracellular matrix interactions. ILK is a central component of focal adhesions and regulates many cellular processes critical for cancer progression, including proliferation, survival, epithelial–mesenchymal transition, migration, invasion, and angiogenesis. The activity and expression of ILK are controlled by a network of intracellular and intercellular processes that result in aberrant ILK expression and signaling in many human malignancies. While the causes of pathologic ILK expression may be varied and remain to be fully elucidated, accumulating evidence supports the targeting of ILK for therapeutic intervention in cancer. Currently, agents designed to interfere with aberrant ILK expression and activity are being evaluated in preclinical models. Interrogation of the ILK interactome using cutting-edge proteomic strategies is also uncovering novel interactions and cellular functions of ILK that may have important implications for the development of effective agents for cancer therapy.

Keywords Integrin-linked kinase • extracellular matrix • focal adhesion • cancer • therapeutic target • proliferation • survival • migration • invasion • angiogenesis • epithelial-mesenchymal transition

Introduction

Since its discovery in 1996, a multitude of investigational strategies have been utilized to interrogate and understand the biological functions of Integrin-Linked Kinase (ILK), a major cytoplasmic effector of integrin-mediated cellular communication (Hannigan et al. 1996, 2005). To date, these studies have defined ILK as a multifunctional intracellular effector of cell–matrix interactions that regulates many cellular

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer*,
DOI 10.1007/978-1-4419-0814-8_11, © Springer Science+Business Media, LLC 2010

processes critical for cancer progression, including proliferation, survival, epithelial–mesenchymal transition, migration, invasion, and angiogenesis. Furthermore, ILK protein expression is elevated in many human malignancies and increased expression is associated with poor patient prognosis. While the causes of aberrant ILK expression may be varied and remain to be fully elucidated, accumulating evidence suggests that its oncogenic capacity derives from the regulation of several downstream targets that provide cell proliferative, survival, and migratory signals, providing support for ILK as a relevant therapeutic target in human cancer (Hannigan et al. 2005; McDonald et al. 2008a). A recent analysis of the ILK interactome using proteomic strategies has revealed several novel interactions and has uncovered unexpected cellular functions of ILK that may have important implications for the development of effective agents for cancer therapy (Dobreva et al. 2008; Fielding et al. 2008b; McDonald et al. 2008a).

Structure of Integrin-Linked Kinase

ILK was discovered in a yeast-two-hybrid screen performed to identify proteins capable of binding the cytoplasmic tail of β-integrins (Hannigan et al. 1996). A single *ILK* gene is highly evolutionarily conserved and homologs of ILK have been identified and characterized in invertebrate (Zervas et al. 2001; Mackinnon et al. 2002) and vertebrate organisms (Sakai et al. 2003; Yasunaga et al. 2005; Bendig et al. 2006; Knoll et al. 2007). The *ILK* gene is located at the distal tip of the short arm chromosome 11, at the junction of bands 11p15.5 and 11p15.4 (Hannigan et al. 1997). The full length protein, together with its 5′ and 3′ untranslated regions, is encoded by 13 exons and the translation initiation site is located within exon 1 (Melchior et al. 2002). The translated product is 452 amino acids in length and exhibits a highly conserved tripartite structure that underpins its multifunctional capacity (Hannigan et al. 2005). ILK comprises four amino-terminal ankyrin repeats, a central plekstrin homology (PH)-like domain, and a carboxy-terminal kinase domain. The ankyrin repeats bind several adaptor and signaling proteins, while the PH-like domain supports the binding of phosphoinositides (Delcommenne et al. 1998; Pasquali et al. 2007). The catalytic domain of ILK harbors the serine/threonine (Ser/Thr) kinase activity (Hannigan et al. 1996) and is also responsible for binding a number of proteins, including several adaptor proteins connected with the actin cytoskeleton and, at the distal end, the cytoplasmic tails of β-integrins (Hannigan et al. 2005; Legate et al. 2006). These interactions will be more completely described in the following section.

Multiprotein Complex at Focal Adhesions

ILK is a central component of a macromolecular protein complex situated at focal adhesions (Fig. 11.1) which are highly specialized adhesive structures that connect the actin cytoskeleton to the extracellular matrix (ECM) through

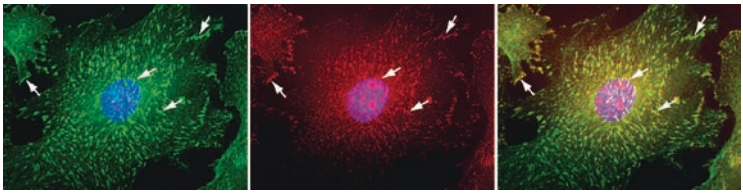

Fig. 11.1 Localization of ILK at focal adhesions. Cultured mammalian vascular smooth muscle cells have been stained for ILK (*left panel*, green) and paxillin (*middle panel*, red). The merged image (*right panel*) demonstrates colocalization of the two proteins (yellow). ILK and paxillin are clearly colocalized at focal adhesions in these cells (*arrows*)

interactions with integrins. ILK functions both as an adaptor protein that serves as a platform to connect integrins to the actin cytoskeleton through a variety of protein–protein interactions, and as a hub that regulates several downstream signaling cascades triggered by ECM-mediated engagement of integrins or binding of growth factors to specific receptors (Fig. 11.2). The ankyrin repeats bind particularly interesting new cysteine–histidine protein (PINCH), a LIM-only adaptor protein that localizes with ILK to focal adhesion sites (Tu et al. 1999). The interaction has been mapped to the LIM1 domain of PINCH and the amino-terminal ankyrin repeat of ILK (Velyvis et al. 2001). Two isoforms of PINCH, PINCH-1 and PINCH-2 (Zhang et al. 2002a; Braun et al. 2003), have been identified and both proteins bind ILK, albeit in a mutually exclusive manner. ILK-associated phosphatase (ILKAP), a PP2C family protein phosphatase which appears to suppress ILK signaling, also interacts directly with the amino-terminal ankyrin repeat of ILK (Leung-Hagesteijn et al. 2001). Meanwhile, the PH-like domain in the central region of ILK functions to bind phosphorylated inositol lipid products of PI3K (Hannigan et al. 2005). Indeed, a recent proteomics strategy has now validated the direct and specific binding of ILK to phosphatidylinositol 3,4,5, triphosphate (PIP_3) (Pasquali et al. 2007). The interaction of PIP_3 with ILK may serve to localize ILK to the plasma membrane, but is also required for PI3K-dependent activation of ILK and subsequent activation of Akt (Delcommenne et al. 1998; Lynch et al. 1999; Persad et al. 2001a). These observations provide a mechanism for the established PI3K-dependency of ILK activity.

The kinase domain of ILK is arguably the most dynamic region of this multifunctional protein, supporting a growing number of protein–protein interactions as well as harboring the catalytic core of the molecule (Fig. 11.2). The distal carboxy-terminal portion of ILK directly interacts with β1 and β3-integrins (Hannigan et al. 1996), although the exact site of interaction has not been mapped (Hannigan et al. 2005). The kinase domain also forms interactions with several actin-binding adaptor proteins, linking ILK and integrins to the actin cytoskeleton. Both α-parvin/CH-ILKBP/actopaxin and β-parvin/affixin interact with ILK, possibly in a mutually exclusive fashion (Zhang et al. 2004). Binding occurs through calponin homology 2 (CH2) domains present on the parvins (Tu et al. 2001; Yamaji et al. 2001) and the α-parvin–ILK interaction is partially dependent on PIP_3 (Attwell et al. 2003). The heterotrimeric complex formed by ILK, PINCH and the parvins, termed the IPP,

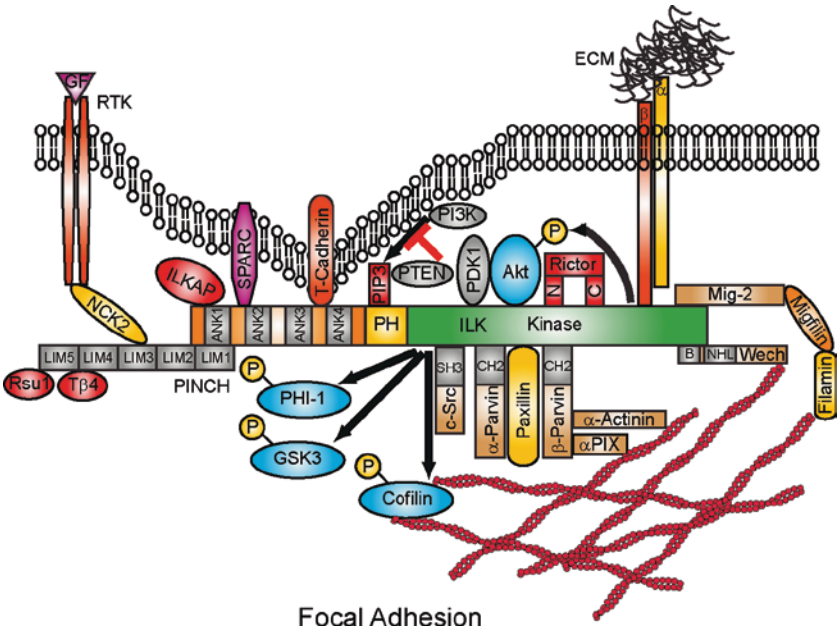

Fig. 11.2 ILK and its interacting protein partners at focal adhesions. ILK supports a vast array of protein-protein interactions that have important functional consequences. It should be noted that the regulation of these interactions is highly dynamic and it is unlikely that all proteins associate with ILK simultaneously. Specific details regarding the nature of these interactions are described in the text. *Rsu1* Ras suppressor 1; *Tβ4* Thymosin β4; *RTK* receptor tyrosine kinase; *GF* growth factor; *ILKAP* Integrin-linked kinase-associated phosphatase; *SPARC* secreted protein acidic and rich in cysteine; *PHI-1* phosphatase holoenzyme inhibitor-1; *GSK3* glycogen synthase kinase-3; *PIP3* phosphatidylinositol 3,4,5, triphosphate; *PTEN* phosphatase and tensin homolog deleted in chromosome 10; *PI3K* phosphatidylinositol-3-kinase; *PDK1* protein-dependent kinase 1 N, amino-terminal; *C* carboxy-terminal; *P* phosphorylation site; *ECM* extracellular matrix; *SH3* src homology 3; *CH2* calponin homology 2; *ANK* ankyrin; *PH* plekstrin homology; *ILK* integrin-linked kinase; *PINCH* particularly interesting new cysteine–histidine protein; *α-PIX* activating PAK-interactive exchange factor α

regulates focal adhesion stability and dynamics (Legate et al. 2006). Interestingly, RNA interference (RNAi)-mediated knockdown of one protein induces proteasome-mediated degradation of the others, demonstrating the interdependence of expression of these proteins (Hannigan et al. 2005; Legate et al. 2006). Furthermore, the CH2 domains of α-parvin bind to the LD1 and LD4 domains of paxillin, another ILK-binding protein. Paxillin interacts with ILK through the first leucine-rich domain (LD) of paxillin and a defined paxillin-binding site in the kinase domain of ILK (Nikolopoulos and Turner 2001, 2002). The kinase domain of ILK also binds Mig-2/Kindlin-2, a PH and FERM domain-containing protein that connects ILK to actin through migfilin and the actin-binding protein filamin (Mackinnon et al. 2002; Xu et al. 2006). Finally, a highly conserved regulator of integrin-mediated adhesive processes in Drosophila, Wech, interacts with the ILK kinase domain through its

B-box/coiled-coil and NHL domains (Loer et al. 2008). A murine orthologue of Wech colocalizes with ILK in muscle tissue where it is proposed to regulate the link between integrins and the cytoskeleton (Loer et al. 2008).

In addition to proteins that mediate interactions with the cytoskeleton, ILK can associate with several proteins implicated in cell signaling (Fig. 11.2). For example, both Akt and PDK-1 have been shown to associate with ILK (Persad et al. 2001a; Barry and Gibbins 2002; McDonald et al. 2008b). Interestingly, Ser^{343}, a residue situated within the activation loop of ILK, is required for these interactions (Persad et al. 2001a). A direct interaction between Rictor and ILK has recently been described (McDonald et al. 2008b). This interaction requires the ILK kinase domain and the amino-terminal and carboxy-terminal portions of Rictor. Secreted protein acidic and rich in cysteine (SPARC), a matricellular protein that mediates cell-ECM interactions, is also reported to bind ILK (Barker et al. 2005). While the binding regions involved in this interaction and the exact location with respect to the plasma membrane remain to be elucidated, the interaction is intriguing because the data indicate that SPARC and ILK exist as part of a membrane-associated complex that has access to the extracellular environment (Barker et al. 2005). Similarly, immunoprecipitation and confocal microscopy have been used to demonstrate an interaction between Glycosylphosphatidylinositol- (GPI) -anchored T-cadherin and ILK in the lamellopodia of migratory endothelial cells (Joshi et al. 2007), linking GPI-anchored proteins present on the outer membrane surface with ILK signaling. Finally, a physical association between the SH3 domain of activated c-Src and the kinase domain of ILK has also been described recently (Kim et al. 2008).

The interactions between ILK and its various binding partners suggest direct functional capacity and link it to several additional proteins that produce an array of biological consequences. For example, in addition to binding ILK, PINCH binds thymosin β4 (Tβ4) via its LIM4 and 5 domains, thereby coupling Tβ4 to ILK. Thymosin β4 upregulates ILK expression and activity (Bock-Marquette et al. 2004; Huang et al. 2007) and may stabilize the ILK–PINCH interaction (Huang et al. 2007). The LIM5 domain of PINCH-1 also binds the Ras suppressor, Rsu-1 (Dougherty et al. 2005), and this protein in now known to colocalize with ILK at focal adhesions and co-purify with the ILK–PINCH complex in non-transformed cells (Dougherty et al. 2008). Interestingly, the association of Rsu-1 with the IPP complex is dramatically reduced following Ras transformation and data suggest full-length Rsu-1 may function to stabilize the IPP complex at focal adhesions (Dougherty et al. 2008). In contrast, ILKAP, which binds ILK directly, negatively regulates its kinase activity and signaling capacity (Leung-Hagesteijn et al. 2001). A direct interaction between PINCH-1 and Nck-2, an adaptor protein that associates with PDGF and IGF receptors, has also been demonstrated using yeast-two-hybrid and in vitro assays (Tu et al. 1998), providing a potential link between integrins and growth factors through ILK.

Several interactions illustrate the interdependency of adaptor and kinase functions of ILK. The association of ILK with the parvins and paxillin couple ILK signaling capacity to the actin cytoskeleton. Phosphorylation of the CH2 domain of β-parvin by ILK is required for the interaction between β-parvin and the actin-binding

protein, α-actinin. Similarly, inhibition of ILK activity results in the loss of paxillin (Hannigan et al. 2005, 2007; Legate et al. 2006). β-Parvin also binds to α-PIX, connecting ILK with activation of the small GTPases Rac and Cdc42, and cell migration (Filipenko et al. 2005). The ILK-c–Src complex is responsible for the Ser^{3} phosphorylation of cofilin and the inhibition of its actin-severing activity (Kim et al. 2008). Finally, the ILK–Rictor interaction is required for promoting Akt Ser^{473} phosphorylation in the ILK complex (McDonald et al. 2008b).

The Dual Functionality of ILK

The preceding discussions bring one to the conclusion that the scaffolding and signaling functions of ILK are distinct, but not mutually exclusive aspects of its biology. Thus, the concept of dual functionality of ILK has emerged (Hannigan et al. 2005; Legate et al. 2006). As the concept suggests, the biological importance of ILK rests both on its role in the regulation of cytoskeletal dynamics and its ability to function as a Ser/Thr kinase.

Cytoskeletal Dynamics

Focal adhesions are a major point of integrin attachment in cells. The interactions between ILK and proteins such as PINCH and the parvins are important for cell behaviors involving actin cytoskeleton rearrangements (Fig. 11.2). Depletion of either PINCH-1 or ILK gene expression impairs cell spreading and migration (Fukuda et al. 2003), as does overexpression of either the LIM1 domain of PINCH or the ankyrin repeat domain of ILK (Velyvis et al. 2001; Zhang et al. 2002b). Truncation of the ILK ankyrin repeat responsible for binding PINCH inhibits targeting of ILK to focal adhesions, demonstrating the necessity of the ILK–PINCH interaction in subcellular localization of ILK. PINCH-2 also interacts with ILK, a process that may inhibit PINCH-1–ILK interactions by competitively binding ILK (Zhang et al. 2002a; Braun et al. 2003).

Interactions between ILK, α-parvin and β-parvin also facilitate actin cytoskeleton-dependent processes such as spreading and migration. Overexpression of β-parvin inhibits cell spreading (Yamaji et al. 2001) and ILK-mediated β-parvin phosphorylation is required for the β-parvin-α–actinin interaction (Yamaji et al. 2004). The depletion of α-parvin increases β-parvin expression and results in Rac activation, further suggesting a role for ILK-β–parvin interactions in Rac-mediated cytoskeletal dynamics. Current data suggest that α-parvin and β-parvin have differential regulatory roles in their interaction with ILK with respect to adhesion-mediated survival signaling. Thus, either interference with the α-parvin–ILK interaction or overexpression of β-parvin promotes apoptosis (Zhang et al. 2004). Accordingly, β-parvin expression is reduced in breast cancer cell lines and advanced

breast cancer specimens relative to normal tissue, and β-parvin expression in breast cancer cells increases cell adhesion and inhibits ILK-mediated GSK-3β phosphorylation, anchorage-independent growth and invasion (Mongroo et al. 2004).

Importantly, functional protein–protein interactions mediated by ILK are regulated both by ILK protein expression and ILK activity, further supporting the concept of functional interdependence. Silencing of ILK protein expression, PTEN-mediated inhibition of ILK activity or treatment with small molecule inhibitors of ILK results in dissociation of ILK–parvin complexes and decreases cell adhesion in prostate cancer cells (Attwell et al. 2003). In these cells, α-parvin increases ILK-actin cytoskeleton interactions, ILK activity and GSK-3β phosphorylation, while disruption of ILK activity abrogates localization of α-parvin and paxillin to focal adhesions. Inhibition of ILK activity also impairs the ILK-paxillin association (Nikolopoulos and Turner 2001). Therefore, the ability of ILK to participate in the regulation of cytoskeletal reorganization relies on its adaptor and catalytic functions.

Ser/Thr Kinase Activity

The Ser/Thr kinase activity of ILK has previously been a source of substantial controversy. The "atypical" structure of the kinase domain of ILK (Hannigan et al. 2005) and the existence of reports demonstrating the non-essential nature of its kinase activity in invertebrates have caused some investigators to challenge the physiological relevance of ILK kinase function (Legate et al. 2006). Although the ILK kinase domain contains several conserved subdomains, including the ATP-coordinating lysine residue in subdomain two and the invariant glutamine in subdomain eight, ILK, like other putative "pseudokinases", lacks the magnesium (Mg^{2+})-coordinating DFG motif. However, the recent high resolution crystal structure of the "pseudokinase" CASK, which like ILK also lacks the aspartic acid in the DFG motif (DFG→GFG), has revealed that CASK can function as a Mg^{2+}-independent kinase (Mukherjee et al. 2008). These revelations have upset the kinase catalysis paradigm and are prompting a rethinking of "pseudokinase" functions (Mukherjee et al. 2008). It is, therefore, highly likely that the kinase domain of ILK may also provide phosphotransferase activity via a unique mechanism that will only be revealed when the crystal structure of the ILK kinase domain is solved. Many biochemical studies have provided very strong evidence for the ability of ILK to phosphorylate substrates in a specific manner. Collectively, these studies have identified ILK substrates, described the structural and functional basis for its kinase activity and demonstrated the existence of regulators of ILK catalytic function, clearly demonstrating that ILK is a biologically relevant Ser/Thr kinase (Fig. 11.2).

Initial assays for ILK kinase activity utilized the prototypic kinase substrate myelin basic protein (MBP) (Hannigan et al. 1996). Subsequent observations that ECM-mediated or growth factor-induced stimulation of ILK activity occurs in a

PI3K-dependent manner (Delcommenne et al. 1998) spurred investigation of its role as an upstream regulator of PI3K effectors such as Akt and GSK-3β. Importantly, there is a structural basis that underpins the PI3K-dependent regulation of ILK activity. The PH domain contains a consensus sequence for binding of phosphoinositides (Hannigan et al. 2005) and PIP_3, but not PIP_2, binds directly to ILK (Pasquali et al. 2007) and stimulates the kinase activity of purified ILK in vitro (Delcommenne et al. 1998). Numerous studies have now verified that ILK participates in PI3K-dependent regulation of these two kinases (Delcommenne et al. 1998; Lynch et al. 1999; Persad et al. 2000, 2001a; Tan et al. 2001; Cruet-Hennequart et al. 2003; Agouni et al. 2007; Dillon et al. 2007; Liu et al. 2007b). Expression of wild type ILK, but not a kinase-deficient mutant of ILK, increases Akt Ser^{473} phosphorylation and inhibits GSK-3β phosphorylation (Delcommenne et al. 1998). Moreover, recombinant ILK phosphorylates GSK-3β in vitro (Delcommenne et al. 1998), confirming its identification as an ILK substrate. ILK, Akt and ^{Ser473}P-Akt immunoprecipitate together (Persad et al. 2001a; McDonald et al. 2008b), and in-gel kinase assays confirm that ILK phosphorylates Akt (Persad et al. 2001a). Depletion of ILK and Rictor, a defining member of the mTORC2 complex implicated in Akt Ser^{473} phosphorylation (Sarbassov et al. 2005), in breast and prostate cancer cell lines results in the inhibition of Akt Ser^{473} phosphorylation and the induction of apoptosis (McDonald et al. 2008b). Rictor and ILK interact directly and siRNA-mediated silencing of Rictor inhibits the amount of ^{Ser473}P-Akt in the ILK complex, suggesting that Rictor facilitates the ability of ILK to phosphorylate Akt (McDonald et al. 2008b). Deletion of PINCH-1 also inhibits Akt Ser^{473} phosphorylation, suggesting that interacting proteins may be critical in modulating ILK kinase function.

Although Akt and GSK-3β are the best studied of its substrates, ILK activity has been linked to the phosphorylation of several other physiologically relevant molecules. ILK has been purified as a calcium independent myosin light chain kinase (MLCK) using in gel kinase assays and mass spectrometry-based strategies (Deng et al. 2001; Wilson et al. 2005). ILK phosphorylates and activates inhibitors of protein phosphatases, including KEPI, CPI-17 and PHI-1 (Deng et al. 2002; Erdodi et al. 2003). It also phosphorylates and inhibits myosin phosphatase targeting subunit 1 (Muranyi et al. 2002). These biochemical studies suggest that ILK plays a role in myosin-based cell movement, the dysfunction or deregulation of which may be important in the oncogenic processes such as metastasis (Hannigan et al. 2005). ILK also phosphorylates α-NAC, resulting in the regulation of c-Jun transcriptional activity (Quelo et al. 2004). In addition, Notch1-IC, a transmembrane protein that functions in the regulation of cell fate (Mo et al. 2007), is a known substrate of ILK. Phosphorylation of Notch1-IC by ILK is important for its degradation via the Fbw7 ubiquitin ligase pathway (Mo et al. 2007). Finally, ILK has recently been reported to phosphorylate cofilin, thereby modulating its actin-severing action and linking cytoskeletal organization to ILK activity (Kim et al. 2008).

In further support of the function of ILK as a kinase, several phosphatases that act as negative regulators of ILK activity have been identified. A number of studies have demonstrated that PTEN and MMAC1, lipid phosphatases and tumor suppressors

that inhibits PI3K signaling, inhibit ILK-mediated signal transduction. Cells devoid of these tumor suppressors maintain constitutively active ILK and have increased levels of Akt Ser^{473} phosphorylation (Morimoto et al. 2000; Persad et al. 2000, 2001b; Obara et al. 2004). Transfection of PTEN phenocopies introduction of a dominant negative ILK mutant into these cells, downregulating ILK activity and Akt Ser^{473} phosphorylation. ILKAP also functions to inhibit ILK activity and signaling (Leung-Hagesteijn et al. 2001; Kumar et al. 2004). ILKAP is downregulated in melanoma cell lines, whereas increased ILK activity and protein correlate with poor prognosis (Dai et al. 2003). Stomach cancer associated-phosphatase 1 (SAP-1) inhibits ILK kinase activity and Akt phosphorylation indirectly, possibly by disrupting focal adhesions (Takada et al. 2002). Finally, DAB2, a candidate tumor suppressor downregulated in ovarian and colorectal carcinomas (Mok et al. 1998; Kleeff et al. 2002), decreases the anoikis resistance of breast cancer cells and downregulates ILK activity (Wang et al. 2001). The presence of multiple regulators of ILK activity speaks about the importance of the ILK kinase function, particularly as it relates to cancer progression.

Overexpression and Increased Activity of ILK in Human Malignancies

ILK is ubiquitously expressed at various levels in normal tissues with the heart and the brain having particularly high levels of expression. Its promoter contains a major transcriptional start site 138 base pairs (bp) upstream of exon 1 and the minimal sequence conferring promoter activity is a 349 bp segment located immediately upstream of exon 1 (Melchior et al. 2002). The promoter does not contain TATA or CAAT boxes, but it has a high GC content, CpG islands and binding sites for SP-1 and NFκB (Melchior et al. 2002), suggesting that the protein is transcriptionally regulated. Indeed, ILK gene expression is transcriptionally regulated by PPARβ (Tan et al. 2004b). Furthermore, the 5' promoter region of the ILK gene contains hypoxia responsive elements (HRE) that bind hypoxia-inducible factor (HIF) transcription factor complexes and drive HRE-luciferase gene expression in reporter assays (Lee et al. 2006b; Abboud et al. 2007).

Although the transcriptional regulation of ILK remains to be fully elucidated, its expression is elevated in many human malignancies. Indeed, immunohistochemical analyses have confirmed the presence of elevated levels of ILK protein relative to normal tissue controls in many cancers, including those of the skin (melanoma) (Dai et al. 2003), gastrointestinal tract (Bravou et al. 2003; Ito et al. 2003; Marotta et al. 2003; Bravou et al. 2006; Huang et al. 2007), head and neck (Younes et al. 2005, 2007), lung (Takanami 2005; Okamura et al. 2007; Watzka et al. 2008), pancreas (Sawai et al. 2006), ovary (Ahmed et al. 2003) and prostate (Graff et al. 2001). Importantly, the degree of ILK expression correlates with the tumor stage and grade in many of these malignancies (Bravou et al. 2003, 2006; Takanami 2005), and strong ILK expression is often a poor prognostic indicator (Graff et al. 2001;

Dai et al. 2003; Takanami 2005; Yau et al. 2005; Okamura et al. 2007). The causes of elevated ILK expression remain to be thoroughly examined, but current evidence suggests that control of its expression is multifaceted.

ILK gene amplification or mutations have not yet been identified in relation to human cancer, but its expression is stimulated by a wide variety of soluble ligands, including endothelin-1 (ET-1) (Rosano et al. 2006), Cyr61 (Xie et al. 2004), transforming growth factor beta (TGF-β) (Li et al. 2003), CXCL12 (Jones et al. 2007), connective tissue growth factor (CTGF) (Liu et al. 2007b) and osteopontin (OPN) (Mi et al. 2006). Many of these factors are produced by the tumor cells themselves or are present in the tumor microenvironment and potentially contribute to the elevated expression of ILK in malignant tissues. Colon cancer cells overexpressing Tβ4 also exhibit increased levels of ILK expression, although the increase has been attributed to enhanced stabilization of ILK through more efficient complexation with PINCH (Huang et al. 2007) as opposed to direct transcriptional control.

Levels of ILK protein may also be enhanced by the aberrant environmental conditions present in cancerous lesions. For example, ILK expression is upregulated by hypoxia (Lee et al. 2006b; Abboud et al. 2007). Viral infection may also play a role, as HCMV infection of prostate cancer cells was found to elevate β1-integrin expression, cellular adhesion and ILK expression (Blaheta et al. 2006). Processes related to disease progression, especially steps in progression to metastasis, also regulate ILK expression. In particular, Epitheial to Mesenchymal transition (EMT) is associated with increased expression of ILK (Liu et al. 2007a, b, 2008a).

In addition to its overexpression, modulation of ILK function may also be controlled through regulation of its kinase activity. A number of mediators with links to cancer progression have been reported to influence the activity of ILK. SPARC and Cyr61, two cysteine-rich matricellular proteins, regulate ILK activity in glioma cells (Xie et al. 2004; Shi et al. 2007). The growth factor angiopoietin-2 (Ang-2) stimulates ILK activity and results in modulation of Akt and GSK-3β phosphorylation, leading to promotion of breast cancer cell migration and invasion (Imanishi et al. 2007). Similarly, growth-promoting ligands such as insulin and IGF-1 are also implicated in the regulation of ILK activity (Delcommenne et al. 1998). GPI-anchored proteins can also modulate ILK activity, as T-cadherin in endothelial cells influences Akt and GSK-3β phosphorylation through ILK (Joshi et al. 2007). Finally, a role for elevated levels of ILK protein and activity has been demonstrated in a mouse model of chemically induced skin tumorigenesis (Segrelles et al. 2002). In this model, H-ras mutations and elevated expression/activity of cyclin D1 are critical for tumor formation. PI3K-dependent upregulation of Akt activity is an early and sustained event in the development of skin cancer in these animals (Segrelles et al. 2002, 2006, 2007). Increased expression and activity of ILK correlates with sustained Akt activity in developing papillomas and, in particular, during conversion to invasive, squamous cell carcinomas (Segrelles et al. 2002). Collectively, these findings suggest that targeted downregulation of ILK expression, or suppression of its functional capacity, may slow or halt cancer progression.

ILK is a Vital Component of Cancer-Associated Signaling Pathways

The strategic localization of ILK to focal adhesions, together with its dual functionality related to cytoskeletal dynamics and regulation of intracellular signaling, positions it as a key regulator of cellular processes critical to oncogenic progression. Over the past decade, investigations of ILK functions have revealed important clues regarding the role of ILK in several processes important in tumor progression, including cell cycle progression, survival, EMT, adhesion, migration and invasion, and angiogenesis (Fig. 11.3). The following sections elaborate on the experimental evidence for a role of ILK in each of these processes.

Proliferation and Cell Cycle Progression

Increased proliferation and derangements in cell cycle progression are hallmarks of malignant cells. Early work on ILK established it as a critical component of pathways regulating these processes (Fig. 11.3). Overexpression of ILK in intestinal epithelial cells (IECs) correlates with several indices of proliferation, including elevated cyclin D1 expression levels, increased cyclin D1-CDK4 activity, retinoblastoma protein hyperphosphorylation and increased numbers of cells in S phase (Radeva et al. 1997). In contrast, RNAi-mediated attenuation of ILK expression in colon or prostate cancer cell lines inhibits cyclin D1 expression and cyclin D1 is reduced in colitis-associated tumors arising in mice containing targeted deletion of ILK in intestinal epithelial cells (Tan et al. 2004a; Assi et al. 2008). Inhibition of ILK activity with small molecules in glioblastoma xenografts results in significantly lower levels of proliferation relative to untreated animals, most likely due to the blockage of the cell cycle at G2-M (Edwards et al. 2008). Constitutive activation of ILK also stimulates cell cycle progression, and inhibition of ILK activity in cancer cells results in the inhibition of cyclin D1 expression and G1/S cell-cycle arrest (Li et al. 1997; D'Amico et al. 2000; Persad et al. 2000), illustrating the importance of the kinase function of ILK in regulating proliferation. The SPARC-related protein, secreted modular calcium-binding protein-2 (SMOC-2), has been reported to regulate cyclin D1 expression by growth factors and maintain ILK activity during the G1 phase of the cell cycle (Liu et al. 2008b), further implicating ILK kinase activity in the regulation of cell cycle progression. Indeed, studies suggest that the regulation of cell proliferation by ILK involves kinase-based targeting of GSK-3β, an established ILK substrate. Phosphorylation and inhibition of GSK-3β by ILK results in the activation of transcription factors that induce cyclin D1 expression, including AP-1 (Troussard et al. 2000), cyclic-AMP-responsive-element-binding protein (CREB) (D'Amico et al. 2000) and TCF/LEF (Persad et al. 2001b). Conversely, PTEN-mediated suppression of ILK activity reduces GSK-3β-controlled β-catenin nuclear translocation and cyclin D1 expression (Persad et al. 2001b). Moreover, the inhibitory effects of ILKAP on ILK activity also suppress cyclin D1 expression through the ILK-GSK-3β-β-catenin axis (Kumar et al. 2004).

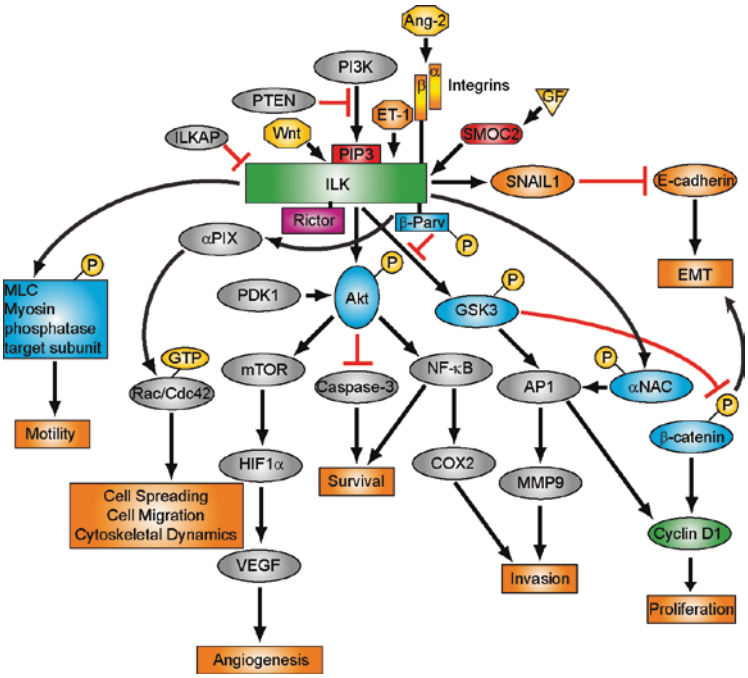

Fig. 11.3 ILK-mediated signal transduction in cancer cells. ILK is a key regulator of several cell signaling pathways important in the progression of cancer. As with the dynamic interplay associated with ILK–protein interactions, it is probable that cancer cells selectively and differentially regulate these pathways, and that the precise pathways manipulated are cell- and context-dependent. Details of the pathways and the mechanisms by which ILK regulates these processes can be found in the text. *Ang-2* angiopoietin-2; *PI3K* phosphatidylinositol-3-kinase; *GF* growth factor; *ILKAP* integrin-linked kinase-associated phosphatase; *SPARC* secreted protein acidic and rich in cysteine; *PHI-1* phosphatase holoenzyme inhibitor-1; *GSK3* glycogen synthase kinase-3; *PIP3* phosphatidylinositol 3,4,5, triphosphate; *PTEN* phosphatase and tensin homolog deleted in chromosome 10; *PDK1* protein-dependent kinase 1; P, phosphorylation site; *ILK* integrin-linked kinase; *α-PIX* activating PAK-interactive exchange factor α; *SMOC2* secreted modular calcium-binding protein-2; *ET-1* endothelin-1; *β-Parv* β-parvin; *EMT* epithelial-to-mesenchymal transition; *NF-κB* nuclear factor-κB; *mTOR* mammalian target of rapamycin; *AP-1* activator protein 1; *MMP9* matrix metalloprotease 9; *COX2* cyclooxygenase-2; *HIF1α* hypoxia-inducible factor-1α; *MLC* myosin light chain; *GTP* guanine triphosphate; *VEGF* vascular endothelial growth factor

ILK-mediated effects on proliferation may also occur by a GSK-3β-independent mechanism, as the JUN transcriptional co-activator, αNAC, is a direct target of ILK kinase activity (Quelo et al. 2004).

Survival

In addition to increased proliferation, elevated survival capacity represents a long-standing benchmark by which cancer cells are defined. Initial experiments

demonstrated that overexpression of ILK in mammary epithelial cells results in resistance to anoikis (Attwell et al. 2000), a form of cell death that occurs under conditions of cell detachment (Fig. 11.3). ILK-mediated activation of Akt under conditions of ILK overexpression also prevents anoikis in human mesenchymal stem cells (Benoit et al. 2007), suggesting the possibility that malignant stem cells may acquire an ILK-based survival phenotype. Furthermore, inhibiting ILK activity or expressing dominant negative Akt in the mammary epithelial cell model induces caspase 3 activity and anoikis, suggesting that ILK-driven Akt activation is a survival mechanism used by these cells (Attwell et al. 2000). In fact, inhibition of ILK in many cancer cell lines inhibits Akt Ser^{473} phosphorylation and stimulates anoikis or apoptosis (Attwell et al. 2000; Morimoto et al. 2000; Persad et al. 2000, 2001a; Duxbury et al. 2005; Younes et al. 2005; Troussard et al. 2006; Abboud et al. 2007; Edwards et al. 2008; McDonald et al. 2008b). ILK activity is critical for the survival of leukemic cells in contact with bone marrow-derived stromal mesenchymal stem cells (MSC) (Tabe et al. 2007). Co-culture of leukemic cells with MSC induces, amongst others, the ILK/Akt pathway, while inhibition of ILK specifically inhibits stroma-induced Akt and GSK-3β phosphorylation and induces apoptosis (Tabe et al. 2007). In turn, leukemic cells induce reciprocal activation of ILK/Akt that is abrogated with the inhibition of ILK activity. These findings indicate that ILK-mediated signaling is critical for the survival of leukemic cells within the bone marrow microenvironment and suggest that targeting ILK may be effective in eliminating leukemic cells from this host niche by affecting both cell populations (Tabe et al. 2007). In addition, ILK-mediated Akt Ser^{473} phosphorylation and NF-κB activation are also critically involved in parathyroid hormone-related protein (PTHrP)-induced survival in human renal cell carcinoma (Agouni et al. 2007). Thus, the ability of ILK to phosphorylate Akt on Ser^{473}, thereby fully activating Akt, is a major mechanism by which ILK facilitates cell survival.

EMT

The conversion of epithelial cells to a mesenchymal phenotype, a process known as epithelial-mesenchymal transition (EMT), is associated with invasion and metastasis of malignant cells (Lee et al. 2006a; Hugo et al. 2007). The process is characterized by altered morphology, loss of epithelial markers and concomitant acquisition of mesenchymal markers. Studies have demonstrated that overexpression of ILK in epithelial cells leads to EMT (Fig. 11.3) (Somasiri et al. 2001; Li et al. 2003). Importantly, ILK overexpression results in the loss of E-cadherin expression (Novak et al. 1998; Wu et al. 1998), a widely used marker of EMT, while exogenous expression of E-cadherin in ILK-overexpressing cells rescues the epithelial phenotype (Somasiri et al. 2001). A similar phenomenon occurs in vivo, with the expression of ILK in mammary epithelial cells in transgenic mice resulting in diminished E-cadherin expression and induction of EMT in ILK-induced tumors (White et al. 2001). ILK regulates the expression of a transcriptional repressor of

E-cadherin, SNAIL (Tan et al. 2001; Barbera et al. 2004; McPhee et al. 2008), providing a mechanistic rationale for the role of ILK in EMT.

A series of recent studies has investigated the role of ET-1 and ILK in mediating EMT in ovarian carcinoma. ET-1 stimulation of ovarian cancer cells induces an invasive, fibroblastic phenotype characterized by downregulation of E-cadherin, increased expression of Snail, β-catenin and mesenchymal markers, and reduced activity of the E-cadherin promoter (Rosano et al. 2005). Data suggest that induction of EMT by ET-1 involves ILK activation through receptor-mediated and integrin-mediated mechanisms. Activation of ET A receptor [ET(A)R] by ET-1 results in activation of ILK, leading to negative regulation of GSK-3β, stabilization of Snail and β-catenin, and regulation of transcriptional events that foster EMT (Rosano et al. 2005). Meanwhile, ET-1 stimulation of β1 integrin expression results in increases in adhesion-induced ILK activity (Rosano et al. 2006). Inhibition of such ILK activity inhibits the phosphorylation of downstream effectors, including GSK-3β and Akt, while blockade of ET-1-mediated ILK activity inhibits MMP activity, motility and invasiveness (Rosano et al. 2006). Antagonism of the ET(A)R in ovarian carcinoma xenografts suppresses EMT markers and tumor growth. In ovarian carcinoma xenografts, a specific ET(A)R antagonist downregulates EMT markers, inhibits ILK expression, Akt and GSK-3β phosphorylation, and reduces tumor growth (Rosano et al. 2005, 2006).

In addition to ET-1, a number of other soluble mediators have been implicated in ILK-mediated EMT. Colon cancer cells overexpressing Tβ4 undergo an EMT that involves the upregulation of ILK through stabilization of its interaction with PINCH (Huang et al. 2007). Overexpression of Tβ4 results in loss of E-cadherin expression and accumulation of β-catenin. β-Catenin accumulation has been attributed to the inhibition of GSK-3β by ILK, possibly through Akt activation (Huang et al. 2007). Human kidney epithelial cells stimulated with CTGF upregulate their expression of ILK (Liu et al. 2007b), an effect that is partially dependent on MEK/ERK1/2 and PI3K signaling pathways (Liu et al. 2007b). CTGF also triggers EMT in these cells and results in decreased E-cadherin expression and increased expression of α-smooth muscle actin, an effect that is downregulated in cells treated with siRNA (Liu et al. 2008a). Finally, transforming growth factor-β (TGF-β)-induced EMT, an important step in kidney fibrosis and metastasis, involves ILK-mediated signaling events (Li et al. 2003, 2007; Shimizu et al. 2006). These studies are of potential importance in cancer biology since TGF-β is cited as an important factor in progression of cancer (Buck and Knabbe 2006; Prud'homme 2007).

Emerging evidence suggests a clinically relevant role for ILK in the process of EMT and in the treatment of drug-resistant malignancies. A study of human colon cancer has revealed that ILK expression is elevated in metastatic lesions and that β-catenin activation, E-cadherin downregulation and Akt-FKHR pathway activation correlate with ILK expression and tumor progression parameters (Bravou et al. 2006), suggesting a connection between ILK and EMT in human cancer. Using human hepatocellular carcinoma as a model, hepatoma cell lines with a mesenchymal phenotype were found to be significantly more resistant to EGFR inhibitors compared to cell lines with an epithelial phenotype (Fuchs et al. 2008). Mesenchymal cell lines resistant to EGFR inhibitors show elevated levels of ILK expression,

Akt activation and STAT3 activation (Fuchs et al. 2008). Introduction of kinase inactive ILK reduces ILK and Akt activity, and increased the sensitivity of these cell lines to EGFR inhibitors in vitro and in vivo in a xenograft model (Fuchs et al. 2008).

In addition to loss of E-cadherin, the process of EMT leads to the loss of β-catenin from adherens junctions, cytoplasmic accumulation and translocation into the nucleus. Overexpression of ILK results in a similar redistribution of β-catenin and activation of the TCF transcription factors (Novak et al. 1998; Somasiri et al. 2001), leading, among other events, to fibronectin deposition (Wu et al. 1998). These observations, in particular the accumulation of nuclear β-catenin, prompted the suggestion that ILK may be a downstream target of the Wnt signaling pathway. In fact, inhibition of ILK activity in mammalian cells directly modulates the Wnt signaling pathway by diminishing the stabilization and nuclear translocation of β-catenin, and β-catenin/Lef-mediated transcription (Oloumi et al. 2006). ILK activity also affects the more chronic, PI3K-dependent effects of Wnt-signaling on GSK-3β phosphorylation (Oloumi et al. 2006).

Adhesion, Migration, Motility, Invasion

The primary goal of EMT in cancer progression is the production of tumor cells with altered adhesive properties and an increased ability to migrate, invade and promote metastasis. Inhibiting ILK activity or expression leads to a reduction in cell migration (Fig. 11.3) (Attwell et al. 2003). AP-1 mediated upregulation of MMP9 expression is regulated by ILK, and inhibition of ILK activity or MMP9 activity reduces invasion into Matrigel by highly invasive human glioblastoma cells as well as ILK-overexpressing mammary epithelial cells (Troussard et al. 2000), suggesting that ILK-mediated increases in MMP9 expression and activity contribute to this phenotype. ILK is also required for activation of Rac and Cdc42 and downstream actin reorganization in epithelial cells (Filipenko et al. 2005). The ILK-binding protein β-parvin can interact with α-PIX, a guanine nucleotide exchange factor for Rac and Cdc42, suggesting a mechanism for ILK-mediated regulation of these small GTPases (Rosenberger et al. 2003; Filipenko et al. 2005). Growth factor-mediated stimulation of ILK is also important in cancer cell motility, as Ang-2 mediates Tie-2-deficient breast cancer cell migration and invasion through a β-integrin/ILK/Akt, GSK-3β/Snail/E-cadherin signaling pathway (Imanishi et al. 2007). Xenograft studies show that Ang-2 overexpression in breast cancer cells promotes metastasis to the lymph nodes and lungs, linking ILK-regulated signaling pathways with the metastatic process in vivo (Imanishi et al. 2007).

Angiogenesis

Recent investigations have yielded important insights into the role of ILK in tumor angiogenesis (Fig. 11.3). Cells overexpressing ILK or harboring constitutively

active ILK produce large amounts of VEGF, a key mediator in the formation of tumor vasculature (Tan et al. 2004a). Importantly, the production of VEGF by cancer cells can be suppressed by inhibiting ILK activity or expression (Tan et al. 2004a). VEGF expression is mediated by ILK through regulation of hypoxia inducible factor 1α (HIF-1α) and suppression of Akt-induced phosphorylation of mTOR (Tan et al. 2004a). ILK-mediated production and secretion of VEGF by tumor cells leads to binding of VEGF by nearby endothelial cells, with consequent effects on survival, proliferation and migration of endothelial cells, all critical functions for neovascularization. Inhibition of ILK in VEGF-treated endothelial cells reduces cell migration and proliferation, as well as blood vessel formation. Moreover, inhibitors of ILK activity in vivo also leads to inhibition of blood vessel formation and tumor growth (Kaneko et al. 2004; Tan et al. 2004a; Younes et al. 2005). Expression of VEGF and HIF-1α, the percentage of blood vessel mass and the number of functional blood vessels decline when established glioblastoma xenografts are treated with inhibitors of ILK activity (Edwards et al. 2008), further demonstrating the importance of ILK in regulating tumor angiogenesis.

ILK as a Therapeutic Target in Cancer

The experimental evidence presented in the previous sections demonstrates that ILK is a critical mediator of multiple cellular processes involved in the development and progression of cancer. It is, therefore, reasonable to suggest that ILK would be a highly relevant target for cancer therapeutics. In fact, there is considerable interest in the development of agents that target ILK expression and activity. There is now considerable evidence that ILK represents an important, druggable target with the potential to achieve beneficial results in the clinical setting.

Inhibition of ILK Expression in Cancer Cells

Novel insights into the mechanistic and functional consequences of manipulating ILK expression on cancer growth and progression have been realized with the development of targeted deletion strategies. The use of RNAi and antisense oligonucleotides (AS) to block ILK production in malignant cells has confirmed the role of ILK in several of the oncogenic processes described above and has revealed the therapeutic potential of downregulating ILK expression in tumors. For example, siRNA-mediated depletion of ILK in cancer cells impairs Akt Ser^{473} phosphorylation (Tan et al. 2004a; Duxbury et al. 2005; Edwards et al. 2006; Liu et al. 2006; Basaki et al. 2007; McDonald et al. 2008b) and results in the induction of apoptosis (Duxbury et al. 2005; Shi et al. 2007; McDonald et al. 2008b). Similarly,

treatment of glioblastoma cells with ILK AS reduces ILK expression and Akt Ser^{473} phosphorylation (Edwards et al. 2005, 2006), and induces apoptosis (Edwards et al. 2005). Furthermore, treatment of mice bearing established glioblastoma xenografts with ILK AS slows tumor growth relative to untreated animals, demonstrating the requirement of ILK expression for the escalation of tumor volume (Edwards et al. 2005). Downregulation of ILK also impairs migration and invasion of cancer cells (Liu et al. 2006, 2007; Shi et al. 2007; Wong et al. 2007). The disruption of cell motility by interfering with ILK expression may be related to cytoskeletal and adhesive changes, as ILK-depleted melanoma cells exhibit defects in adhesion and spreading coupled with a reduction in stress fiber formation (Wong et al. 2007). Stable knockdown of ILK in melanoma cells also impairs the growth of ILK depleted xenografts (Wong et al. 2007), again highlighting the necessity of ILK expression for tumor progression in vivo. Collectively, these studies illustrate the dependence of cancer cells on the kinase and adaptor functions of ILK for oncogenic processes such as survival and invasion, and indicate that AS and RNAi may be effective strategies for interfering clinically with ILK-mediated tumor progression.

Investigations are now focused on the potential for enhancing therapeutic efficacy and overcoming chemoresistance through the use of agents that inhibit ILK expression together with conventional chemotherapy or other targeted therapies. Results are very encouraging. RNAi-induced suppression of ILK expression in gemcitabine-resistant pancreatic adenocarcinoma cells induces caspase-3-mediated apoptosis and renews the sensitivity of these cells to gemcitabine (Duxbury et al. 2005) Significant rescue of gemcitabine resistance in the presence of ILK depletion is achieved with the introduction of activated Akt (Duxbury et al. 2005), indicating the pro-survival role of ILK in establishing chemoresistance in some tumor cells. Simultaneous, targeted disruption of survival and proliferative signaling pathways is also being explored. The utilization of ILK AS in combination with either Raf-1 or MEK inhibitors, and the use of ILK siRNA together with a MEK inhibitor, demonstrate synergy in cellular assays based on a glioblastoma model (Edwards et al. 2005). These studies illustrate the potential therapeutic benefit of downregulating the pro-survival function of ILK by manipulating its expression in combination with strategic application of conventional chemotherapy or other targeted agents.

Increasingly, tissue-specific knockout of ILK expression is being used as the background on which the formation of tumors in vivo through induction by oncogenes or chemical carcinogens is being analyzed. These studies provide further validation of the requirement of ILK in tumor progression. Carcinogen-induced and colitis-associated intestinal tumor formation is markedly diminished in the ILK-null genetic background (Assi et al. 2008), and breast tumors induced by the Neu oncogene are delayed significantly in animals lacking ILK in the mammary gland (Dr Bill Muller, personal communication). Downregulation of cyclin D expression is a major attribute of ILK depletion in the intestinal model, whereas inhibition of Akt Ser^{473} phosphorylation is observed in the mammary tumor model, illustrating the varied roles of ILK in different tumor environments in vivo.

Pharmacologic Inhibition of ILK Function

While inhibition of ILK expression using siRNA and AS are invaluable strategies for dissecting the role of ILK in tumor progression, use of these technologies in the clinical setting remains a significant obstacle. Furthermore, by virtue of their mechanism of action, these modalities interfere with protein expression, potentially leading to undesired consequences. Potent, specific small molecule inhibitors of ILK activity have been developed and exist as critical tools for validating the biological functions of ILK and interrogating the therapeutic efficacy of specifically inhibiting ILK activity. Importantly, pharmacologic inhibition of ILK kinase activity results in significant downregulation of Akt Ser^{473} phosphorylation in several models of human cancer (Persad et al. 2000; Cruet-Hennequart et al. 2003; Tan et al. 2004a; Koul et al. 2005; Yau et al. 2005; Younes et al. 2005, 2007; Liu et al. 2006; Rosano et al. 2006; Troussard et al. 2006; Tabe et al. 2007; Edwards et al. 2008). Although active ILK derived from normal epithelial and mesenchymal cells is sensitive to the kinase suppressive effects of ILK inhibitors, cellular Akt Ser^{473} phosphorylation remains unaffected, suggesting that normal cells may be able to circumvent ILK inhibition, while tumor cells become addicted to ILK (Troussard et al. 2006).

The downstream effects of inhibitor-mediated suppression of ILK activity in cancer cells involve the cellular processes known to be regulated by ILK. Thus, pharmacologic perturbation of ILK in vitro and in vivo reduces proliferation (Koul et al. 2005; Younes et al. 2005, 2007; Edwards et al. 2008), stimulates apoptosis (Yau et al. 2005; Younes et al. 2005, 2007; Liu et al. 2006; Troussard et al. 2006; Tabe et al. 2007; Edwards et al. 2008), inhibits metastatic processes (Troussard et al. 2000; Koul et al. 2005; Rosano et al. 2005, 2006) and interferes with angiogenesis (Tan et al. 2004a; Koul et al. 2005; Edwards et al. 2006). ILK inhibitors also represent the first small molecule inhibitors of pathological Wnt signaling, as inhibiting ILK activity abrogates Wnt-induced β-catenin stabilization and nuclear translocation (Oloumi et al. 2006). Importantly, the ILK inhibitors are well-tolerated in animal models and are not toxic at biologically active doses, even when used in combination with conventional chemotherapeutics (Tan et al. 2004a; Yau et al. 2005; Younes et al. 2005; Liu et al. 2006; Edwards et al. 2008).

Data are now emerging that suggest that inhibition of ILK activity may be a novel strategy for overcoming chemoresistance in tumors. The introduction of kinase-inactive ILK into EGFR inhibitor-resistant, mesenchymal hepatoma cell lines in vitro and hepatic xenografts in vivo increases the sensitivity of these tumor cells to the EGFR inhibitors erlotinib, gefitinib, and cetuximab (Fuchs et al. 2008). Inhibition of ILK may also be effective in overcoming acquired resistance to agents such as imatinib. In chronic mylogeneous leukemia (CML), both tumor cells and CML progenitor cells from imatinib-treated patients express elevated levels of CXCR4, potentially promoting migration of CML cells to bone marrow stroma, and resulting in quiescence and survival of this cell population (Jin et al. 2008). Inhibition of ILK activity or blockade of CXCR4 signaling abrogates these protective effects and overrides imatinib

resistance in the leukemic cells (Jin et al. 2008). This finding is particularly interesting, given that CXCL12, the ligand for CXCR4, is known to stimulate ILK expression (Jones et al. 2007). Finally, targeting ILK in combination with radiotherapy may also improve treatment efficacy when dealing with radioresistant tumors. Integrin-dependent modulation of cancer cell radiosensitivity is attributed in part to ILK-dependent activation of RhoB, and targeting ILK leads to the regulation of radiation-induced mitotic cell death in glioblastoma (Monferran et al. 2008). Collectively, these studies demonstrate that specific inhibitors of ILK kinase activity result in growth arrest and apoptosis in vivo, and provide support for targeting ILK in human cancer, particularly in the combination setting.

New Interacting Partners

A consistent theme in ILK biology is the presence of a complex network of protein –protein interactions critical for engaging ILK-mediated control of cell spreading, migration, growth, cell cycle progression and survival. The past decade has seen substantive progress in the elucidating the identity and functional ramifications of these interactions, but our understanding of how these interactions interdigitate and mediate such an array of vital cellular processes remains incomplete. Efforts to more completely characterize the ILK interactome have lead to the application of a proteomic strategy based on stable isotope labeling with amino acids in cell culture (SILAC) technology to identify ILK–protein interactions (Dobreva et al. 2008). This proteome-wide analysis of cytoskeletal-associated ILK complexes has yielded many novel interactions and is now revealing exciting and unexpected cellular functions of ILK (Dobreva et al. 2008). Some of these interactions have initiated new areas of active investigation, most notably the identification of a novel complex between ILK and Rictor (McDonald et al. 2008b), and the discovery of a new role of ILK in centrosome biology (Fielding et al. 2008b).

ILK–Rictor Complex

The identification of Rictor as an ILK-binding protein is a particularly intriguing observation (Fig. 11.3) (Dobreva et al. 2008; McDonald et al. 2008b). Rictor has been described as a requisite member of mTORC2, a mammalian TOR complex reported to mediate Akt Ser^{473} phosphorylation (Sarbassov et al. 2005). Together with the original identification of Rictor as a regulator of actin cytoskeletal dynamics (Sarbassov et al. 2004), data point to overlapping functions of ILK and Rictor. Further investigation has revealed that the interaction is direct, present under endogenous conditions and especially prominent in membrane extensions of cancer cells (McDonald et al. 2008b). Akt and ^{Ser473}P-Akt are present in ILK immunoprecipitates, and siRNA-mediated depletion of Rictor, or expression of an amino-terminal

fragment of Rictor, reduces the amount of ^{Ser473}P-Akt in the ILK complex. In contrast, global inhibition of mTOR expression enhances ILK-associated Akt Ser^{473} phosphorylation in the cancer cells examined. It is noteworthy that the ILK–Rictor interaction is the first report of a complex between Rictor and proteins not associated with mTORC2. A further interaction between Rictor and myosin 1c to form a complex that mediates cortical actin dynamics has now been reported (Hagan et al. 2008), and confirms the capacity of Rictor to interact with proteins independent of its association with mTORC2.

The identification and characterization of the ILK–Rictor complex is of consequence in understanding the mechanism by which Akt Ser^{473} phosphorylation is regulated in cancer cells. It is clear that Rictor associates with a multiprotein complex containing ILK and active Akt that is independent of, but co-exists with mTORC2. In some cancer cell types, when mTOR function is dysregulated or depleted, phosphorylation of Akt on Ser^{473} is induced, and this phosphorylation requires ILK and Rictor. The results of this study also suggest that the ILK–Rictor interaction is required for promoting Akt Ser^{473} phosphorylation in the ILK complex. Finally, the observation that disruption of mTORC1 and mTORC2 function stimulates cancer cells to make available other kinase complexes to regulate Akt phosphorylation suggests that the ILK–Rictor complex may play a role in the development of resistance to mTOR inhibitors and that inhibition of ILK activity concomitant with disruption of mTOR function may be necessary if therapeutic efficacy is to be realized.

New Role in Spindles/Centrosomes

Among the more unanticipated findings revealed in the proteomic analysis of the ILK interactome is the association between ILK and several centrosomal and mitotic spindle proteins such as ch-TOG, α-tubulin, β-tubulin and RUVBL-1 (Dobreva et al. 2008). These findings have been interpreted to mean that ILK also exists as part of a complex in centrosomes and plays a functional role in mitosis. Detailed characterization of these interactions demonstrated that ILK is present in FAs and centrosomes simultaneously in the interphase cells (Fielding et al. 2008b). ILK colocalizes with ch–TOG and RUVBL1 in centrosomes, while α-parvin is absent from the centrosome. The differential localization of these ILK-binding partners suggests that ILK forms distinct complexes in the two separate subcellular spaces. Inhibition of ILK activity or expression induces dramatic defects in mitotic spindle organization and inhibits Aurora A-TACC3/ch–TOG interactions (Fielding et al. 2008a, b), indicating an important functional role for ILK in mitotic spindle assembly.

Summary

ILK is a complex, multidomain protein that dynamically regulates signals derived from cell–ECM interactions or from growth factor receptor stimulation through a combination of intermolecular interactions and specific phosphotransferase activity.

The activity and expression of ILK are controlled by a network of intracellular and intercellular processes that result in aberrant ILK expression and signaling in many human cancers. As a consequence of its regulatory actions, ILK plays an important role in controlling an array of biological processes critical for the progression of malignant disease, including proliferation, survival, EMT, migration, invasion and angiogenesis. A large body of evidence now exists to support ILK as a novel target for therapeutic intervention in cancer and agents designed to interfere with aberrant ILK expression and activity are being evaluated. Finally, interrogation of the ILK interactome using cutting-edge proteomic strategies is uncovering novel interactions and cellular functions of ILK that may have important implications for the development of effective agents for cancer therapy.

Acknowledgments This research was funded by grants from the Canadian Institutes of Health Research (CIHR), National Cancer Institute of Canada with funds from the Terry Fox Foundation and the Canadian Breast Cancer Research Alliance with special funding support from the Canadian Breast Cancer Foundation and the Cancer Research Society.

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Chapter 12
Focal Adhesion Kinase with the Interacting Proteins and Signaling in Cancer

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Abstract Human cancer is characterized by a process of tumor cell motility, invasion, and metastasis. One of the critical tyrosine kinases that is linked to these processes of tumor invasion and survival is the Focal Adhesion Kinase (FAK). Our laboratory was the first to isolate FAK from human tumors, and in our initial report, we demonstrated that FAK mRNA was upregulated in invasive and metastatic human breast and colon cancer samples. We identified several binding partners of FAK in cancer cells, such as RIP, VEGFR-3, p53, and IGFR proteins. Recently, we have cloned FAK promoter and have found that FAK promoter contains p53 binding sites, and that p53 inhibits FAK transcription and regulates its expression in tumor samples. In addition, we have found that N-MYCN binds FAK promoter and induces FAK transcription in neuroblastoma cells. Thus, we will review FAK structure and discuss novel proteins and pathways interacting with FAK and discuss the role of FAK-mediated signaling in tumorigenesis (especially in breast, colon, pancreatic, and neuroblastoma cancers). Thus, this review will be focused on FAK signal transduction pathways and FAK-targeted therapy, linking signaling from extracellular matrix to the nucleus in cancer cells.

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R. Zent and A. Pozzi (eds.), *Cell-Extracellular Matrix Interactions in Cancer,*
DOI 10.1007/978-1-4419-0814-8_12, © Springer Science+Business Media, LLC 2010

Keywords Focal adhesion Kinase • Cancer • Apoptosis • Tumorigenesis

Introduction

Focal Adhesion Kinase (FAK) was discovered more than 15 years ago as a protein that plays a critical role in intracellular processes of cell spreading, adhesion, motility, survival, and cell cycle progression. One of the critical tyrosine kinases that is linked to the processes of tumor invasion and survival is the FAK. The FAK gene encodes a nonreceptor tyrosine kinase that localizes at contact points of cells with extracellular matrix and is activated by integrin (cell surface receptor) signaling. The FAK gene was first isolated from chicken embryo fibroblasts transformed by v-src (Schaller et al. 1992). Our laboratory was the first to isolate FAK gene from human tumors, and we demonstrated that FAK mRNA was upregulated in invasive and metastatic human breast and colon cancer samples (Weiner et al. 1993). At the same time, matched samples of normal colon and breast tissue from the same patients had almost no detectable FAK expression. This was the first evidence that FAK might be regulated at the level of gene transcription, as well as other mechanisms (such as gene amplification). Subsequently, we have demonstrated upregulation of FAK at the protein level in numerous types of human tumors, including colon, breast, thyroid, ovarian, melanoma, and sarcoma (Owens et al. 1995; Owens et al. 1996; Judson et al. 1999; Cance et al. 2000; Lark et al. 2003; Lightfoot et al. 2004) (Fig. 12.1a). In addition, we have found novel interaction of FAK with several binding partners, such as: RIP (Kurenova et al. 2004), linking FAK with the death-receptor pathways; p53 (Golubovskaya et al. 2005), linking FAK with the apoptotic/survival nuclear pathways (Golubovskaya and Cance 2007; Cance and Golubovskaya 2008); VEGFR-3 (Garces et al. 2006), linking FAK with lymphagenesis and angiogenesis; and IFG-1R (Liu et al. 2008), linking FAK with the insulin growth factor receptor pathway, critical in pancreatic cancers. In addition, we have cloned the regulatory promoter region of the FAK gene, and confirmed transcriptional upregulation in cancer cell lines (Golubovskaya et al. 2004). We have found that the FAK promoter contains p53 binding sites, and that p53 inhibits FAK transcription both in vitro (Golubovskaya et al. 2004) and in vivo (Golubovskaya et al. 2008b). Recently, we demonstrated, that N-MYCN was able to bind FAK promoter and upregulate its expression in neuroblastoma cells (Beierle et al. 2007). Thus, this review will be focused on FAK intracellular signaling in cancer, linking signaling from extracellular matrix to the nucleus. We will discuss the role of FAK expression, localization, activity, protein–protein interaction, and survival signaling in the development of cancer. We will discuss the FAK structure, function, binding partners and the novel FAK crosstalk pathways in the junction of death and growth factor receptors and apoptotic and survival pathways. Then, we will pay attention to novel therapeutics approaches to target these interaction and pathways in cancer.

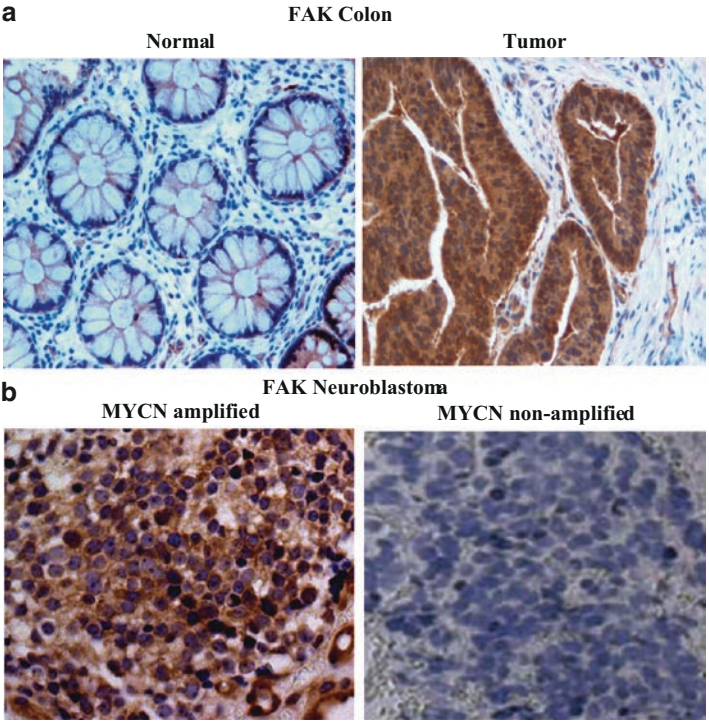

Fig. 12.1 *Focal Adhesion Kinase is overexpressed in tumor samples*. (**a**) Immunohistochemical staining is shown for colon cancer samples. *Left panel*: normal tissue, *right panel*: matched tumor tissue from the same patient. (**b**) FAK protein detection in formalin-fixed, paraffin embedded human neuroblastoma specimens. Antibody to $p125^{FAK}$ was utilized to detect FAK staining in human neuroblastoma specimens. *Left panel*: This photomicrograph (40×) shows a *MYCN* amplified INSS stage 4 neuroblastoma specimen with significant staining for $p125^{FAK}$. Right panel shows a *MYCN* nonamplified INSS stage 4 neuroblastoma specimen with no significant $p125^{FAK}$ staining

Structure and Function of Focal Adhesion Kinase protein

Gene and Promoter Structure

First, FAK cDNA encoding 125 kDa protein was isolated from chicken embryo cells (Schaller et al. 1992). The human FAK (also known as PTK2, protein tyrosine kinase 2) gene has been mapped to chromosome 8 (Fiedorek and Kay 1995; Agochiya et al. 1999). Human complete FAK mRNA sequence is a 3,791 bases long sequence (Whitney et al. 1993). We were the first group to isolate human FAK cDNA from primary sarcoma tissue and found increased FAKmRNA in tumor samples when compared with the normal tissue samples (Weiner et al. 1993).

Recently, the genomic structure of FAK has been characterized (Corsi et al. 2006). The gene coding sequence contains 34 exons, and genomic sequence spans 230 kb (Corsi et al. 2006). We were the first group to clone the human FAK

promoter, regulating FAK expression (Golubovskaya et al. 2004). The core promoter contains 600 base pairs and includes many transcription binding sites, such as AP-1, AP-2, SP-1, PU.1, GCF, TCF-1, EGR-1, NF-κ B, and p53 (Golubovskaya et al. 2004). Interestingly, we found two transcription binding sites for p53 in the FAK promoter, and found that p53 can block the FAK promoter activity (Golubovskaya et al. 2004). Recently, mouse promoter has been cloned that was highly homologous to the human promoter and contained the same binding sites (Corsi et al. 2006).

FAK Protein Structure

The FAK protein is a 125 kDa tyrosine kinase (p125^{FAK}) with a large amino-N-terminal domain, exhibiting homology with a FERM (protein 4.1, ezrin, radixin, and moesin) domain with an autophosphorylation site (Y-397), a central catalytic domain, and a large carboxy-C-terminal domain that contains a number of potential protein interacting sites, including two proline-rich domains and FAT domain (Schaller et al. 1994; Schaller and Parsons 1994; Hanks and Polte 1997) (Fig. 12.2).

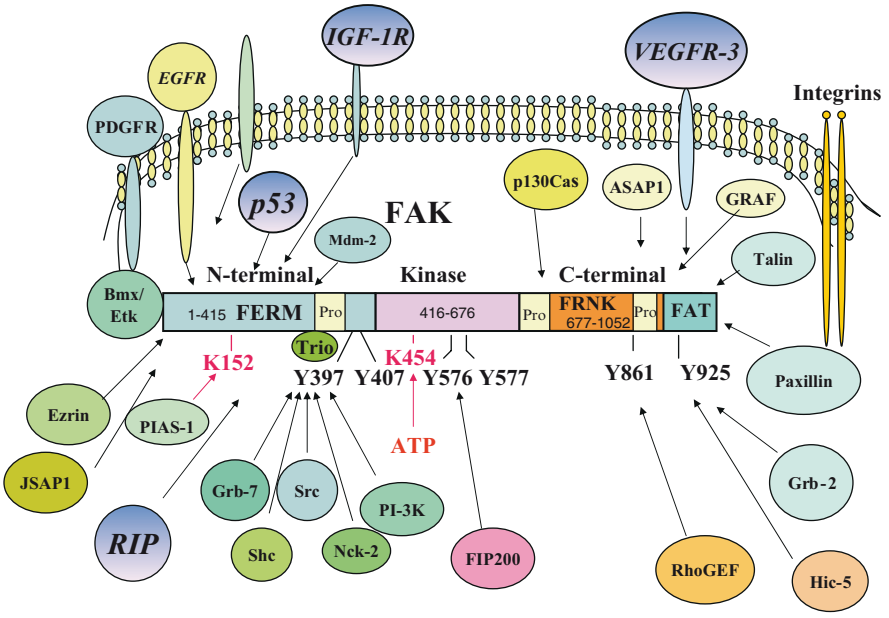

Fig. 12.2 *FAK structure*. FAK has the N-terminal, Kinase domain, and the C-terminal domains. The N-terminal domain has Y-397-Y-autophosphorylation site. The Kinase domain has Y576/577 tyrosines important for catalytic activity of FAK. The C-terminal domain of FAK has Y861 and Y925 tyrosines. Different proteins bind to these domains and involved in motility and survival signaling, The N-terminal domain (205–422 a.a.) of FAK is involved in interaction with Src, RIP, p53, PI3Kinase, PIAS-1, PI3Kinase, Grb-7, EGFR/PDGFR, Ezrin, Bmx, Trio, and others. Kinase domain is involved in binding with FIP200 protein. ASAP, p130Cas, Grb-2, Paxillin, Talin, RhoGEFp190, and other proteins bind C-terminal domain of FAK. Interactions of FAK and other proteins demonstrated by group are shown in Italics

The N-Terminal Domain

The function of the N-terminal, homologous to FERM domain was linked to the binding of integrins, via their β subunits (Schaller et al. 1995). The N-terminal domain (1–415 a.a) of FAK protein contains the major autophosphorylation site Y397-tyrosine, that in phosphorylated form becomes a binding site of SH-2 domain of Src, leading to its conformational changes and activation (Hanks and Polte 1997). Crystal structure of the N-terminal domain of avian FAK, containing FERM domain has been recently shown (Ceccarelli et al. 2006). Interesting negative regulation of FAK function by FERM domain was revealed by (Cooper et al. 2003) and (Lietha et al. 2007). Tyrosine phosphorylation of FAK causes binding and activation of Src that leads to tyrosine phosphorylation Y407 and Y576, Y577 – major phosphorylation sites in the catalytic domain of FAK; Y861, and Y925 (Hanks and Polte 1997; McLean et al. 2005), and then to phosphorylation of FAK binding proteins, such as paxillin and Cas (Schaller et al. 1999). This cascade causes subsequent cytoskeletal changes and activation of RAS-MAPK (mitogen-activated protein kinase) signaling pathways (Hanks et al. 2003; McLean et al. 2005). Thus, FAK-Src signaling complex activates many other signaling proteins, involved in survival, motility, and metastatic, invasive phenotype in cancer cells. Phosphorylated Y397 FAK is able to recruit another important signaling protein, p85 PI3-kinase (phosphoinositide 3-kinase), growth factor receptor bound protein Grb 7, phospholipase Cγ (PLCγ) and others. Thus, the N-terminal domain of FAK binds to the extracellular matrix receptors, integrins, growth factor receptors or important signaling cytoplasmic, cytoskeletal, and nuclear proteins, mediating signaling from the extracellular matrix to the cytoplasm and nucleus and controlling cytoskeletal changes, survival, motility, and invasion.

The Kinase Domain

The central kinase (catalytic) domain of FAK (416–676 amino-acids) is the most conserved domain in vertebrate and nonvertebrate organisms (Corsi et al. 2006). The central catalytic domain of FAK contains Y576 and Y577, major phosphorylation sites, and also K454, ATP binding site (Fig. 12.2). Phosphorylation of FAK by Src on Y576 and Y577 is an important step in the formation of active signaling complex and is required for maximal enzymatic activity of FAK (Calalb et al. 1995). The crystal structure of FAK kinase domain revealed open conformation similar to the fibroblast growth factor receptor-1 (FGFR-1) and vascular endothelial growth factor receptor (VEGFR) (Nowakowski et al. 2002). Recently the mechanism of autoinhibition of Focal adhesion kinase was suggested on the basis of structural studies of FAK FERM domain-kinase domain protein. The N-terminal FERM domain directly binds the kinase domain, blocking access to the catalytic cleft and protecting the FAK activation loop from Src phosphorylation (Lietha et al. 2007).

The C-Terminal Domain

Different proteins can bind to the C-terminal domain of $p125^{FAK}$ (677–1052 amino-acids), including paxillin, p130cas, PI3-kinase, GTP-ase-activating protein Graf, leading to changes in the cytoskeleton and to the activation of Ras-MAP kinase pathway (Schaller and Parsons 1994; Windham et al. 2002; Hanks et al. 2003; Parsons 2003). The carboxy-terminal domain of FAK contains sequences responsible for its targeting to focal adhesions, also known as the FAT domain. Alternative splicing of FAK results in autonomous expression of the C-terminal part of FAK, FAK-related non-kinase (FRNK) (Richardson and Parsons 1995). The crystal structure of the C-terminal domain of FAK, FAT, has been determined recently by several groups (Hayashi et al. 2002; Magis et al. 2009). It forms a four-helix bundle that resembles those found in two other proteins involved in cell adhesion, alpha-catenin and vinculin FAT domain mediates signaling through Grb-2 binding to Y925 site of FAK (Arold et al. 2002).

FAK Function in Cancer Cells

FAK has numerous functions in cell survival, motility, metastasis, invasion, and angiogenesis (Fig. 12.3).

Survival

FAK plays a major role in survival signaling and has been linked to detachment-induced apoptosis or anoikis (Frisch et al. 1996). It has been shown that constitutively activated forms of FAK rescued epithelial cells from anoikis, suggesting that FAK can regulate this process (Frisch et al. 1996; Frisch and Ruoslahti 1997; Frisch 1999b; Frisch and Screaton 2001; Windham et al. 2002). Similarly, both FAK antisense oligonucleotides (Xu et al. 1996; Smith et al. 2005), as well as dominant-negative FAK protein (FAK-CD), caused cell detachment and apoptosis in tumor cells (Xu et al. 1996; Xu et al. 1998; Xu et al. 2000; van De Water et al. 2001; Golubovskaya et al. 2002; Beviglia et al. 2003; Gabarra-Niecko et al. 2003; Golubovskaya et al. 2003; Park et al. 2004). The antiapoptotic role of FAK was also demonstrated in FAK-transfected FAK/HL60 cells that were highly resistant to apoptosis induced with etoposide and hydrogen peroxide compared with the parental HL-60 cells or the vector-transfected cells (Sonoda et al. 2000; Kasahara et al. 2002). HL-60/FAK cells activated the AKT pathway and NF-κ B survival pathways with the induction of inhibitor-of-apoptosis proteins, IAPs (Sonoda et al. 2000). We have demonstrated that EGFR and Src signaling cooperate with FAK survival signaling in colon and breast cancer cells (Golubovskaya et al. 2002; Park et al. 1999; Golubovskaya et al. 2003; Park et al. 2004). We have also demonstrated that simultaneous inhibition of Src and FAK or EGFR and FAK pathways was able to increase apoptosis in cancer cells (Golubovskaya et al. 2002; Golubovskaya et al. 2003). Thus, cancer cells use cooperative function of kinases and growth factor receptor signaling to increase survival.

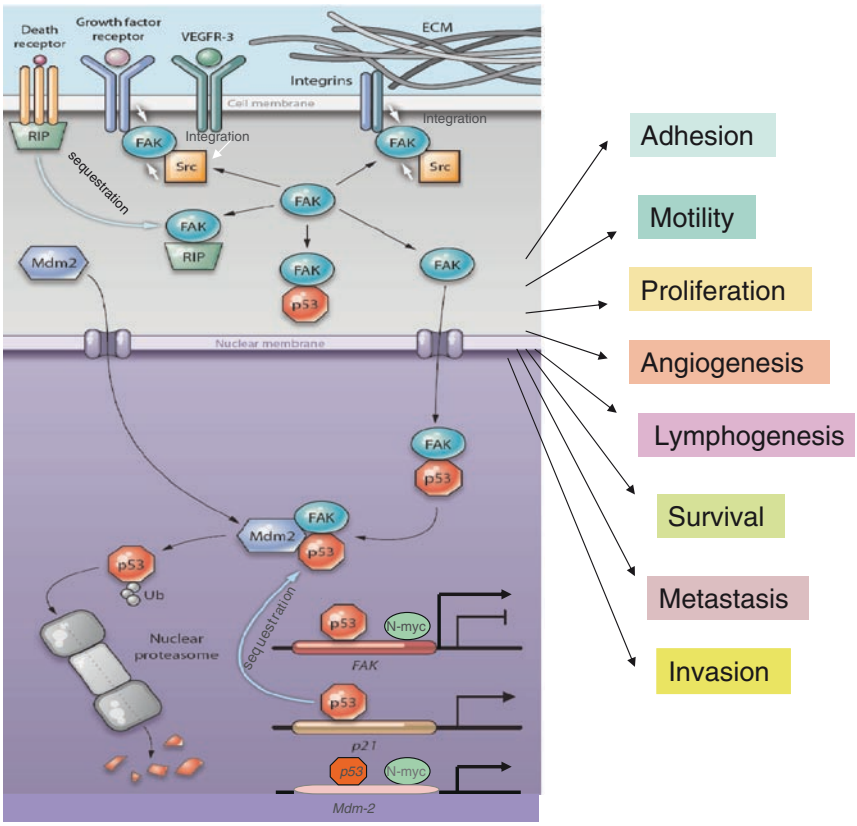

Fig. 12.3 Sequestration and Integration Model of FAK functions in cells and signal transduction pathways from extracellular matrix to the cytoplasm and nucleus. Focal Adhesion Kinase integrates signals from growth factor receptors (EGFR, IGFR), vascular endothelial growth factor receptors (VEGFR-3), Src and integrins to control motility, survival, proliferation, metastasis, lymphangiogenesis and angiogenesis (integration arrows). Numerous binding partners of FAK mediate this signaling. FAK sequesters pro-apoptotic proteins, such as RIP and p53 from apoptotic signaling (sequestration arrows). P53 binds FAK promoter and inhibits its transcription. MYCN protein binds FAK promoter and induces FAK transcription. MYCN also binds Mdm-2 promoter, as p53 and induces Mdm-2 transcription. FAK sequesters p53, binds Mdm-2 protein and degrades p53 by ubiquitination. There is a feedback loop in FAK-p53 regulation. Thus, FAK mediates signaling from extracellular matrix to the cytoplasm and nucleus. Part of the model was discussed in detail (Cance and Golubovskaya, 2008)

Motility

FAK has also been shown to be important for cell motility (Hauck et al. 2001; Schaller 2001; Hanks et al. 2003; Schlaepfer and Mitra 2004). FAK-null embryos exhibit decreased motility in vitro (Illc et al. 1995). Furthermore, enforced expression of FAK stimulated cell migration (Hildebrand et al. 1993; Sieg et al. 1999). Cell migration is initiated by protrusion at the leading edge of the cell, by the formation of peripheral adhesions, exertion of force on these adhesions, and then the release of the adhesions at the rear of the cell (Tilghman et al. 2005). FAK is involved in the regulation of

migration, although the precise mechanism of this FAK-regulated migration is unclear. FAK has been shown to be required for the organization of the leading edge in migrating cells by coordinating integrin signaling in order to direct the correct activation of membrane protrusion (Tilghman et al. 2005). SH2 domain of Src, targeting Src to focal adhesions and Y397 activity has been shown to be important for motility (Yeo et al. 2006). PI3 kinase has been also shown to be critical for FAK-mediated motility in Chinese hamster ovary (CHO) cells (Reiske et al. 1999). Tumor suppressor gene PTEN, encoding phosphatase has been shown to interact with FAK, caused its dephosphorylation and blocked motility (Tamura et al. 1998). Moreover, Y397 FAK was important for PTEN interaction with FAK (Tamura et al. 1999). Overexpression of FAK reversed the inhibitory effect of PTEN on cell migration (Tamura et al. 1998).

Metastasis, Invasion, and Angiogenesis

Activation of FAK is linked to invasion and metastasis signaling pathways. FAK was important in Erb-2/Erb3-induced oncogenic transformation and invasion (Benlimame et al. 2005). Inhibition of FAK in FAK-proficient invasive cancer cells prevented cell invasion and metastasis processes (Benlimame et al. 2005). In addition, FAK has been shown to be activated in invading fibrosarcoma and regulated metastasis (Hanada et al. 2005). Inhibition of FAK with dominant-negative FAK-CD disrupted the invasion of cancer cells (Hauck et al. 2001). We have also shown that high FAK expression in breast cancers associated with tumor aggressive phenotype (Lark et al. 2005). Subsequently, we analyzed FAK expression in preinvasive ductal carcinoma in situ, DCIS tumors and detected protein overexpression in preinvasive tumors (Lightfoot et al. 2004), suggesting that FAK survival function occurs as an early event in breast tumorigenesis.

Overexpression of FAK in vascular endothelial cells promoted angiogenesis in transgenic mice (Peng et al. 2004). Overexpession of FAK induced human retinal endothelial cell (HREC) migration and in vivo angiogenesis (Kornberg et al. 2004). FAK activity and phosphorylation of Y925 site of FAK promoted angiogenic switch during tumor progression (Mitra et al. 2006). FAK-Grb2-MAPK signaling has been shown to be important for promoting angiogenesis. Furthermore, inhibition of FAK resulted in disruption of angiogenesis (Mitra et al. 2006). FAK and Src catalytic activities are important to promote VEGF-dependent angiogenesis (Mitra and Schlaepfer 2006). Thus, FAK is involved in angiogenesis and plays a major role in tumorigenesis.

FAK-Protein Binding Partners and Signaling

The FAK and N-, Central and C-Terminal Domain Binding Complexes

FAK has numerous binding partners in the N-terminal, Central and C-terminal domains (Fig. 12.2). The N-terminal domain of FAK contains one proline-rich domain, and the C-terminal domain of FAK contains another two proline-rich domains that

are sites of binding proteins, containing SH3 domains. The C-terminal part of C-terminal domain of FAK (853–1012 a.a) called FAT (Focal adhesion targeting domain) domain that is necessary for targeting of FAK to focal adhesion complexes through binding with different proteins (paxillin, talin, Rho, etc). We classified all binding partners by the binding to the specific domain of FAK (N, Central or C-terminal) described all these binding partners in detail in the recent review (Golubovskaya and Cance 2007). In the present review we will focus on four recent novel binding partners ones, found by our group (RIP, VEGFR-3, p53, and IGF-1R) (Fig. 12.3) and will discuss novel FAK and N-myc signaling in neuroblastoma (Fig. 12.3).

FAK and RIP

It has been shown that the constitutively activated forms of FAK rescued epithelial cells from anoikis, suggesting that FAK can regulate this process (Frisch et al. 1996; Frisch and Ruoslahti 1997; Frisch 1999a; Frisch and Screaton 2001; Windham et al. 2002). Tumor cells have been postulated to be resistant to anoikis to allow them to survive during the processes of invasion and metastasis that require anchorage-independent survival (Hanahan and Weinberg 2000; Frisch and Screaton 2001; Grossmann 2002; Douma et al. 2004). Intriguingly, there was evidence for the involvement of death-receptor-related, death-domain-containing proteins in anoikis (Frisch 1999a; Rytomaa et al. 1999), however, the linkage of the signaling pathways to the death receptors remained unknown (Frisch and Screaton 2001) until when we found the interaction of FAK with the Receptor Interacting Protein (RIP), a key mediator of tumor necrosis factor-induced NF-κB and JNK activation (Kurenova et al. 2004). We have shown that RIP provides proapoptotic signals in FAK-deficient cells and concluded that the ability of FAK to suppress apoptosis is mediated by binding to receptor-interacting protein RIP. RIP–serine–threonine kinase with the C-terminal death domain (DD) is a 74 kDa protein constitutively expressed in many tissues and also inducible after TNF-alpha stimulation (Stanger et al. 1995; Kelliher et al. 1998; Meylan and Tschopp 2005). Through DD RIP interacts with the death domains of cell surface receptors of the Tumor Necrosis Factor (TNF) superfamily and death domain adaptor proteins (Stanger et al. 1995; Chinnaiyan et al. 1996; Hsu et al. 1996; Duan and Dixit 1997) and is a major component of the death receptor complex (Baud and Karin 2001; Chen and Goeddel 2002).

It is well established that RIP mainly acts as cell survival factor to protect against TNF-induced apoptosis via NF-κB activation (Liu et al. 1996; Kelliher et al. 1998; Martinon et al. 2000; Baud and Karin 2001; Karin and Lin 2002; Blonska et al. 2004). But opposing the function of RIP as a cell-death inducer is also well documented. It has been shown that RIP is required for Fas-induced caspase-independent (Holler et al. 2000) and caspase-dependent (Barcia et al. 2003) cell death in T-cells and for TNF-induced necrotic cell death (Lin et al. 2004), RIP is essential for ROS-induced cell death (Shen et al. 2004), it is important for DNA damage-induced, p53-independent cell death (Hur et al. 2006), it also provides apoptotic

signals in cells with attenuated FAK and in anoikis (Kurenova et al. 2004). RIP protein has dual survival and apoptotic functions: it can recruit CRADD (apoptosis) and NF-κ B (survival/proliferation) (Thakar et al. 2006). Hence, RIP has a dual function and is capable of either inducing apoptosis or activating cellular survival signals and it has been proposed that RIP is one of the switches between the cell survival and apoptosis (Martinon et al. 2000). However, the mechanisms by which such diverse functions are selected remain unclear. Cleavage of RIP by caspase-8 during TNF, Fas or TRAIL-induced apoptosis generate C-terminal fragment of RIP, which possess strong proapoptotic activity. This was suggested as a mechanism, leading to death pathway (Lin et al. 1999; Kim et al. 2000; Martinon et al. 2000). We have found RIP physically bound to FAK in vivo and in vitro and have shown that RIP provides proapoptotic signals that are suppressed by its binding to FAK (Kurenova et al. 2004). Thus, FAK sequesters RIP from apoptotic signaling (Fig. 12.3). We have identified that RIP binds to the N-terminal domain of FAK (Kurenova et al. 2004). We also narrowed down the binding region in RIP to its death domain. We have found that RIP disappears from the FAK-RIP complex after 4–5 h of treatment and at the same time we have seen an increase of RIP-FADD complex, which is the main component of the DISC (Kim et al. 2000; Schneider-Brachert et al. 2004). These changes in complex distributions are accompanied by rapid FAK tyrosine dephosphorylation and following degradation.

Our data well correlate with the recent finding from Tadashi lab that FAK determines the pathway leading to death or survival in TNF-alpha/actinomycin D (Act D)-stimulated fibroblasts (Takahashi et al. 2007). Authors found that FAK−/− cells are more sensitive to TNF-alpha-induced apoptosis in the presence of Act D when compared with the FAK+/− cells. They also analyzed the dynamic of formation of prosurvival complex I comprising TNFR1, TRADD, RIP, and TRAF2 and proapoptotic complex II, the death-inducing signaling complex (DISC), which contains TNFR1, TRADD, RIP, and FADD, and procaspase-8 proteins. Coimmunoprecipitation assays revealed that RIP is included in complex I in FAK+/− cells, and FAK was associated with RIP. On the other hand, RIP is included in DISC in FAK−/− cells. They concluded that FAK might be a key protein in the formation of complex I and the activation of NF-κB (Takahashi et al. 2007). These data shed light on previously published work on TNF-alpha-mediated activation of NF-κB which depends on the association of RIP and FAK (Funakoshi-Tago et al. 2003). It was shown that TNF-induced NF-κB DNA binding activity and activation of IκB kinases (IKKs) were markedly impaired in FAK−/− cells. Association of RIP with TRAF2 were not observed in FAK−/− cells, resulting in a failure of RIP to recruit the IKK complex. Interestingly, TNF-induced interleukin (IL)-6 production was nearly abolished in $FAK^{-/-}$ fibroblasts, whereas a normal level of production was obtained in $FAK^{+/-}$ or $FAK^{+/+}$ fibroblasts. The reintroduction of wild type FAK into FAK−/− cells restored the interaction of RIP with TRAF2 and the IKK complex and allowed the recovery of NF-κB activation and subsequent IL-6 production, which suggested a novel role for FAK in the NF-κB activation pathway leading to the production of cytokines (Funakoshi-Tago et al. 2003). Recently, it was confirmed and shown that FAK promoted TNF-alpha-stimulated MAPK activation needed for maximal IL-6 production

(Schlaepfer et al. 2007). TNF-alpha-stimulated the FAK catalytic activation and IL-6 production were inhibited by the FAK N-terminal, but not the FAK C-terminal domain overexpression. Analysis of FAK(−/−) fibroblasts stably reconstituted with wild type or various FAK point mutants showed that FAK catalytic activity, Tyr-397 phosphorylation, and the proline-rich region of FAK (Pro 712/713) were required for TNF-alpha-stimulated MAPK activation and IL-6 production (Schlaepfer et al. 2007). The authors speculate that FAK–RIP interaction is involved in cytokine production.

Although RIP possesses serine/threonine kinase activity, the biological significance of kinase activity is poorly understood (Hsu et al. 1996; Kim et al. 2001). RIP kinase activity is not required for NF-κB activation (Ting et al. 1996; Devin et al. 2000). Only lately some data emerged that kinase activity of RIP is important for an alternative, caspase-8 independent cell death pathway of necrosis (Holler et al. 2000; Vanden Berghe et al. 2004) and is also required for the TNF-mediated activation of ERK (Devin et al. 2003; Lee et al. 2003). Based on our recent data, we speculate that RIP is involved in the regulation of the balance between Tyr- and Ser-phosphorylated forms of FAK and thus in the turnover of FAK in focal adhesions, its kinase activity and the balance between cell attachment/detachment.

FAK and VEGFR-3

Vascular endothelial growth factor (VEGF) is one of the known angiogenic and lymphangiogenic growth factors, stimulating the formation of new blood vessels or angiogenesis. FAK has been shown to play a major role in vasculogenesis. It has been shown that VEGF induced tyrosine phosphorylation of FAK in human umbilical vein endothelial cells (HUVEC) and other endothelial cell line (Abedi and Zachary 1997). VEGF-induced stimulation of FAK phosphorylation was also demonstrated in cultured rat cardiac myocytes that was accompanied by subcellular translocation of FAK from perinuclear sites to the focal adhesions and increased association with the adaptor proteins Shc, Grb-2, and c-Src (Takahashi et al. 1999). VEGF-induced phosphorylation of FAK was induced in human brain microvascular endothelial cell (HBMEC) (Avraham et al. 2003). Furthermore, inhibition of FAK with the dominant-negative inhibitor FRNK (FAK-related non-kinase) or the C-terminal FAK (FAK-CD) significantly decreased HBMEC spreading and migration (Avraham et al. 2003; Avraham et al. 2004). In addition, angiogenic inhibitor endostatin blocked VEGF-induced activation of FAK (Kim et al. 2002).

The vascular endothelial growth factor receptors (VEGFRs) are a family of receptor tyrosine kinases that play key roles in angiogenesis and lymphangiogenesis. VEGFR-1 (flt-1) and VEGFR-2 (flk-1/kdr) are primarily located on vascular endothelial cells and when activated by their cognate ligands play a major role in tumor angiogenesis (Otrock et al. 2007).

VEGFR-3 or Flt4 is a receptor tyrosine kinase playing a major role in lymphangiogenesis (Plate 2001; Alitalo and Carmeliet 2002) and has also been linked to tumorigenesis. VEGFR-3 is activated by its specific ligands, VEGF-C and VEGF-D which promote cancer progression (Skobe et al. 2001; Achen and Stacker 2006).

The VEGFR-3 protein is divided into two domains: a 775-amino acid, N-terminal extracellular domain and a C-terminal intracellular kinase domain further divided into two regions, TK1 and TK2, by a 65-amino acid sequence (Aprelikova et al. 1992; Pajusola et al. 1992). The TK1 domain contains the ATP-binding site and the TK2 domain contains the tyrosine kinase catalytic site as well as the site of receptor autophosphorylation at Y1068 (Pajusola et al. 1992; Salameh et al. 2005). Mutation of Y1068 completely eliminated ligand-induced receptor phosphorylation and abrogated all VEGFR-3 functions. Tyrosine 1063 promoted cell survival through its interaction with Crk I/II promoting MKK4-dependent activation of c-Jun N-terminal kinase with resultant increased expression of c-Jun. The tyrosines 1230 and 1231 promoted cell proliferation, migration and survival through their association with Grb2 resulting in activation of AKT and ERK 1,2 (Fournier et al. 1995).

The VEGFR-3 receptor gene mapped to the long arm of chromosome 5 and alternative spicing of the primary VEGFR-3 gene leads to the expression of two VEGFR-3 isoforms (Galland et al. 1992). The long form (VEGFR-3-L, 1363 amino acids) differs from the short form (VEGFR-3-S, 1298 amino acids) by the presence of a 65 amino acid stretch at the distal end of the cytoplasmic tail. Both forms of VEGFR-3 formed tumors after subcutaneous implantation of stably-transfected NIH3T3 cells in mice (Fournier et al. 1995). Ligand-induced activation of VEGFR-3 led to its physical interaction with RAS-activating adapter protein Grb2 and its physical interaction and phosphorylation of the adaptor protein Shc, which is the only described substrate for VEGFR-3 tyrosine kinase activity (Fournier et al. 1996).

To explore the role of FAK in lymphangiogenesis and angiogenesis, we have performed phage display assay to map protein–protein interactions with the C-terminal domain of FAK and demonstrated that VEGFR-3 binds to the C-terminal and FAT domains of FAK (Garces et al. 2006). We synthesized TAT-conjugated peptide, containing 12 amino-acid binding site of VEGFR-3, introduced it into the cancer cells and caused cell detachment and displacement of FAK from focal adhesions (Garces et al. 2006). Thus, this approach can be used for specific targeting protein–protein interactions and for future cancer therapy. Recently, we demonstrated that VEGFR3 overexpression in the absence of ligand expression promotes breast cancer cell survival in vitro and tumor formation in vivo in a mouse subcutaneous xenograft model of human breast cancer (Kurenova et al. 2009a). VEGFR-3 overexpression promoted proliferation, motility, survival, and, most importantly, tumorigenicity of breast cancer cells. Thus, FAK–VEGFR3 interaction provide essential survival signals for cancer cells and targeting the FAK–VEGFR3 interaction may have a potential role in developing novel molecular therapeutics to treat human tumors.

FAK and p53

The first indirect link of FAK and p53 was provided by (Ilic et al. 1998). The authors showed that the extracellular matrix survival signals mediated by FAK suppressed p53-directed apoptosis (Ilic et al. 1998). We have shown direct binding of FAK and

p53 in different cancer cells (Golubovskaya et al. 2005). The N-terminal domain of p53 (1–92 a.a.) interacts with the N-terminal domain of FAK (Golubovskaya et al. 2005). Previously, we have shown that p53 can bind FAK promoter and inhibit its luciferase activity (Golubovskaya et al. 2005). Moreover, FAK can block p53 transcriptional activity of p21, BAX, and Mdm-2. Thus, there is a feedback loop mechanism of regulation of these two proteins (Cance and Golubovskaya 2008) (Fig. 12.3). The recent report confirmed binding of the N-terminal domain of FAK, p53, and Mdm-2 and provided a novel mechanism of FAK-mediated ubiquitination of p53 in the nucleus (Lim et al. 2008) (Fig. 12.3). These data link FAK with the p53 tumor suppressor signaling that we will discuss below.

Structure and Function of p53 Protein

P53 is a tumor suppressor gene, which is located at chromosome 17p13 region, spans 20 kb and contains 11 exons (Levine 1997). The p53 protein is a phosphoprotein transcription factor that binds to 5'Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py3' (Pu-purine; Py-pyrimidine) consensus DNA sequence in the promoters of the genes and activates their transcription (Farmer et al. 1992). The p53 gene encodes 393 amino-acid protein. The promoter of p53 lacks TATA box and contains various binding sites for known transcription factors, such as NF-κ B, Sp1 or c-Jun (Bouchet et al. 2006).

P53 protein contains three domains: an acidic N-terminal, transcriptional activating domain (1–92 a.a.), Central, DNA-binding domain (102–292 amino-acids) and C-terminal (102–292 amino-acids), tetramerization domain (325–393 amino-acids). The p53 protein contains many sites for phosphorylation by different kinases: ATM, Chk2, ATR, JNK, MAPK, CKI, CKII).

p53 Mutations in Cancer Cells

It is known that the p53 tumor suppressor is the most frequent target for genetic alterations in human cancers and is mutated in almost 50% of all tumors (Baker et al. 1990; Sidransky et al. 1992; Hollstein et al. 1999; Willis and Chen 2002; Wang et al. 2003). Inactivation of p53 gene is a critical step in tumorigenesis (Fearon and Vogelstein 1990). Following induction by variety of cell stresses such as DNA damage, hypoxia, presence of activated oncogenes, p53 upregulates a set of genes that can promote cell death and growth arrest, such as p21, GADD45, cyclin G, Bax, reviewed in (Giaccia and Kastan 1998). Recently, it has been shown that p53 can repress promoter activities of a number of antiapoptotic genes and cell-cycle genes (survivin Hoffman et al. 2002), cyclin B1, cdc2, (Taylor and Stark 2001; Wu et al. 2001), cdc25 c (Krause et al. 2000), stathmin (Johnsen et al. 2000), Map4 (Murphy et al. 1996), bcl-2 (Wu et al. 2001).

Among reported mutations, 75% are missense mutations, with 80% of them located in the DNA-binding domain of p53 (Bouchet et al. 2006), and 30% of the

mutations are in 5 hot-spot codons: 175, 245, 248, 273, and 282). Arginine residues (248 and 273) involved in interaction of p53 with DNA, and arginines (175 and 282) stabilize DNA-binding sequence (Bouchet et al. 2006). Wild type p53 binds to promoters differently, for example, p53 activates p21 promoter with higher affinity than Bax promoter (Bouchet et al. 2006). Some p53 mutants are able to transactivate different genes, such as EGFR, MDR1, c-Myc, PCNA, IGF-2, or VEGF, providing growth-promoting phenotypes and drug-resistance (Bouchet et al. 2006).

P53 Binds FAK Promoter

Our group was first to clone human FAK promoter and to find two p53 binding sites in the FAK promoter (Golubovskaya et al. 2004). We have shown that p53 can bind FAK promoter and inhibit its transcriptional activity in vitro by EMSA (Golubovskaya et al. 2004) and in vivo by ChIP (chromatin immunoprecipitation) assay (Golubovskaya et al. 2008b) (Fig. 12.3). In addition, several other transcription factors, such as SP-1, AP-2, TCF-1, and NF-κB were shown to be present in the FAK promoter. NF-κB protein has been shown to be linked to p53 pathway (Benoit et al. 2006). For example, activation of Cox-2 transcription required cooperation of NF-κB and p53 (Benoit et al. 2006). Thus, regulation of FAK promoter can also include association of these two transcription factors, thus providing additional indirect p53-regulated FAK expression mechanism.

Moreover, while wild type inhibited FAK promoter activity, mutant p53 did not inhibit FAK promoter activity, as wild type did. The recent global analysis of p53 transcription factor binding sites demonstrated that the induction of HCT116 colon cancer cells with 5-fluorouracil transcriptionally down-regulated FAK (Wei et al. 2006). Thus, the authors suggested that p53 can suppress metastasis through down-regulation of metastasis-related genes, as FAK. Recently, we have shown that p53 can regulate FAK expression in human cancer cells (Golubovskaya et al. 2008b). FAK mRNA and protein were increased in primary colon and breast tumors with mutant p53 versus wild type p53 tumors (Golubovskaya et al. 2008b). In addition, population-based study of 600 breast cancer tumors demonstrated high correlation between FAK overexpression and p53 mutation (Golubovskaya et al. 2009).

Direct FAK and p53 Protein Binding

In our recent report, we have demonstrated that the N-terminal transactivation domain of (1–92 a.a.) of p53 physically directly associated with the N-terminal domain of FAK (Golubovskaya et al. 2005) (Fig. 12.3). In addition, there have been several reports on the localization of the N-terminal part of FAK in the nucleus (Stewart et al. 2002; Beviglia et al. 2003; Jones and Stewart 2004; Lobo and Zachary 2000; Stewart et al. 2002). Furthermore, the N-terminus of FAK was shown to cause apoptosis in breast cancer cell lines (Beviglia et al. 2003) and its nuclear localization was regulated by caspase inhibitors in endothelial cells (Lobo and Zachary 2000). In addition, p53 has been reported to be localized in the cytoplasm (Chipuk and Green 2004).

P53 directly activated Bax and released proapoptotic molecules, activating multidomain proteins in the cytoplasm. This mechanism required 62–91 residues in the proline-rich N-terminal domain of p53 (Chipuk and Green 2004). Consistent with these findings, recently we demonstrated that 7 amino-acid from proline-rich region of p53 was involved in interaction with FAK (Golubovskaya et al. 2008a). The recent report demonstrated that FAK binds also Mdm-2 and causes p53 proteosomal degradation in the nucleus (Lim et al. 2008) (Fig. 12.3). Thus, understanding of the mechanism and functions of FAK/p53-interaction may ultimately have important implications for targeted cancer therapy.

Feedback Model of FAK-p53 Protein Interaction

We have shown that p53 can suppress FAK transcription (Golubovskaya et al. 2004; Golubovskaya et al. 2008b). Recently, global characterization of 65,572 p53 ChIP DNA fragments was done in HCT116 colorectal cancer cell line, treated with 5-fluorouracil for 6 h that activated p53 (Wei et al. 2006). The authors identified novel targets of p53, that are involved in cell adhesion, migration and metastasis, and PTK2 or FAK was one of these kinases (Wei et al. 2006). Interestingly, in HCT116 cells, treated with 5-fluorouracil that increases p53 level, PTK2 (FAK) expression was also inhibited (Wei et al. 2006).

We have also shown that FAK can suppress transcriptional activity of p53 through its interaction (Fig. 12.3), as p53-mediated activation of p53-targets: p21, Mdm-2 and Bax was blocked by overexpression of FAK (Golubovskaya et al. 2005). Thus, p53 can regulate FAK (by inhibiting transcription, and in turn, FAK can regulate p53 by sequestering it from apoptotic signaling and then ubiquitination that decreases p53 transcriptional functions (Lim et al. 2008). Thus FAK and p53 can be regulated through a comprising a feedback mechanism (Cance and Golubovskaya 2008) (Fig. 12.3). Mutations of p53 that are frequently found in cancers, can lead to upregulation and overexpression of FAK (Golubovskaya et al. 2009). Thus, novel mechanisms of FAK survival function, FAK and wild type or mutant p53 interactions remain to be discovered during carcinogenesis.

FAK and IGF-1R

There is an evidence in the literature that FAK and IGF-1R interact, thus, we will discuss this crosstalk signaling in pancreatic cancer.

IGF-1R and Signaling Pathways

Both the IGF-1 (insulin growth factor) and insulin receptors are heterotetrameric transmembrance glycoproteins with intrinsic tyrosine kinase activity. Following ligand binding, both receptors undergo phosphorylation and thus activate insulin

receptor substrate-1 (IRS-1), which then initiates a cascade of events that have mitogenic and metabolic effects (Vincent and Feldman 2002). Insulin, unlike IGF-1, is produced by the beta cells of the Islet of Langerhans and is primarily involved in glucose homeostasis and the regulation of metabolic pathways. The role of insulin in tumorigenesis is less clear; in pancreatic cancer, its effect appears to be its ability to activate the IGF-1 receptor (Korc 1998).

The best defined pathway by which IGF-1R signaling can prevent apoptosis is mediated by phosphoinositide 3-kinase (PI3K) signaling to Akt. Tyrosine phosphorylation of IRS-1 by IGF-1R leads to PI3K activation by binding of the SH2 domain of its regulatory subunit to IRS-1 leading to an increase in phosphatidylinositol 3,4,5-trisphosphate (PIP3). The proteins Akt/PKB and phosphoinositide-dependent kinase-1 (PDK-1) are then bound by PIP3. Residue Thr308 on Akt/PKB is then phosphorylated by PDK-1. Activated Akt/PKB plays a key role in the prevention of apoptosis. It phosphorylates and inactivates several proteins that are involved in apoptosis including Bad (Bcl-2 family member). Akt/PKB also can prevent the initiation of the caspase cascade through phosphorylation and inactivation of caspase-9. In addition to the inhibition of proapoptotic transcription factors, the activity of Akt/PKB also increases the levels of antiapoptotic proteins including Bcl-2 and Bcl-X. With activation of Akt/PKB, the expression of the antiapoptotic transcription factor NF-κ B is also increased (Vincent and Feldman 2002).

The mitogen-activated protein (MAP) kinases are also activated by IGF-1R and are involved in the regulation of apoptosis in different cell types (Dews et al. 2000). One principal MAPK pathway involves the extracellular signal-regulated kinase (ERKs) ERK1 and ERK2. Upon IGF-1R autophosphorylation, the protein Shc is recruited to the IGF-1 receptor and becomes phosphorylated on tyrosine residues. Activated Shc then binds the adaptor Grb2 in an IRS-1 independent manner, leading to activation of the Ras-ERK pathway (Kim et al. 1998). This pathway has been shown to be important in fibroblasts in regulating the machinery of apoptosis in detachment-induced death or anoikis (Valentinis et al. 1999). Similar to the Akt pathway, the downstream target of ERK that prevents apoptosis may be Bad.

IGF-1R and Cancer

Several members in the IGF family signaling pathway, including IGF-1, IGF-1R, IGF-2R, and IRS-1 are overexpressed in cancer including pancreatic malignancy (Bergmann et al. 1995, 1996; Ishiwata et al. 1997). Several studies support the significance of the IGF-1 receptor-mediated mitogenic signal in cancer cells. Both IGF-1 receptor antisense oligonucleotides and anti-IGF-1R antibodies have been shown to inhibit the proliferation of human pancreatic cancer cells. Overexpression of IRS-1 in pancreatic cancer contributes to increased activation of the IGF-1R signaling pathway (Bergmann et al. 1995, 1996).

Interaction Between FAK and IGF-1R

It has been shown that FAK activates proliferation and inhibits apoptosis through PKC and the PI3K-Akt pathway which results in induction of cyclin D3 expression and CDK activity (Yamamoto et al. 2003). Therefore, activation of either FAK or IGF-1R induces the PI3K-Akt pathway and cell survival. Clearly, there is crosstalk and redundancy in the signaling via these tyrosine kinases, but the pathways diverge as well. Induction of the IGF-1R results in MAPK pathway activation which is independent of FAK phosphorylation and activation (Arbet-Engels et al. 1999). Recently, it has been shown that a member of the MAPK pathway (MEK kinase 1) binds to FAK, linking FAK to possible activation of this pathway (Yujiri et al. 2003). It has been shown that FAK is activated by IGF-1R (Baron et al. 1998), and that IRS-1 is a substrate for FAK(Lebrun et al. 1998) and that FAK activity regulates IRS-1 mRNA levels (Lebrun et al. 2000). Furthermore, it has been shown that FAK participates in integrin-mediated phosphorylation of the insulin receptor (El Annabi et al. 2001).

We have demonstrated by coimmunoprecipitation and confocal studies that FAK and IGF-1R physically interact in human pancreatic cancer cells and that these cells have survival signals operative through FAK and IGF-IR activities (Liu et al. 2008). We have shown through the use of models in fibroblast and cancer cells, and with the use of multiple inhibitors including transient expression of a FAK dominant negative (Ad FAK-CD), FAK knockdown with siRNA, stable expression of an IGF-1R dominant negative, a selective small molecule inhibitor of IGF-1R (AEW-541) and a novel small molecule kinase inhibitor of both FAK and IGF-1R (TAE226) that dual inhibition of both kinases synergistically induces cell detachment, decreases cell viability and increases apoptosis (Liu et al. 2008). In addition, their interaction is dependent on tyrosine phosphorylation as inhibitors of phosphorylation prevent binding of FAK and IGF-1R.

There have been no previous studies which have examined the effect of strategies that target FAK and IGF-1R interactions in human pancreatic cancer cells. Utilizing multiple inhibitors of IGF-1R (dominant negative and kinase inhibitors) and FAK (dominant negative, siRNA, and kinase inhibitor) we have shown that inhibition of the activity of both tyrosine kinases resulted in a synergistic decrease in cell proliferation, increase in cell detachment and increase in apoptosis. The mechanism for this synergistic effect appears to be through pathways that involve ERK and Akt. TAE226 (Novartis FAK inhibitor) which inhibited both FAK and IGF-1R tyrosine kinase activities, led to inhibition of cell growth and increased apoptosis at lower doses than individual FAK or IGF-1R kinase inhibitors suggesting that it is the kinase activity of FAK and IGF-IR that is important for cell survival (Liu et al. 2008).

FAK and N-MYCN Signaling in Neuroblastoma

Since N-MYCN overexpression is major event in neuroblastoma, we will discuss N-MYCN and FAK signaling in this childhood disease.

Overview of MYCN Oncogene

The *MYCN* oncogene plays a major role in human oncogenesis. The N-MYCN protein functions primarily as a transcription factor and is known to bind to the specific DNA E-box sequence, CACGTG (Alex et al. 1992). Abnormal expression of *MYCN* is associated with neuroblastoma, the most common extracranial solid tumor of childhood. The exact function and gene targets of *MYCN* have not been completely defined (Stanton et al. 1986), and a thorough investigation of which target genes are required for the proliferation and tumorigenesis of neuroblastoma have not been published (Adhikary and Eilers 2005). However, there is significant interest in this arena and some progress is being made with recent studies showing in vitro (Harris et al. 2002; Manohar et al. 2004; Thomas et al. 2004) and in vivo (Adhikary and Eilers 2005) binding of MCYN to the genes that it regulates. For example, N-MYCN has been shown to bind to the gene promoters for various oncogenes involved in cellular proliferation, differentiation, and survival and to increase the expression of these targets. These genes include the proto-oncogenes high mobility group A1 (*HMGA1*) (Giannini et al. 2005) and *Pax-3* (Harris et al. 2002), the multidrug resistance-associated protein gene (*MRP1*) (Manohar et al. 2004), and the protein nestin (Thomas et al. 2004). There are also a number of negative transcriptional targets for N-MYCN in neuroblastoma including the growth-inhibitory gene *Ndrg1* (Li and Kretzner 2003) and leukemia inhibitory factor (*LIF*) (Hatzi et al. 2002).

MYCN Oncogene and Neuroblastoma

The strongest adverse prognostic indicator in human neuroblastoma is gene amplification of greater than ten copies of the *MYCN* oncogene (Brodeur et al. 1984). Amplification of this gene occurs in about 20% of neuroblastomas (Brodeur et al. 1984) and is associated with both increased recurrence of disease and decreased survival. Numerous studies have demonstrated the importance of *MYCN* in neuroblastoma tumorigenicity. The level of MYCN expression has been shown to correlate with the growth and proliferation of neuroblastoma cells in vitro (Schweigerer et al. 1990). Interruption of *MYCN* induces cellular differentiation in neuroblastoma cells. Nara and colleagues showed that after blocking *MYCN*, neuroblastoma cells developed multidirectional neurite extension and increased cellular and nuclear size, findings consistent with differentiation (Nara et al. 2007). Downregulation of *MYCN* with antisense oligonucleotides resulted in a decrease in both cellular proliferation and anchorage independent growth in the cells (Negroni et al. 1991). Other authors have utilized small interfering RNAs (siRNAs) to silence MYCN. Woo et al demonstrated that *MYCN* interference resulted in a decrease in the number of neuroblastoma cells in the S-phase of the cell cycle (Woo et al. 2008). In addition, other investigators have utilized this method to silence *MYCN* and have shown decreased neuroblastoma cell growth and an increase in neuroblastoma cell apoptosis (Kang et al. 2006). Finally, transgenic mice with *MYCN* overexpression develop spontaneous neuroblastomas (Weiss et al. 1997).

MYCN and Focal Adhesion Kinase (FAK)

An association between *MYCN* amplification and tumorigenicity through cell adhesion, motility, and invasiveness has been implied in some studies. *MYCN* amplified human neuroblastoma cells have increased cellular motility and invasiveness and decreased attachment when compared with *MYCN* nonamplified cell lines, but no mechanistic explanation for these observations has been elucidated. The association between *MYCN* amplification and cellular motility and invasiveness implied a potential relationship between FAK and *MYCN*, since FAK is a key protein involved in cellular motility. However, until recently, there were no data demonstrating the expression of FAK in human neuroblastoma specimens Our recent examination of human neuroblastoma specimens with various INSS stage and *MYCN* amplification status revealed a link between FAK and N-MYCN in neuroblastoma, as we found an increase in the expression of FAK protein, as detected by immunohistochemistry, in *MYCN* amplified human neuroblastomas compared with those tumors that were not *MYCN* amplified (Beierle et al. 2008a) (Fig. 12.1b). Recently, Wu and others demonstrated that FAK activity is required to promote integrin stimulated neuroblastoma motility through $\alpha 5\beta 1$ but not for $\alpha 4\beta 1$ integrin (Wu et al. 2008). These data provide evidence that FAK is involved in neuroblastoma cell adhesion. Recently, the *FAK* promoter has been cloned (Golubovskaya et al. 2004) and evaluations have demonstrated E-box sequences on the *FAK* promoter that are the potential binding sites for N-MYCN (Beierle et al. 2007). We have shown that N-MYCN binds FAK promoter in vitro and in vivo and upregulates FAK expression (Beierle et al. 2007) (Fig. 12.3). Understanding of the effects of *MYCN* upon the FAK promoter in neuroblastoma impacts our understanding of the role of FAK in tumorigenesis in other tumor types. *MYCN* is reported to be amplified in human melanoma and sarcomas and is associated with poor outcomes in these tumor types. FAK has also been shown to be overexpressed in human sarcoma and melanoma tumors. These data provide evidence that N-*MYCN* is potentially a transcriptional regulator of FAK in these tumor types as well, but as of yet the role of FAK here has not been fully investigated.

MYCN, Mdm-2, FAK, and p53

Since FAK and p53 proteins associate (Cance and Golubovskaya 2008) we would like to review the connection of MYCN with Mdm-2 and p53 signaling. Most neuroblastomas have wild type p53 and only minority of this pediatric malignancy contain mutant p53 (Hosoi et al. 1994). Thus, neuroblastomas should escape p53-driven apoptosis/cell death in response to stress and balance N-MYCN-regulated functions in mediating proliferation and cell death pathways (Hogarty 2003). It has been shown recently that N-MYCN directly binds not only to FAK promoter (Beierle et al. 2007), but also to Mdm-2 promoter and upregulates Mdm-2 transcription (Slack et al. 2005), thus connecting M-myc with FAK-p53-Mdm-2 pathways (Fig. 12.3).

The authors also demonstrated that elevated mdm-2 levels in neuroblastoma caused genomic instability through centrosome amplification (Slack et al. 2007). In addition, MYCN amplification impaired p53 functions to regulate duplication of centrosomes and promoted tumorigenesis. In conclusion, novel therapeutic approaches, such as Mdm-2, FAK inhibitors can be future approach for neuroblastomas.

FAK-Targeted Therapy

FAK Inhibitors

Recently, FAK has been proposed to be a new therapeutic target (McLean et al. 2005). Several in vitro approaches used to down-regulate FAK-adenoviral FAK-CD (dominant-negative FAK) (Xu et al. 2000), antisense oligonucleotides (Smith et al. 2005) and siRNA for FAK (Golubovskaya et al. 2009; Han et al. 2004). The melanoma cells treated with antisense oligonucleotides lost their attachment and underwent apoptosis (Xu et al. 1996; Smith et al. 2005). The same effect was observed with Ad-FAK-CD in different cancer cells. While breast cancer cells underwent apoptosis by down-regulation of FAK with FAK-CD, normal MCF-10A and HMEM cells did not undergo apoptosis (Xu et al. 2000). These inhibitors applications are limited due to the cell toxicity in vivo. Thus, developing small-molecule drugs is critical for future FAK-targeting therapy, involving kinase inhibitors (Golubovskaya et al. 2009; Hockwald et al., 2009) and drugs, targeting FAK-protein interactions.

There were no pharmacological inhibitors, reducing FAK kinase activity. Recently Novartis Inc. developed novel FAK inhibitors down-regulation its kinase activity (Choi et al. 2006). The novel Novartis FAK inhibitor, TAE-226 recently was employed in brain cancer and effectively inhibited FAK signaling and caused apoptosis in these cells (Shi et al. 2007). We also used TAE-226 inhibitor in breast (Golubovskaya et al. 2007), neuroblastoma (Beierle et al. 2008b) and pancreatic cancer cells (Liu et al. 2008) and found that this inhibitor can effectively cause apoptosis in these different types of cancer. Although, it can inhibit also other signaling pathways in addition to FAK, such as IGF-1R. Another, ATP-targeting site inhibitor of FAK, Pfizer-PF-573,228 has been recently described (Slack-Davis et al. 2007). Another Pfizer inhibitor PF-562,271 with high specificity in inhibiting FAK activity has been shown to be effective in tumor xenograft models in vivo (Roberts et al. 2008) and on bone tumors (Bagi et al. 2008) and is now is in clinical trials. The future detail studies will be needed to address specificity of these drugs.

Targeting Protein–Protein Interactions

One of the approaches to inhibit FAK function can be targeting its protein–protein interaction with its binding partners, such p53. Small-molecule drugs can be found either through high-throughput screening or through database searches using protein crystal structures.

Small molecule drug inhibitors are effectively used to target p53 protein–protein interactions, particularly with Mdm-2 protein (Vassilev 2005). The first potent inhibitors targeting p53–Mdm-2 interaction have been identified by high-throughput screening followed by structure-based optimization (Vassilev 2005). The screening identified nutlins that represent a class of cis-imidazole analogues that bind to the p53 pocket interacting with Mdm-2. The same strategy can be used to target interaction with FAK.

Recently we used the crystal structure of the N-terminus and FAT domain of FAK, and screened 240,000 small molecules from the NCI bank for their ability to target this binding site. We identified several potential lead compounds and tested them on human breast, colon, and melanoma cell lines VEGFR-3–FAK (Kurenova et al. 2009b) and FAK-IGF-1R (Zheng et al. 2009) or VEGFR-3–FAK binding and to induce cancer cell death. Our data suggest that small molecule inhibitors of FAK–protein interaction can be identified as lead compounds to provide the basis for specific novel cancer therapeutic agents. Such compounds will be valuable experimental tools for further analyses of FAK function. Furthermore, they might prove useful pharmaceutically to perturb FAK signaling in cancerous cells overexpressing FAK.

Summary

Thus, understanding of FAK biology during tumorigenesis, mechanisms of its upregulation in different tumors, angiogenesis, motility, and especially mechanisms of its direct physical interaction with protein binding partners and their downstream signaling pathways will be critical in developing targeted therapeutics. Studies with peptide inhibitors already have indicated that blockade of specific protein–protein interactions has therapeutic promise for treating a variety of diseases, including cancer (Akhter et al. 1998; Aramburu et al. 1999; Chen et al. 1999; May et al. 2000; Aarts et al. 2002; van Rooij et al. 2002). Small molecule drugs are particularly attractive as inhibitors of intracellular protein–protein interactions due to the ability to modify their structures to achieve optimal target binding. As we further define the mechanisms of FAK signaling in cancer cells, we will identify the optimal sites for targeting this protein and disrupting its signaling to cause apoptosis in human tumors. Thus, targeting of FAK–protein interactions can be important in cancer treatment programs.

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