Biology of Extracellular Matrix 13
Series Editor: Nikos K. Karamanos

Donald Gullberg
Johannes A. Eble *Editors*

Integrins in Health and Disease

Key Effectors of Cell-Matrix and Cell-Cell Interactions

Biology of Extracellular Matrix

Volume 13

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Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

All chapters are systematically reviewed by the series editor and respective volume editor(s).

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Donald Gullberg • Johannes A. Eble
Editors

Integrins in Health and Disease

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Preface

Integrins belong to a family of cell adhesion receptors with affinity for extracellular matrix (ECM) proteins and cell surface molecules involved in cell–cell interactions. They are evolutionary old, but have only been characterized at the molecular level since 1986 with the expression cloning of cDNA encoding the chick integrin β1 subunit. The name "integrin" was coined for this receptor to denote their importance for maintaining the integrity of the cytoskeletal-ECM linkage. In the years leading up to the cloning of the β1 subunit, several groups had been working on various cell surface proteins, which at the time included position specific (PS) antigens in *Drosophila*, late antigens of activation (VLA) on immune cells, cell surface receptors on lymphoid and myeloid cells, and platelet glycoproteins. With the cloning of the cDNAs encoding these proteins, it became clear that they were related to fibronectin receptors isolated by using RGD peptides or cell adhesion blocking antibodies, and that they all belonged to the same protein family. The two last integrins subunits to be cloned in this family were the integrin α10 and α11 chains. Since the 80s and the identification of integrins, enormous progress has been made in the integrin field. Thanks to genetic knockout models, the role of integrins during embryogenesis has been largely clarified. The phenotypes of individual integrins vary and for integrins with no embryonic phenotype we do not yet know with certainty whether this reflects that their function is postnatally limited or that some sort of compensation mechanism has kicked in. In the latter case, another integrin takes over the function of the deleted integrin. Compensation mechanisms are further complicated by integrin crosstalk with other cell surface receptors, opening for several indirect effects. Extensive autocrine and paracrine signaling further complicates interpretation of what individual integrins do, especially in disease processes.

In the current book, a group of authors have contributed chapters on different aspects of integrin function in health and disease. Below we briefly summarize the chapters in this volume.

In Chap. "Integrins α1β1 and α2β1: The Generalist Collagen Receptors," the two collagen-binding integrins, α1β1 and α2β1, are introduced. Collagens are the most abundant protein in our body. They serve as ECM scaffold, to which cells can adhere

directly via collagen-binding integrins, among which the two integrins, α1β1 and α2β1, are most ubiquitously expressed in cells of most tissues. Beyond long-known facts, this chapter provides new insights of these two collagen-binding integrins in angiogenesis and immunity. The laminin-binding integrin α3β1 is in the focus of Chap. "Roles for Integrin α3β1 in Development and Disease." Recent years have uncovered several signal pathways of this integrin, which explain the symptoms of its deficiency or loss on epithelial cells. The pleiotropic effects of this integrin in various tissues and in different pathological situations, such as cancer, make it a key member of the integrin family and a potential pharmaceutical target. In Chap. "Integrins: Key Targets in Tissue Fibrosis and Tumor Stroma," a summary of the ample literature covering different aspects of integrin function in different tissue-and tumor fibrosis models is covered, including TGFβ-activating integrins, collagen-binding integrins, and α5β1 integrin. In Chap. "Integrins in Cardiac Form, Function, and Disease," the role of integrins in heart physiology and pathology is analyzed in some detail. Historically there has been an interest in defining the role of integrins on cardiomyocytes and a prominent role of α7 integrin and the β1D splice variant has been established in heart muscle cells. Currently there is an interest in defining integrins involved in cardiac fibroblasts with a profibrotic role in heart disease. An overview of the changing integrin profile in heart disease models and the finding of a synergistic profibrotic role of syndecan-4 and integrin α11β1 is described. In Chap. "Integrin α8 and Its Ligand Nephronectin in Health and Disease," the interesting story of how integrin α8β1 and nephronectin both have evolved to have a central role in kidney physiology and pathology during development and in fibrosis, is told. In the kidney glomeruli, the α8β1 integrin seems to be protective in the fibrotic kidney by a mechanism suggested to potentially involve a dampening of TGF-β signaling. In Chap. "αv Integrin-Dependent TGFβ Activation in Cancer: A Brief Update," an update on the status of TGFβ-activating integrins and clinical trials targeting these are presented. In Chap. "α11β1, a Mesenchymal Collagen-Binding Integrin with a Central Role in Tissue- and Tumor Fibrosis," the role of integrin α11β1 in tissue and tumor fibrosis is summarized. There is currently a great interest in finding specific biomarkers for fibroblasts subsets, and α11 appears to be a good candidate for defining a subset of myofibroblastic cells in fibrotic conditions. Among the tissues, which is mechanically challenged the most, is cartilage. Chondrocytes, along with their unique ECM architecture, are well adopted to the mechanical forces. In pathological conditions, such as osteoarthritis and rheumatoid arthritis, the importance of normal levels of integrins in cartilage/bone can be demonstrated using animal models, as will be presented in Chap. "Integrins in Pathological Tissue Remodeling of Joints." Chapter "α4 Integrins in Immune Homeostasis and Disease" summarizes the major findings regarding α4 integrins (α4β1 and α4β7) over the last decades, including their structures, extracellular ligands, intracellular adaptor proteins, and functions in homeostasis and diseases, including cancer, multiple sclerosis (MS), inflammatory bowel disease (IBD), and other autoimmune diseases. Integrins on immune cells is the topic of Chap. "β2-Integrins in Immunity: New Roles for Old Players." Members of the β2 integrin subfamily are typically found on cells of the innate and adaptive immune systems. They are presented, along with their ligands,

signaling pathways, and functions in immune cells, as deficiency of β2 integrins results in the very severe immune disorder of leukocyte adhesion deficiency (LAD). In Chap. "Structure and Function of the Leukocyte Integrin αMβ2," a careful review of the structure of αMβ2 describes the αMβ2 protein structures and biophysical measurements that underpin current understanding of diverse ligand recognition through the metal-ion-dependent adhesion site (MIDAS). Early on, thrombocytes have been instrumental in integrin research. Even today, these anuclear cell fragments are still an attractive research object because of their crucial role in hemostasis, but also in thrombosis and occlusive blood vessel diseases. Chapter "Platelet Integrins: Critical Mediators of Hemostasis and Pathological Thrombus Formation" highlights platelet biology in the light of integrins. Particularly, the platelet integrin αIIbβ3 is crucial for platelet activation and thrombus formation. It is not only a receptor to mechanically tighten the primary platelet aggregate, but also conveys signals from outside to the inside of platelets. Moreover, platelet aggregation requires an activation of the platelet integrins by intracellular signals. Both inside-out and outside-in signaling will be discussed, as well as novel integrin-related roles of platelets in metastasis and immunity. Chapter "Integrins as Receptors for Bacterial and Viral Pathogens" covers another aspect of integrin biology. Being exposed on the cell surface, integrins may also serve as a docking site for extracellular pathogens, such as bacteria and viruses. These pathogens subvert the integrins to be endocytosed by the eukaryotic host cells. In intracellular vesicles, the pathogens evade the host's immune reaction. Therefore, preventing pathogens from binding to integrins may be a therapeutic way to curb certain infectious diseases.

In the original plan for the book, additional chapters were included, but largely due to pandemic-related issues, several authors failed to submit their chapters. Having said this, we still foresee that the book will be an important document over some central themes and state of the art in the integrin field in the beginning of the twenty-first century. Yet, it is hard to foresee which chapters will remain relevant in another decade. On the other hand, we would be delighted to see the field move forward and the chapters quickly becoming outdated as new knowledge is assembled. Such a development would in turn necessitate another edition of this book in another decade. Finally, we hope you will enjoy reading the book as much as we have enjoyed assembling the chapters.

Bergen, Norway Donald Gullberg
Münster, Germany Johannes A. Eble

Contents

Part I Knockouts and Animal Models

Integrins α1β1 and α2β1: The Generalist Collagen Receptors 3
Jyrki Heino and Elina Siljamäki

Roles for Integrin α3β1 in Development and Disease 27
C. Michael DiPersio and Whitney M. Longmate

Part II Fibrosis and Cancer

Integrins: Key Targets in Tissue Fibrosis and Tumor Stroma 99
Devin Veerman and Jai Prakash

Integrins in Cardiac Form, Function, and Disease 135
Ana Maria Manso, Andreas Romaine, Geir Christensen,
and Robert S. Ross

Integrin α8 and Its Ligand Nephronectin in Health and Disease 185
Carole L. Wilson, Chi F. Hung, and Lynn M. Schnapp

αv Integrin-Dependent TGFβ Activation in Cancer: A Brief Update . . . 217
John F. Marshall, Pantelitsa Protopapa, and Natalie Allen

Part III Human Disease

**α11β1: A Mesenchymal Collagen-Binding Integrin with a Central
Role in Tissue and Tumor Fibrosis** 235
Cédric Zeltz and Donald Gullberg

Integrins in Pathological Tissue Remodelling of Joints 255
Thomas Pap, Beate Eckes, and Adelheid Korb-Pap

α4 Integrins in Immune Homeostasis and Disease 273
ChangDong Lin, ShiHui Wang, and JianFeng Chen

β2-integrins in Immunity: New Roles for Old Players 309
Liisa M. Uotila, Heidi Harjunpää, and Susanna C. Fagerholm

Structure and Function of the Leukocyte Integrin αMβ2 357
Gregers Rom Andersen and Jonas Emsley

Part IV Integrins in Hemostasis and Immune Control

Platelet Integrins: Critical Mediators of Haemostasis and Pathological Thrombus Formation 381
Timo Vögtle and Bernhard Nieswandt

Integrins as Receptors for Bacterial and Viral Pathogens 427
Maria Mrakovcic, Samriti Sharma, and Petra Dersch

About the Editors

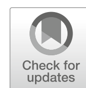
Donald Gullberg has a BSc in Chemistry from Uppsala University, Sweden, and earned his PhD on a thesis entitled "Structural and functional characterization of collagen receptors on primary rat hepatocytes and cardiac fibroblasts" in 1990 under the supervision of Prof. Kristofer Rubin at the Department of Medical and Physiological Chemistry. During 1990–1992, both he and Marion Kusche-Gullberg conducted postdoctoral research with Professors John and Lisa Fessler at the Molecular Biology Institute, UCLA. In his laboratory, ITA11 was identified and characterized in the 1990s. He was appointed to a professorship at the University of Bergen Department of Biomedicine in 2004 and has continued to study ITA11 in the context of tissue and tumor fibrosis. He has spent two inspiring sabbaticals at UCSF (with Prof. Dean Sheppard, MD, 2010–2011, with Prof. Valerie Weaver, PhD, 2017–2018).

Johannes A. Eble After having studied biochemistry at the University of Tübingen, Johannes A. Eble gained an intense training on extracellular matrix and collagen-binding integrins during his doctorate thesis at the Max Planck-Institute of Biochemistry in Martinsried close to Munich. In 1994, he gained his doctorate degree from the Ludwig Maximilians-University, Munich, under Prof. Dr. Klaus Kühn's supervision. During 1995–1998, he intensified his knowledge of integrins under Prof. Martin Hemler's supervision at the Dana-Farber Cancer Institute of Harvard Medical School in

Boston, where he developed the technique of recombinant expression of soluble integrins, especially of the laminin-332-binding ITGA3 integrin. After his return to Germany, he habilitated at the University of Münster in 2004. In 2008, he was appointed to a professorship of vascular matrix biology at the University of Frankfurt within the Excellence Cluster Cardio-Pulmonary System. Since 2014, he heads the Institute of Physiological Chemistry and Pathobiochemistry of the University of Münster. Although his scientific focus still pivots around integrins, his view on cell adhesion and migration has widened to integrin agonists and antagonists, the former being recombinant integrin-binding minicollagen and mini-laminin-332, the latter being integrin-blocking snake venom toxins. Moreover, redox regulation of integrins and cell-matrix contacts is also included in his research portfolio.

Part I
Knockouts and Animal Models

Integrins α1β1 and α2β1: The Generalist Collagen Receptors



Jyrki Heino and Elina Siljamäki

Abstract Four members of the large integrin family have the capability to directly recognize specific collagenous motifs in proteins. These integrins can be found in all vertebrates, but not in other animals. Thus, direct cell adhesion to collagens has evolved concomitantly with, e.g., the development of musculoskeletal system, closed blood circulatory system, and acquired immunity. Two integrin-type collagen receptors α1β1 and α2β1 are abundantly expressed in vertebrates and a growing pile of evidence indicates their involvement in numerous physiological functions. At the same time, the corresponding knockout mouse models show no obvious developmental defects. Thus, α1β1 and α2β1 are not critical for organogenesis, but they can be considered as generalist integrins that have evolved to support and intensify processes driven by other receptors and proteins.

1 Integrins α1β1 and α2β1: Abundant Expression

Collagen receptor integrins α1β1 and α2β1 were among the first integrins that were found. Studies focused on T lymphocytes unveiled their α subunits as very late activation antigens 1 and 2 (VLA-1, VLA-2) (Hemler et al. 1985) and confirmed that both exist as a heterodimeric complex with β1 integrin subunit. Later in the Cluster of Differentiation (CD) nomenclature these integrins were numbered as CD49a and CD49b, while β1 subunit is CD29. The names of the corresponding genes are ITGA1 and ITGA2. Independently of the lymphocyte studies a major collagen binding receptor on platelets had been described (Santoro 1986) and named as GPIa and later identified to be VLA-2, i.e. α2 integrin. Both α1β1 and α2β1 integrins can be found in many different cell types and tissues. Table 1 is a summary of information available in *Tabula Muris* database (Tabula Muris Consortium et al. 2018). In general, α1β1 is commonly expressed on mesenchymal cell types such as endothelial cells, smooth muscle cells, myofibroblasts, and monocytes, while α2β1

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Table 1 Expression of ITGA1 and ITGA2 in specific cell types. The table is based on the Tabula Muris database. The data show results of single-cell RNA sequencing by FACS-based full length transcript analysis. This table only shows the cell populations expressing >10% ITGA1 or ITGA2

Cell type	ITGA1 expressing cells (%)
Blood cells	Basophil (*marrow*, 40%)
	Blood cell (*trachea*, 13%)
	Classical monocyte (*lung*, 12%)
	Granulocyte monocyte progenitor cell (*marrow*, 11%)
	Leukocyte (*thymus*, 20%; *skin*, 20%; *kidney*, 19%)
	Monocyte (*marrow*, 47%)
	Myeloid cell (*lung*, 16%)
Endothelial cells	Endocardial cell (*heart*, 22%)
	Endothelial cell (*pancreas*, 86%; *trachea*, 65%; *heart*, 62%;
	brain non-myeloid, 57%*; fat*, 52%; *limb muscle*, 47%, *diaphragm*, 45%;
	aorta, 39%*; mammary gland,* 34%*; kidney*, 32%)
	Lung endothelial cell (*lung*, 61%)
Epithelial cells	Basal cell (*mammary gland*, 14%)
	Bladder cell (*bladder*, 15%)
	Enteroendocrine cell (*large intestine*, 10%)
	Epithelial cell of hepatic sinusoid (*liver*, 90%)
	Epithelial cell of large intestine (*large intestine*, 16%)
	Hepatocyte (*liver*, 31%)
	Large intestine goblet cell (*large intestine*, 14%)
	Pancreatic acinar cell (*pancreas*, 16%)
Inflammatory cells	DN1 thymic pro-T cell (*thymus*, 19%)
	Natural killer cell (*liver*, 44%)
	Professional antigen presenting cell (*aorta*, 17%)
Muscle cells	Cardiac muscle cell (*heart*, 26%)
	Smooth muscle cell (*heart*, 52%)
Pericytes	Brain pericyte (*brain non-myeloid*, 79%)
Stem cells	Mesenchymal stem cell (*trachea*, 27%; *limb muscle*, 15%)
Stromal cells	Fibroblast (*aorta*, 24%; *heart*, 15%)
	Myofibroblast cell (*heart*, 83%)
	Pancreatic stellate cell (*pancreas*, 47%)
	Stromal cell (*lung*, 18%)

Cell type	ITGA2 expressing cells (%)
Blood cells	Basophil (*marrow*, 36%)
	Leukocyte (*skin*, 13%)
Endothelial cells	Lung endothelial cell (*lung*, 10%)
Epithelial cells	Basal cell of epidermis (*skin*, 24%; *tongue,* 23%)
	Bladder urothelial cell (*bladder*, 65%)
	Enterocyte of epithelium of large intestine (*large intestine*, 13%)
	Epithelial cell of large intestine (*large intestine*, 27%)
	Large intestine goblet cell (*large intestine*, 34%)
	Luminal epithelial cell of mammary gland (*mammary gland*, 16%)

(continued)

Table 1 (continued)

Cell type	ITGA2 expressing cells (%)
Inflammatory cells	B cell (*marrow*, 18%)
	Immature natural killer cell (*marrow*, 31%)
	Immature NK T cell (*marrow*, 24%)
	Macrophage (*spleen*, 40%)
	Mature natural killer cell (*marrow*, 47%)
	Natural killer cell (*lung*, 30%; *liver,* 18%; *fat*, 13%)
	Pre-natural killer cell (*marrow*, 64%)
Stem cells	Basal cell (*mammary gland*, 19%)
	Stem cell of epidermis (*skin*, 30%)

is abundant on epithelial cells. However, under different physiological and pathological conditions the two integrins can also be expressed at the same time on the same cell (Table 1).

2 Ligands: Not Only Collagen Receptors

Despite the fact that α1β1 and α2β1 are usually called collagen receptors they also have numerous non-collagen ligands. Both receptors can recognize different members of the laminin family (Calderwood et al. 1997; Orian-Rousseau et al. 1998; Languino et al. 1989; Elices and Hemler 1989). Extracellular matrix (ECM) molecules that have been reported to interact with α2β1 integrin include proteoglycans, such as decorin (Guidetti et al. 2002), lumican (Zeltz et al. 2010), and endorepellin, a fragment of perlecan (Bix et al. 2004). Tenascin C (Sriramarao et al. 1993), chondroadherin (Camper et al. 1997), matrix metalloproteinase 1 (Dumin et al. 2001), E-cadherin (Whittard et al. 2002), C-reactive protein (Kim et al. 2018a), and collectin-family members, namely C1q complement protein, mannose-binding lectin, and surfactant protein, have been indicated to bind to α2β1 integrin (Zutter and Edelson 2007). Compared to the collagens these ligands often bind to α2β1 with significantly lower avidity. However, if the physiological concentrations of ligands are high, the interactions may still be important regulators of integrin function. For example, the low avidity binding of the plasma protein histidine-rich glycoprotein (HRG) to α2β1 can significantly suppress adhesion, spreading, and migration of endothelial cells on collagen I (Roche et al. 2015).

Integrin α1β1 can, in addition to collagens and laminins, recognize ECM proteins such as matrilin-1 (Makihira et al. 1999) as well as galectin-8, a β-galactoside binding lectin (Cárcamo et al. 2006). Furthermore, among α1β1 ligands there are also transmembrane proteins such as semaphorin 7A (Sema 7A) (Pasterkamp et al. 2003). Sema7A is expressed on neural cells, activated T-cells, platelets, skin keratinocytes and fibroblasts.

Although α1β1 and α2β1 are both collagen receptors they seem to have different mechanisms and priorities in their interaction with the various members of the collagen family (Heino 2007). There are no less than 28 different collagen subtypes (Ricard-Blum 2011) and for obvious reasons it has not been possible to test them all in binding assays with integrins. High avidity interaction of α2β1 with collagen I, the most abundant fibril-forming collagen, has been studied in detail in numerous laboratories. Other fibril-forming collagens recognized by α2β1 include collagens I, II, III, V, and XI. Furthermore, basement membrane collagen IV, FACIT (fibril-associated collagen with interruptions in triple-helix) collagens IX and XVI, network-forming collagens VIII and X, beaded-filament forming collagen VI, and anchoring filaments forming collagen VII are ligands for α2β1. The transmembrane collagen XIII seems to interact with α2β1 with a low avidity only, whereas negative binding results have been reported with FACIT collagen XIV and the COL15 domain of the transmembrane collagen XVII (Reviewed in Heino 2007).

While α2β1 has been considered to especially favour fibril-forming collagens, α1β1 binds with highest avidity to basement membrane collagen IV. This receptor has also been reported to recognize network-forming collagen VIII, FACITs IX and XVI, as well as transmembrane collagen XIII (Reviewed in Heino 2007).

The collagen recognition mechanisms of α1β1 and α2β1 also have differences. The interaction of α2β1 with a special triple-helical motif that contains an amino acid sequence GFOGER (O = hydroxyproline) (Knight et al. 1998) has been analysed at atomic level (Emsley et al. 2000). In collagen the critical interaction sites are formed by phenylalanine residues in the trailing and the middle strands and glutamic acid and arginine residues in the middle strand (Emsley et al. 2000). Other GFOGER-like α2β1 recognition motifs include GROGER, GLOGER, GMOGER, GLSGER, GQRGER, GLOGEN, GAOGER, GVOGEA, GFKGER, GLQGER, and GASGER (Xu et al. 2000; Siljander et al. 2004; Kim et al. 2005; Raynal et al. 2006; Hamaia et al. 2012; Zwolanek et al. 2014). The consensus integrin binding sequence is often presented as G*XX'*GER (*X* and *X'* can be any amino acid residue). Interestingly, many of these motifs contain hydroxyproline residues that are essential for high avidity interactions. Thus, proline hydroxylation and the activity of the critical enzymes, namely collagen prolyl 4-hydroxylases, may be important regulators of cell adhesion to collagens (Rappu et al. 2019). Proline hydroxylation may also regulate integrin binding by promoting the stability of the triple-helical conformation (Sipilä et al. 2018; Rappu et al. 2019). Furthermore, the hydroxylation of proline residues seems to be more critical for α1β1 than for α2β1 binding (Perret et al. 2003). In general, very little is known about the potential role of extracellular post-translational modifications in the regulation of ECM interactions. Still, for example, inflammation-related citrullination of arginine residues in collagen can affect integrin-mediated cell adhesion (Sipilä et al. 2014). Collagen glycosylation may also play a role (Stawikowski et al. 2014).

Like α2β1, α1β1 can also recognize the triple-helical motif formed by GFOGER-like sequence. However, α1β1 also binds with high avidity to a very different triple-helical motif found in collagen IV. In this site α1β1 recognizes two aspartic acid

residues and one arginine residue, all located in different α-chains (Saccà et al. 2003). Thus, α1β1 may bind to fibril-forming and basement membrane collagens with a different mechanism.

Despite the fact that the coevolution of collagens and integrins has resulted in specific high avidity integrin binding motifs in fibril-forming collagens, it is questionable, whether cells are using this mechanism in adhesion to mature collagen fibrils (Woltersdorf et al. 2017). Integrin α2β1 can bind to fibrillar collagen I in in vitro assays (Jokinen et al. 2004; Taubenberger et al. 2007). It has been shown that GPO triplets provide local conformational flexibility in collagen fibrils and some of the high affinity integrin binding motifs occur close to the conserved GPO sites. This observation may explain the mechanism of direct integrin binding to collagen fibrils (Chow et al. 2018). Furthermore, the reconstruction of type I collagen fibrils has unveiled conformational changes on fibril surfaces, which may allow cryptic binding sites, e.g. integrin binding motifs, to be exposed (Zhu et al. 2018). However, given the fact that in tissues collagen fibrils are usually covered with ECM glycoproteins and proteoglycans it is probable that the anchorage of cells to collagen fibrils most often takes place via indirect mechanisms.

Proteolytic degradation of ECM and plasma proteins generates biologically active peptides that have the ability to inhibit angiogenesis and tumour growth via binding to cell surface integrins (Ricard-Blum and Vallet 2019). Integrin α1β1 is a cellular receptor for some of collagen IV derived peptides, such as arresten (Colorado et al. 2000; Nyberg et al. 2008; Aikio et al. 2012) and canstatin (Roth et al. 2005). Integrin α1β1 binding to arresten prevents angiogenesis by inhibiting endothelial cell proliferation, migration, and tube formation, and the binding also has pro-apoptotic functions (Colorado et al. 2000; Nyberg et al. 2008; Boosani et al. 2009). In addition to anti-angiogenic activity, canstatin mediates tumour cell adhesion in an α1β1 integrin-dependent manner (Roth et al. 2005).

3 Functions: Development

Integrin-mediated cell adhesion regulates cell morphology, locomotion, metabolism, survival, differentiation, and growth. In general, the knockout mouse phenotypes of different integrin subunits reflect their evolutionary history. For example, the deficiency of integrin α5, a fibronectin receptor, leads to early embryonic lethality (Yang et al. 1993). Integrin α5 or its orthologs that also recognize arginine–glycine–aspartic acid motifs in ECM proteins are found in basically all metazoans and they may represent the oldest existing type of an integrin, while the collagen receptor integrins are only found in the vertebrates (Johnson et al. 2009). Therefore, it is not very surprising that α1 (Gardner et al. 1996) and α2 null mice (Chen et al. 2002; Holtkötter et al. 2002) have no obvious developmental defects in their tissues or organs.

It is also important to notice that mice are relatively small mammals and it is possible that development defects that remain indistinguishable in mouse would be

more prominent in larger animals. Indeed, this is the case in knockout phenotype of α10 integrin, a collagen receptor with more limited expression pattern than α1 or α2. In mouse a mild and transient delay in the development of long bones has been reported (Bengtsson et al. 2005). However, in dog inherited defect of this gene leads to impaired bone growth and short legs also in adulthood (Kyöstilä et al. 2013). Furthermore, it is possible that large and long-living mammals would develop ageing-related phenotypes. The main known in vivo functions of α1β1 and α2β1 are summarized in Table 2.

4 Functions: Cell Proliferation

The α1 deficient mice are not fully normal since their skin is hypocellular, proposing that stem cell proliferation may be affected (Pozzi et al. 1998). The healing of bone fractures is compromised in α1 null mice due to the defected proliferation of bone marrow mesenchymal stem cells (Ekholm et al. 2002). Accordingly, α1 deficient animals seem to develop age-dependent and post-traumatic osteoarthritis (Zemmyo et al. 2003; Shin et al. 2016). The important role of α1β1 integrin in the maintenance of bone metabolism is also proposed based on the observation that polymorphism in integrin α1 gene is associated with osteoporosis and related fracture risk in Korean females (Lee et al. 2007). These observations are in accordance with the idea that one of the functions of α1β1 is to support the proliferation of specific mesenchymal stem cells populations.

The role of α2 integrin in the regulation of cell proliferation is still unclear, since it has been reported to either promote or suppress cell proliferation. The function of α2β1 may well be dependent on cell type or experimental system. Especially three-dimensional cell culture conditions, contracting collagen gels, or cell adhesion to fibrillar collagen may trigger α2β1 signalling that promotes growth arrest. In these in vitro experiments the inhibition in cell proliferation has been connected, e.g., to the accumulation of cyclin/cyclin-dependent kinase inhibitor p27kip (Henriet et al. 2000; Fringer and Grinnell 2001; Koyama et al. 1996). Furthermore, in three-dimensional spheroid cultures of prostate cancer cells α2β1 inhibits cell proliferation (Ojalill et al. 2018).

Integrin α2 null animal models have not been able to give clear evidence supporting participation of this integrin in the regulation of cell division. Interestingly, in mice in ageing bone α2β1 seems to have an opposite role when compared to α1β1. While the deficiency of α1β1 promotes osteoarthritis (Zemmyo et al. 2003), the α2 null mice seem to be resistant to age-related bone deterioration (Stange et al. 2013). It is not clear whether accumulation of collagen and other bone matrix proteins in the lack of α2 is due to enhanced cell proliferation or increased ECM production per cell or diminished degradation. In skin epidermis basal keratinocytes express α2β1, but it is not clear whether the receptors are active or bound to ligands. During human mucosal wound healing the expression and location of α2 suggest that it may participate in cell migration (Larjava et al. 1993). However, healing

Table 2 Summary of main functions of α1β1 and α2β1 collagen receptor integrins

Tissue/target	Function of α1β1 integrin	Cell type	Reference
Angiogenesis	Promotes tumour vascularization by regulating matrix metalloproteinase and angiostatin production	Endothelial cells	Pozzi et al. (2000)
Bone	Promotes undifferentiated mesenchymal cell proliferation and cartilage production	Mesenchymal stem cells	Ekholm et al. (2002)
	Protects against age-dependent osteoarthritis	Chondrocytes	Zemmyo et al. (2003)
	Protects against post-traumatic osteoarthritis	Chondrocytes	Shin et al. (2016)
Retina	Localizes to the basal aspect of retinal pigment epithelial cells; Protects against retinal degeneration; Required for transducin translocation in photoreceptor cells	Epithelial cells	Peng et al. 2008
ECM remodelling	Controls collagen turnover and fibrosis by modulating reactive oxygen species production	Mesangial cells	Chen et al. (2004)
Immunity	Mediates effector T cell suppression via adhesion to collagen IV	Effector CD4+ T cells	Eckert et al. (2020)
	Promotes cutaneous CD8+ T_{RM} persistence; Regulates CD8+ T_{RM}-mediated immunity	CD8+ T_{RM} cells	Bromley et al. (2020)
	Modulates CD8+ T cell localization and motility	CD8+ T cells	Melssen et al. (2021)
	Marker of lung-resident NK cells	Natural killer cells	Cooper et al. (2018)
	Binding to collagen XIII mediates peripheral blood monocyte adhesion and migration	Monocytes	Dennis et al. (2010)
Inhibitors	**Effect of α1β1 integrin inhibition**	**Cell type**	**Reference**
α1-blocking antibody	Inhibits endothelial cell attachment to collagen I and IV, and laminin-1; Inhibits VEGF-driven angiogenesis; Suppresses tumour growth in a combination with α2-blocking antibody	Endothelial cells	Senger et al. (1997), Senger et al. (2002)
	Inhibits inflammatory responses and cartilage destruction	Leukocytes	de Fougerolles et al. (2000)
	Reduces atherosclerosis and induces a stable plaque phenotype	Macrophages	Schapira et al. (2005)
	Inhibits antigen-induced airway responses	Leukocytes	Abraham et al. (2004)
Tissue/target	Function of α2β1 integrin	Cell type	Reference
Platelets	Mediates adhesion to collagen I	Platelets	Chen et al. (2002), Holtkötter et al. (2002)
	Influences platelet size; Mediates platelet adhesion to soluble collagen I	Megakaryocytes	Habart et al. (2013)

(continued)

Table 2 (continued)

Tissue/target	Function of α2β1 integrin	Cell type	Reference
Angiogenesis	Inhibits wound neovascularization; Regulates matrix metalloproteinase production	Endothelial cells	Grenache et al. (2007)
	Mediates anti-angiogenic activity of endorepellin; Necessary for endorepellin recruitment to the vasculature	Endothelial cells	Nyström et al. 2009, Woodall et al. (2008)
	Inhibits tumour angiogenesis via regulation of VEGFR1 expression	Endothelial cells	Zhang et al. (2008)
Retina	Mediates retinal neovascularization; Mediates oxygen-induced retinopathy by regulating Müller cell VEGF production	Müller retinal cells	Madamanchi et al. (2014)
ECM remodelling	Contributes to glomerular injury by regulating collagen IV synthesis	Mesangial cells	Borza et al. (2012)
Immunity	Regulates binding to collagen I and II; Stimulates production of Th17 cytokines	Th17 cells	Boisvert et al. (2010)
	Required for the migration of memory CD4 T cell precursors; Mediates CD4 T cell binding to collagen II	CD4 T cells	Hanazawa et al. (2013)
	Modulates CD8+ T cell relocalization and motility	CD8+ T cells	Melssen et al. (2021)
	Required for NK cell proliferation	Natural killer cells	Stotesbury et al. (2020)
	Mediates polymorphonuclear leukocyte trafficking in extravascular tissue	Leukocytes	Werr et al. (2000)
	Required for mast cell activation, cytokine production, and normal inflammatory responses	Peritoneal mast cells	Edelson et al. (2004)
Inhibitors	**Effect of α2β1 integrin inhibition**	**Cell type**	**Reference**
α2-blocking antibody	Inhibits endothelial cell attachment to collagen I; Inhibits VEGF-driven angiogenesis; Suppresses tumour growth in a combination with α1-blocking antibody	Endothelial cells	Senger et al. (1997), Senger et al. (2002)
	Inhibits inflammatory responses and cartilage destruction	Leukocytes	de Fougerolles et al. (2000)
	Limits TH17 cell differentiation and promotes T_{REG} cell induction	CD4+ T cells	Breuer et al. (2019)
	Inhibits extravascular trafficking of leukocytes in inflammatory bowel disease	Leukocytes	Gillberg et al. (2013)
	Suppresses inflammation of experimental autoimmune encephalomyelitis	–	Tsunoda et al. (2007)

related re-epithelialization is not affected in α2 null mice (Grenache et al. 2007; Zweers et al. 2007). In normal and wounded epidermis the controversy between prominent presence of α2 and lack of knockout phenotype has not yet been solved.

During wound healing increased angiogenesis has been reported in α2 null mice (Zweers et al. 2007; Grenache et al. 2007; Woodall et al. 2008; Zhang et al. 2008; Nyström et al. 2009), suggesting that in endothelial cells α2β1 may inhibit proliferation.

5 Functions: Platelets

Integrin α2β1 is one of the main surface proteins on platelets, while α1 is not present. Based on the analysis of α2 null mice the most obvious role of α2β1 integrin is its action as one of the platelet collagen receptors (Chen et al. 2002; Holtkötter et al. 2002). Integrin α2 deficient platelets show reduced response to collagen and the knockout animals have slightly prolonged bleeding time (Sarratt et al. 2005). The epidemiological data related to human thrombotic diseases also supports these observations. Due to genetic polymorphism some individuals have elevated levels of α2β1 integrin on platelets. These persons may have elevated risk for, e.g., myocardial infarction or cerebrovascular stroke (Carlsson et al. 1999; Santoso et al. 1999; Moshfegh et al. 1999). Furthermore, the medical literature knows one individual lacking α2β1 integrin on platelets and suffering from a mild bleeding disorder (Nieuwenhuis et al. 1985). Conditional knockout of α2 in mouse megakaryocytes leads to reduced platelet size (Habart et al. 2013). It is worth to note that platelets also use other mechanisms to bind to collagen. Direct binding of GPVI to collagens and indirect collagen recognition by von Willebrand factor and GPIβ are often more prominent mechanisms than α2β1 mediated adhesion (Nuyttens et al. 2011).

6 Functions: ECM Remodelling

Early cell culture observations suggested that both α1 and α2 could be involved in the remodelling of collagenous extracellular matrix, but with different mechanism (Riikonen et al. 1995; Langholz et al. 1995). Integrin α1β1 was reported to be a negative feedback regulator of collagen synthesis, while α2β1 mediated adhesion increased collagen synthesis. Furthermore, α2 was reported to be a positive regulator of matrix metalloproteinases 1 and 13, which both are major collagenases (Riikonen et al. 1995; Ravanti et al. 1999). In the case of α1 these ideas have been partially supported by knockout phenotypes, since the turnover rate of the skin collagenous matrix seems to be altered (Gardner et al. 1999). Despite the increased collagen synthesis, α1 knockout animals develop no obvious fibrotic disease due to the fact that the lack of α1 also seems to lead to increased production of matrix metalloproteinases (Gardner et al. 1999). In kidney mesangial cells and podocytes express both α1 and α2 and there the connection between collagen receptors and fibrosis is more obvious. Integrin α1 deficient mice are more sensitive to

adriamycin-induced kidney fibrosis than their normal littermates (Chen et al. 2004), supporting the role of α1β1 as a negative regulator of collagen synthesis. In contrary, integrin α2β1 promotes glomerular injury by positively regulating collagen synthesis (Borza et al. 2012). In accordance with these observations, the loss of integrin α2 delays kidney fibrosis in COL4A3 knockout mice, a genetic model of Alport's syndrome (Rubel et al. 2014). Direct crosstalk between the two collagen receptors is supported by the observation, that when integrin α1 and α2 subunits are co-expressed in murine ureteric bud epithelial cells α1 may regulate α2 dependent signalling functions (Abair et al. 2008). Interestingly, α1β1 mediated cellular signals may also play an important role in the pathogenesis of Alport syndrome (Zallocchi et al. 2013).

Collagen receptors can regulate the synthesis of collagen I and additionally in vitro observations suggest that α2β1 on cell surface may regulate the formation of new collagen fibrils (Velling et al. 2002; Li et al. 2003). This is an interesting hypothesis, but it still lacks in vivo conformation. Putative mechanisms of collagen fibril formation, including the potential role of α2β1, have recently been reviewed (Revell et al. 2021; Musiime et al. 2021).

7 Functions: Angiogenesis

Immunohistochemical analysis of murine and human tissues have indicated that microvascular endothelial cells are positive for α1β1 (Senger et al. 1997; Senger et al. 2002). Sprouting tips of neonatal blood vessels express α2β1 integrin (Senger et al. 1997). The expression of both collagen receptors is increased in vascular endothelial growth factor (VEGF) induced angiogenesis (Senger et al. 1997). Still, vasculogenesis and angiogenesis related to normal development are not affected in α1 or α2 deficient mice. In the absence of α1 experimental tumours are less well vascularized and smaller than in the wild-type mice (Pozzi et al. 2000). The effect is considered to be due to elevated plasma matrix metalloproteinase 9 levels and consequent increase in the generation of angiostatin, a potent inhibitor of endothelial cell proliferation, from circulating plasminogen (Pozzi et al. 2000). In contrary, α2 null mice show increased wound and tumour angiogenesis (Zweers et al. 2007; Grenache et al. 2007; Woodall et al. 2008; Zhang et al. 2008; Nyström et al. 2009). Based on these observations, the two collagen receptors seem to have opposite roles. Interestingly, in α1 and α2 double knockout mice wound and tumour angiogenesis is impaired which reflects α1 rather than α2 function (Ghatak et al. 2016).

Pericytes are located in close proximity to endothelial cells of capillaries, arterioles, and venules and they are regulators of vascular development, stability, and remodelling. In in vivo model of autoimmune encephalomyelitis the expression of α2 on pericytes is induced during cerebrovascular remodelling (Tigges et al. 2013). This may be due to the fact that in vitro the pro-inflammatory cytokine tumour necrosis factor strongly promotes pericyte proliferation and migration, and concomitantly induces a switch in pericyte integrins from α1 to α2 (Tigges et al. 2013).

8 Functions: Retina

In the retinal pigment epithelium α1β1 localizes to the basal surfaces of epithelial cells in contact with basement membrane. Integrin α1 null mice develop delayed onset progressive retinal degeneration and thickening of the basal lamina. Following exposure to light dark-adapted α1 deficient mice display delay in transducin translocation. Thus, α1β1 mediated cell adhesion to basement membrane seems to be essential for photoreceptor cell function (Peng et al. 2008).

Integrin α2β1 is also important for retina. It is strongly expressed in activated Müller cells, a type of retinal glial cells. In an oxygen-induced retinopathy model, α2 deficient mice displayed reduced hyperoxia-induced vaso-attenuation, reduced pathological retinal neovascularization, and decreased vascular endothelial growth factor expression as compared to wild-types (Madamanchi et al. 2014). Thus, also α2β1 contributes to the pathogenesis of retinopathy, but with a very different mechanism than α1β1.

9 Functions: Immunity

Collagen receptors α1β1 and α2β1 were originally found in T lymphocytes as activation-dependent antigens (Hemler et al. 1985). Since then their expression in many types of inflammatory cells has been confirmed (Table 1) and their action has been linked to various inflammatory diseases and infections (Table 2).

Both CD4 and CD8 cells express α1 and α2 (Richter et al. 2007). Integrin α1β1 on human CD4-positive T cells is restricted to conventional effectors, while regulatory T cells (Tregs) do not express this receptor. Tregs are a specialized subpopulation of T cells that act to suppress immune response (Goldstein et al. 2007). Myeloid-derived suppressor cells (MDSCs) represent a major population controlling T cell immune responses. Both MDSCs and their targets, CD4-positive effector T cells express α1. Integrin α1β1 mediated adhesion to collagen IV can be a prerequisite for optimal cell–cell contact times that are required for suppression (Eckert et al. 2020).

Th17 cells form a special subclass of CD4 helper cells, defined by interleukin-17 production. Integrin α2β1 is known to be the main collagen receptor on Th17 cells (Boisvert et al. 2010). Type 1 regulatory T cells (Tr1 cells) are CD4 cells defined by their surface expression of α2β1 integrin and LAG-3 (Kim et al. 2018b). Tr1 cells are interleukin 10 producing T cells implicated as key regulators of peripheral immune tolerance. Furthermore, memory CD4 T cells express high levels of α2β1 integrin and their α2 null precursors fail to migrate from blood into their survival niches in the bone marrow. Integrin α2 deficient cells especially fail to transmigrate through sinusoidal endothelial cells (Hanazawa et al. 2013).

Tissue-resident memory T (Trm) cells form a heterogeneous population that provides localized protection against pathogens. They persist at sites of previous

infection, where they provide rapid local protection against pathogen challenge. Integrin α1β1 has been defined as a marker that differentiates CD8 positive Trm cells on a compartmental and functional basis (Cheuk et al. 2017) and it promotes optimal cutaneous $CD8^+$ Trm-mediated immunity (Bromley et al. 2020).

Recently it has been shown that tumour-infiltrating CD8 positive T cells initially express α2, then gain α1, and finally loose α2 over the course of tumour outgrowth. The expression of the two collagen receptors affects T cell trafficking and localization in tumours but via distinct mechanisms (Melssen et al. 2021).

Natural killer (NK) cells play an important role in antiviral resistance. NK cells can express both α1β1 and α2β1 collagen receptors. Integrin α1β1 has been found to be the hallmark of a special subset of tissue-resident NK cells in the human lung (Cooper et al. 2018). Integrin α2β1 is required for optimal NK cell proliferation but is dispensable for protection against Ectromelia virus (ECTV) and Murine cytomegalovirus (MCMV), two well-established models of viral infection in which NK cells are known to be important (Stotesbury et al. 2020).

In monocytes α1β1 is rapidly induced after LPS-activation (Rubio et al. 1995). The important role of collagen receptors for monocyte function has been shown in a mouse model of Alport syndrome, since in kidney endothelial cells collagen XIII can mediate a selective recruitment of α1β1 positive monocytes (Dennis et al. 2010). Intrahepatic macrophages can be either α1 positive or negative. Integrin α1 positive cells express significantly higher levels of maturation and activation markers, while α1 negatives are highly responsive to stimulation with Toll-like receptor ligands (Martrus et al. 2019).

Integrin α2β1 is not expressed in blood polymorphonuclear leukocytes, but it is induced after activation and is necessary in leukocyte trafficking. For example, neutrophils in experimental human skin blisters are α2β1 positive (Werr et al. 2000).

Peritoneal mast cells also express α2β1. In α2 null mice the numbers of peritoneal mast cells remain normal, but α2 deficient cells are not able to support in vivo mast cell dependent inflammatory responses (Edelson et al. 2004).

To conclude, collagen receptors increase the functional diversity of highly specialized T lymphocytes and support many important processes. In addition, they are expressed on monocytes, neutrophils, and eosinophils and may participate in innate immunity. In some cases, integrin action has been connected to ligand recognition, but often the potential role of collagens or other ECM ligands can only be speculated.

10 In Vivo Functions Unveiled by Integrin Inhibitors

Many published reports indicate that by using specific antibodies or selective inhibitors for α1 or α2 it is possible to affect physiological and disease model related cellular functions in mouse (Table 2). These observations indicate the presence of the collagen receptors on critical cells. However, integrins are known to participate in the formation of signalling platforms on cell surface, where they exist in close

contact with other membrane proteins, including many growth factor receptors. Therefore, integrins have the ability to modify the function of other receptors (Reviewed in Ivaska and Heino 2011). Importantly, integrins may also orchestrate endocytosis and trafficking of growth factor receptors and regulate their number on cell surface (Reviewed in Ivaska and Heino 2011). Collagen receptor integrins also act in collaboration with other collagen receptors, such as the discoidin domain receptors 1 and 2 (Xu et al. 2012). Thus, integrin inhibitors may also affect cellular functions that are primarily regulated by non-integrin receptors. Therefore, based on results related to the use of antibodies and other inhibitors it is not always possible to make conclusion about the direct, i.e. ligand binding dependent, functions of integrins. Given these facts, it is not surprising that observations based on the use of antibodies are occasionally in disagreement with the data from knockout mice experiments.

For example, antibodies against α2 can inhibit cancer-related angiogenesis (Senger et al. 1997; Senger et al. 2002) and endorepellin, an inhibitor of angiogenesis, seems to work in an α2β1-dependent manner (Woodall et al. 2008; Bix et al. 2007; Goyal et al. 2011), while α2 null mice show increased tumour angiogenesis (Zweers et al. 2007; Grenache et al. 2007; Woodall et al. 2008; Zhang et al. 2008; Nyström et al. 2009). Interestingly, antibodies against α1 integrin can also inhibit angiogenesis and lymphangiogenesis (Senger et al. 1997, Senger et al. 2002).

In many animal models the use of integrin inhibitors has confirmed the important involvement of α1β1 and α2β1 in inflammation (de Fougerolles et al. 2000; Breuer et al. 2019; Gillberg et al. 2013; Tsunoda et al. 2007; Schapira et al. 2005; Abraham et al. 2004). Special sulfonamide type inhibitors have been developed to selectively recognize either bend/nonactivated or preactivated α2β1 (Nissinen et al. 2012). An inhibitor selective for nonactivated conformation could in ex vivo assays inhibit platelet function whereas an inhibitor for activated α2β1 state reduced in vivo the signs of inflammation in arachidonic acid-induced ear oedema, PAF stimulated air pouch, ovalbumin-induced skin hypersensitivity, adjuvant arthritis, and collagen-induced arthritis animal models (Nissinen et al. 2015).

11 Signalling by α1β1 and α2β1

The basic molecular structures of α1 and α2 integrin subunits are very similar. They are both single-pass type I membrane glycoproteins and both recognize their ligands using an inserted domain (I domain) that is often called an A domain, based on its structural similarity with von Willebrand factor A domain (for atomic structures, see: Emsley et al. 1997, Salminen et al. 1999). The integrin β1, α1 and α2 subunits contain short intracellular domains, transmembrane anchors, and relatively large ectodomains. Extracellularly located are the “leg” parts of the α and β subunits and the “head” part formed by the two subunits together. The leg parts of integrins can bend at the “knees” of the molecules (Reviewed in Luo et al. 2007). The generally accepted model of integrin function recognizes that integrin heterodimers have

several different conformation-based functional states. Nonactivated integrins are in bent conformation but intracellular proteins, such as talin, can bind to the cytoplasmic tail of β1 subunit and consequently straighten the receptor and activate it (Luo et al. 2007).

Ligand binding to preactivated integrins leads to further conformational changes, separation of intracellular tails, and finally binding and activation of signal mediators (Luo et al. 2007). Increase in the levels of phosphatidylinositol-4,5-biphosphate and phosphatidylinositol-3,4,5-triphosphate is supposed to be one of the initial steps that then promotes tyrosine phosphorylation of proteins such as focal adhesion kinase (FAK), p130Cas and Src (Legate et al. 2009). Soon afterwards, Rho-family members of small GTPases are activated (Legate et al. 2009). In the case of platelets under shear stress it is also possible that ligand binding itself, without α2β1 preactivation, triggers the change from bent to activated conformation (Nissinen et al. 2012).

Integrin-mediated activation of intracellular signalling pathways has often been associated to the β1 subunit. Still, α1β1 and α2β1 integrins may have very different effects on cell behaviour. Heterodimer specific signalling mechanisms are still poorly known. Intuitively, different integrin α subunits may have unique features and are each connected to separate signalling pathways. However, some of the results may be explained by the fact that integrins also participate in a complex interplay with many other receptors. Naturally there are also cell-type specific differences. Therefore, it is often difficult to judge, what the roles of direct and indirect mechanisms are. Still, based on published results, it is clear that both α1 and α2 cytoplasmic tails have their own interaction partners. For example, T cell protein tyrosine phosphatase (TCPTP) can bind to α1 intracellular domain and negatively regulate epidermal growth factor (EGF) receptor function (Mattila et al. 2005). Deletion of α2 tail leads to ligand-independent accumulation of the receptor to focal adhesion sites, suggesting that α tails may also control the function of β1 cytoplasmic domain (Kawaguchi et al. 1994).

Both α1β1 and α2β1 can regulate ERK MAP-kinase (Wary et al. 1998; Pozzi et al. 1998; Klekotka et al. 2001), while α2β1 has been associated to the activation of p38 (Ivaska et al. 1999; Klekotka et al. 2001). Interestingly, α2β1 targeted inhibitors, such as vatelizumab, may also inhibit p38 pathway (Breuer et al. 2019). Both α1β1 and α2β1 may also regulate Akt and PI3-kinase pathway (Ivaska et al. 2002; Consonni et al. 2012) and the formation of reactive oxygen species (ROS) (Honoré et al. 2003; Chen et al. 2007; Chen et al. 2010).

In platelets α2β1 signalling has been studied in detail. Regulated signalling proteins include p21 activated kinase (PAK), Pyk2 (a FAK family protein tyrosine kinase), spleen tyrosine kinase (Syk), Src family kinases, PI3K, SH2 domain-containing leukocyte phosphoprotein (SLP-76), and phospholipase Cγ2 (LPCγ2) (Suzuki-Inoue et al. 2001; Inoue et al. 2003; Guidetti et al. 2009; Consonni et al. 2012).

Integrins are also connected to the cytoskeleton and have the potency to mediate mechanical forces from solid ECM to nucleus and by this mechanism regulate the expression of a large number of genes (Zuidema et al. 2020).

12 Conclusions

During the past decades, the two abundant collagen receptors, α1β1 and α2β1, have been connected to numerous biological phenomena, but in none of them these receptors seem to be absolutely required. Instead of specializing to some critical processes they seem to be generalists with a large number of assisting functions. When the two receptors are present at the same time they often mediate opposite effects rather than support each other. Integrin α1β1 is pro-angiogenic and α2β1 anti-angiogenic as an example. Integrin α1β1 also promotes cell proliferation, while α2β1 may cause growth arrest. In the remodelling of ECM α2β1 increases collagen synthesis and α1β1 inhibits it. When expressed on lymphocytes both collagen receptors give their contribution to the specialization and effectivity of acquired immunity, but they also participate in less specific functions of monocytes and granulocytes. On platelets α2β1 is an important, but still not indispensable, mediator of adhesion to collagen.

General views about the physiological significance of collagen receptor integrins very much reflect the information received from the corresponding knockout mice. However, it is possible that small size, light weight, short lifespan, and germ-free environment of laboratory rodents make them less optimal study objects when we want to analyse cell adhesion to ECM, e.g., during immune response and in musculoskeletal and circulatory systems. Integrins α1 and α2 are also involved in many pathological mechanisms relevant to human diseases and they can be considered as potential drug targets.

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Roles for Integrin α3β1 in Development and Disease

C. Michael DiPersio and Whitney M. Longmate

Abstract Integrins are the major cell surface receptors for adhesion to the extracellular matrix, and their roles in normal physiology and disease pathologies have been the focus of intense investigation since they were discovered almost four decades ago. α3β1 is a laminin-binding integrin with particularly important roles in epithelial tissue development, homeostasis, and repair. The clinical importance of α3β1 is evident from patients that inherit mutations in the *ITGA3* gene (which encodes the α3 integrin subunit), who are born with a multi-organ disorder that is often fatal within the first months of life and includes interstitial lung disease, nephrotic syndrome, and a mild form of epidermolysis bullosa. α3β1 also has important roles in wound healing and several types of cancer, including squamous cell carcinoma and breast cancer. In this chapter, we provide a comprehensive overview of α3β1 functions in normal physiology and how changes in these functions contribute to disease pathologies. Throughout our discussion, we highlight preclinical studies using cell culture models or genetically modified mice that have shed light on mechanisms through which α3β1 regulates a range of cell functions such as migration/invasion, proliferation, survival, matrix assembly/remodeling, and gene expression. We also discuss clinical and bioinformatic studies that support the relevance of these mechanisms to human health and disease. Much of our discussion centers on cutaneous wound healing as a paradigm for investigating roles for α3β1 in tissue remodeling. We then extend this discussion into the context of cancer, where α3β1 has either cancer-promoting or -suppressive roles depending on the cancer type/subtype or stage of progression. In both contexts, we emphasize an emerging role for α3β1 as a regulator of the keratinocyte secretome, allowing these cells to modify the tissue microenvironment through matrix remodeling or crosstalk to stromal cells during skin

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development, wound healing, or tumorigenesis. Finally, we discuss the prospect of exploiting α3β1 as a therapeutic target to treat cancer or pathological wounds, taking into consideration the associated challenges that arise from its complex biology.

Abbreviations

AREs	AU-rich elements
BM	Basement membrane
BMP1	Bone morphogenetic protein 1
Brain-2	Brn-2/Oct-7/N-Oct3/POU3F2 transcription factor
CAFs	Cancer-associated fibroblasts
CCN2	Connective tissue growth factor
Cox-2	Cyclooxygenase-2
CSF-1	Colony-stimulating factor-1
DMBA	7,12-Dimethylbenz[a]-anthracene
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
IAPs	Integrin-associated proteins
ILNEB	Interstitial lung disease, nephrotic syndrome, and epidermolysis bullosa
JEB	Junctional epidermolysis bullosa
JNK	c-Jun N-terminal kinase
LM	Laminin
MAPKs	Mitogen-activated protein kinases
MCP-1	Macrophage chemoattractant protein 1
MMP	Matrix metalloprotease
MRP-3	Mitogen-regulated protein 3
MS	Mass spectrometry
NMD	Nonsense-mediated decay
OSCC	Oral squamous cell carcinoma
RGD	Arginine–glycine–aspartic acid motif present in many ECM ligands
RNAi	RNA interference
SCC	Squamous cell carcinoma
SMG	Submandibular salivary gland
TAZ	Transcriptional co-activator with a PDZ-binding domain
TEMs	Tetraspanin-enriched microdomains
TGF-β	Transforming growth factor β
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
TPA	12-O-tetradecanoylphorbol-13-acetate
uPA	Urinary-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor

VEGF	Vascular endothelial growth factor
VLA	Very late antigen
YAP	Yes-associated protein

1 Introduction

Integrin α3β1 is a laminin-binding integrin that has critical developmental and post-developmental roles in a wide range of epithelial and other tissues. The developmental importance of α3β1 in humans has been revealed since 2012 through discovery of more than a dozen inherited mutations in the *ITGA3* gene, which encodes the α3 integrin subunit. Patients with homozygous *ITGA3* mutations are born with a congenital, multi-organ disorder termed interstitial lung disease, nephrotic syndrome, and epidermolysis bullosa (ILNEB), which is usually fatal within the first months of life (Has et al. 2012; He et al. 2016; Kinyo et al. 2021; Liu et al. 2021b; Nicolaou et al. 2012; Yalcin et al. 2015). α3β1 is also upregulated during wound healing and in a variety of cancer types, reflecting important post-developmental roles in normal and pathological tissue remodeling and implicating this integrin as an attractive therapeutic target (Koivisto et al. 2014; Longmate and DiPersio 2014; Schnittert et al. 2018; Subbaram and DiPersio 2011). However, despite its initial discovery more than 30 years ago, α3β1 has been somewhat enigmatic compared with other integrins due to functional complexities and unique traits.

Our goal in this chapter is to provide a comprehensive and integrated overview of the various roles that integrin α3β1 plays in normal physiology, and to discuss how changes in, or loss of, α3β1 functions contribute to human pathologies and diseases. We will begin with a brief historical perspective of α3β1 in the context of the integrin field's development. Next, we will review basic cellular functions that are regulated by α3β1, including cell adhesion and migration, signal transduction, assembly and organization of the extracellular matrix (ECM), and gene regulation, and discuss how the coalescence of these functions is thought to underlie the physiological roles that this integrin plays in normal and pathological processes. We will then discuss roles for α3β1 in developmental morphogenesis and remodeling of specific tissues and organs that were identified through phenotypes of mouse genetic models and pathologies of patients with inherited *ITGA3* mutations. We will also discuss post-developmental roles for α3β1 in cutaneous wound healing and cancer, revealed largely through conditional α3 knockout models in mice, and supported by clinical studies and bioinformatic analyses of human data sets. In these contexts, we will cover an emerging role for α3β1 as a regulator of gene expression programs in "activated" keratinocytes, including genes that determine their repertoire of secreted proteins, known as the secretome, and allow them to modify the tissue microenvironment of developing skin, wounds, or tumors (Longmate and DiPersio 2017; Longmate 2020). Finally, we will discuss α3β1 as a potential therapeutic target to

treat cancer or pathological wounds. Throughout this chapter, where space constraints preclude us from covering a topic in greater detail, we will direct the reader to published reviews.

2 Integrin α3β1: A Historical Perspective

2.1 The Integrin Family of Cell Adhesion Receptors

Integrins are the major class of cell surface receptors for adhesion to the ECM. All members of this diverse receptor family are obligate heterodimers that consist of an α and a β subunit (Hynes 2002b). Integrins were discovered in the mid-1980s, and their importance as a major family of adhesion receptors across cell types emerged following the 1987 Gordon conference entitled "Fibronectin and Related Proteins" (Hynes 1987). This conference was organized by Richard Hynes and pulled together co-discoverers of different integrins from across divergent fields of biology, revealing their importance across diverse cell types and tissues, as well as their evolutionary conservation throughout the animal kingdom (for a review, see Hynes 2004). Subsequently, rapid development of the integrin field was driven by the cloning and sequencing of cDNAs that encode the α or β subunit of a number integrins, including the platelet integrin αIIbβ3, receptors for fibronectin and vitronectin, leukocyte integrins, and several integrins on lymphocytes originally termed very late antigen (VLA) proteins (Argraves et al. 1987; Corbi et al. 1987, 1988; Fitzgerald et al. 1987; Hemler et al. 1987; Poncz et al. 1987; Suzuki et al. 1987; Tamkun et al. 1986). We now know that all metazoan organisms express integrins, reflecting the necessity of robust cell adhesion in the evolution of multicellularity (Hynes and Zhao 2000). Although much of the initial focus was on integrins that bind specifically to the tripeptide arginine–glycine–aspartic acid (RGD) motif that is present in many adhesive proteins of the ECM or blood (e.g., fibronectin, vitronectin, osteopontin, collagens, fibrinogen) (Ruoslahti and Pierschbacher 1987), the true diversity of the integrin family quickly became evident as more integrin heterodimers were discovered, along with an expanded knowledge of their ligand-binding specificities. Indeed, the mammalian genome harbors genes for 18 α subunits and 8 β subunits that can heterodimerize in a limited number of αβ combinations to form 24 functionally diverse integrins with distinct, although often overlapping, ligand-binding specificities (Hynes 2002a). Moreover, some mRNA transcripts that encode α or β subunits can undergo alternative splicing in regions encoding the extracellular or cytoplasmic domains to generate different integrin isoforms with altered adhesion or signaling functions, adding another level of complexity to this diverse family of receptors (de Melker and Sonnenberg 1999).

Within a few years of their discovery, it became clear that integrins are not only cell adhesion receptors, but they also function as transmembrane conduits of both "outside-in" and "inside-out" signal transduction that can regulate a broad range of cellular processes such as motility, proliferation, survival, and gene expression

(Hynes 2002a; Schwartz and Ginsberg 2002). Integrins have no inherent enzymatic activity; rather, their α and/or β cytoplasmic domains interact directly or indirectly with cytoskeletal components and a wide variety of signaling effectors and adaptor proteins (Legate and Fassler 2009; Liu et al. 2000). Through these interactions, integrins transmit both biochemical signals via intracellular signaling pathways and biomechanical signals via the ECM–cytoskeletal connection (Kechagia et al. 2019). "Outside-in" signals in response to extracellular cues are transmitted through the integrin following its binding to ECM or other extracellular ligand to stimulate intracellular pathways that activate multiple signaling effectors such as mitogen-activated protein kinases (MAPKs; e.g., ERK1/2, JNK), Rho family GTPases (e.g., Rac1, Cdc42), and others. Integrins can also stimulate or support signaling that occurs through other cell surface proteins, such as growth factor receptors (Schwartz and Ginsberg 2002). "Inside-out" signaling is transmitted through the integrin following interactions of its cytoplasmic domain with factors that modulate the integrin's binding affinity and/or avidity for an ECM ligand (Hynes 2002a; Schwartz and Ginsberg 2002).

Integrins often signal from where they concentrate at sites of cell attachment to the ECM, such as focal adhesions (e.g., α5β1, αv integrins) or hemidesmosomes (α6β4) where they mediate the transmembrane linkage between the ECM outside the cell and the actin cytoskeleton or intermediate filament system, respectively, inside the cell (Kadry and Calderwood 2020). Some integrins can also signal from sites of cell–cell adhesion (LaFoya et al. 2018) or lipid rafts (Lietha and Izard 2020), and it is well established that crosstalk between distinct, integrin-mediated cell adhesion complexes (for example, between cell–ECM contacts and cell–cell adherens junctions) is an important mode of regulating mechanically transduced signals (Zuidema et al. 2020). Many integrins signal in cooperation with other cell surface molecules that cohabitate these sites of cell adhesion. Such integrin-associated proteins (IAPs) include growth factor receptors, G-protein coupled receptors, members of the tetraspanin family, caveolin, and urokinase-type plasminogen activator receptor (uPAR) (Berditchevski 2001; Chapman et al. 1999; Comoglio et al. 2003; Del Pozo and Schwartz 2007; Guo and Giancotti 2004; Hemler 2005; Maldonado and Hagood 2021; Sarker et al. 2020; Stipp 2010). In some cases, IAPs can confer ligand-independent signaling functions to integrins, such as α6β4 (Ramovs et al. 2017) and α3β1 (discussed in this chapter). Through their adhesion and signaling functions, integrins have fundamentally important roles in a variety of physiological processes including development, inflammation, thrombosis, angiogenesis, and tissue repair (Bergmeier and Hynes 2012; Dzamba and DeSimone 2018; Guidetti et al. 2019; Hynes 2002b; Koivisto et al. 2014; Longmate and DiPersio 2014; Mezu-Ndubuisi and Maheshwari 2021). Moreover, alterations in integrin adhesion and/or signaling functions play key roles in a wide range of developmental and adult pathologies (Hynes 2002a; Schnittert et al. 2018; Winograd-Katz et al. 2014), including wound pathologies (DiPersio et al. 2016), fibrosis (Maldonado and Hagood 2021), and cancer (Desgrosellier and Cheresh 2010; Hamidi and Ivaska 2018).

2.2 Integrin α3β1

α3β1 was among the first integrins to be discovered in the late 1980s by several groups (Hemler et al. 1987; Hynes et al. 1989; Takada et al. 1988; Tsuji et al. 1990; Wayner and Carter 1987; Wayner et al. 1988), and the cDNA sequence for the human α3 subunit was published in 1991 (Takada et al. 1991). While some integrin α subunits can pair with more than one β subunit, the α3 subunit pairs exclusively with β1 (Hynes 2002a). The mRNA for the α3 subunit can be alternatively spliced to encode two isoforms, α3A or α3B, that have very different cytoplasmic domains (Tamura et al. 1991). The vast majority of cells and tissues that express α3β1 utilize the α3A subunit (de Melker et al. 1997; Tamura et al. 1991). In contrast, there are only a few published studies of α3B expression or function (de Melker et al. 1997; DiPersio et al. 2001), and physiological roles for the rare α3B isoform have been elusive. α3β1 is prominently expressed in epithelial tissues of the skin, kidney, intestine, lungs, bladder, and stomach, but it is also expressed in other tissues including skeletal muscle, the neuromuscular junction, and vascular endothelial cells (Avraamides et al. 2008; Hodivala-Dilke et al. 2003; Kreidberg 2000; Luque 2004; Ramovs et al. 2017; Ross et al. 2017). As we discuss below, α3β1 is a major receptor for cell adhesion to certain laminin (LM) isoforms in the basement membranes (BMs) that underlie or surround these tissues (Longmate and DiPersio 2014; Margadant et al. 2010). Interestingly, most adherent cell types in culture express α3β1 (Fradet et al. 1984), which could reflect its upregulation in cells that are actively growing or remodeling their ECM. Indeed, α3β1 is also expressed in a variety of cancer cell types (Kreidberg 2000; Stipp 2010; Subbaram and DiPersio 2011), as we discuss in Sect. 6.

Initially, α3β1 was classified as a promiscuous integrin that binds to several ECM proteins, including fibronectin, collagen, and LM-111 (Elices et al. 1991; Gehlsen et al. 1989; Takada et al. 1988; Wayner and Carter 1987). Early reports included α3β1 among integrins that bind the RGD tripeptide sequence that is present in many ECM ligands (Elices et al. 1991; Hynes 1992). However, α3β1 binding affinity for these ligands was weak compared with other integrins, and some subsequent studies did not corroborate these interactions (Delwel et al. 1994; Elices et al. 1991; Weitzman et al. 1993), suggesting that the list of physiological ligands for α3β1 is less broad than once thought. The initial confusion around α3β1 ligands occurred before the discovery of its physiologically important LM ligands (see below) and probably contributed to the relatively slow pace at which α3β1 adhesion and signaling functions were characterized compared with other integrins that were discovered around the same time. As a result, integrins with better defined ligand-binding specificities, such as α5β1 (a FN receptor), αIIbβ3 (a fibrinogen receptor on platelets), and the β2 integrins on leukocytes became early paradigms for working out mechanisms through which integrins control cell adhesion and adhesion-dependent signal transduction (Hynes 2002a), while such characterization of α3β1 lagged behind (Kreidberg 2000; Tsuji 2004).

In the two decades following the 1979 discovery of LM-111, the first known LM isoform, the true complexity of the ~16 member LM family became clear (Aumailley 2013). By the early 1990s, it was discovered that α3β1 has a strong affinity for a particular LM isoform, LM-332 (originally named kalinin/LM-5/epiligrin) (Carter et al. 1991; Wayner et al. 1993). As additional LM isoforms were discovered, it became clear that α3β1 has higher affinity for a subset of them (e.g., LM-332, LM-511, LM-521) than it does for LN-111 (Delwel et al. 1994; Kreidberg et al. 1996; Margadant et al. 2010; Nishiuchi et al. 2003; Rousselle and Aumailley 1994). With the discovery of these *bona fide* α3β1 ligands, together with phenotypic characterization of the original α3 knockout mouse (DiPersio et al. 1997; Kreidberg et al. 1996), we began to develop a better understanding of the important roles that this interesting integrin plays in various tissues, as we will discuss in Sect. 4. We now know that α3β1 belongs to a subfamily of LM-binding integrins that includes α6β1, α6β4, and α7β1, wherein the involved α subunits (α3, α6, and α7) are closely related evolutionarily (Hynes 2002a). These four integrins show different (although overlapping) binding specificities and affinities for different LM isoforms (Delwel et al. 1994; Margadant et al. 2010; Nishiuchi et al. 2006; Ramovs et al. 2017; Stipp 2010), and their cell signaling functions and developmental roles are largely distinct (De Arcangelis et al. 1999; Ramovs et al. 2017; Stipp 2010). Therefore, the repertoire of LM-binding integrins expressed by a particular cell type will determine its adhesive and signaling responses to the mixture of LMs that it encounters in the basement ECM.

3 Basic Cell Adhesion and Signaling Functions of Integrin α3β1

Before we delve into the roles that α3β1 plays in different physiological or pathological processes, in this section, we will summarize what is known about how this integrin regulates basic cell functions that are likely to underlie these roles—namely, adhesion/migration, signal transduction, matrix assembly, and gene expression. Although we will consider each of these functions separately, they are often linked or integrated with one another in the contexts of intact tissues. In later sections, we will discuss how these α3β1-dependent cell functions collectively contribute to physiological or pathological processes where evidence supports their involvement.

3.1 Regulation of Cell Adhesion and Migration by α3β1

As already mentioned, α3β1 is a strong receptor for certain LM isoforms. However, numerous studies have reported that it also has relatively low affinity interactions with a variety of other ECM proteins (Tsuji 2004). In addition, α3β1 can localize to

focal adhesions in cells cultured on ECM proteins that are not *bona fide* ligands, such as vitronectin (DiPersio et al. 1995). In such cases, recruitment of α3β1 to pre-existing focal adhesions may be driven by lateral interactions with other cell surface proteins, or by binding of its cytoplasmic domain to cytoskeletal components as has been documented for other integrins under certain conditions (LaFlamme et al. 1992; Takada et al. 1992). In the developing cerebral cortex, α3β1 interacts with netrin (a diffusible guidance cue protein) to regulate inter-neuronal migration (Stanco et al. 2009). α3β1 has also been implicated in the maintenance of cell–cell adhesion in some cell types, including endothelial and epithelial cells (Carter et al. 1990b; Chattopadhyay et al. 2003; Kaufmann et al. 1989; Yanez-Mo et al. 1998; Zhang et al. 2003). While we do not rule out the possibility that α3β1 is a weak receptor or a co-receptor for other ECM proteins, here we will consider LMs as the major adhesive ligands for α3β1. All members of the LM family are heterotrimeric glycoproteins consisting of three disulfide-linked polypeptide chains termed α, β, and γ, which are encoded by 11 distinct genes (5 α genes; 3 β genes; 3 γ genes) (Aumailley 2013). Each LM isoform is named according to its α, β, and γ chain numbers (for example, LM-332 consists of the α3, β3, and γ2 chains) (Aumailley et al. 2005). Among the ~16 different LM isoforms, LM-332, LM-511, and LM-521 are the best ligands for α3β1 (Delwel et al. 1994; Kreidberg et al. 1996; Margadant et al. 2010). However, the predominant LM ligand for α3β1 varies between different tissues (for example, LM-332 in the interfollicular epidermis; LMs-511 and -521 in the hair follicle; LMs-511, -521, and -332 in the kidney collecting duct system) (Margadant et al. 2010; Nguyen et al. 2000; Yazlovitskaya et al. 2021).

Integrin α3β1 binds to LM-332 through the LG1-3 globular domains within the C-terminal region of the LM α3 chain (Aumailley et al. 2003; Carter et al. 1991), and early studies using a recombinant LG3 module demonstrated its sufficiency to support α3β1-dependent cell adhesion, migration, and focal adhesion kinase (FAK) activation (Shang et al. 2001). Studies using cultured keratinocytes with genetic ablation of α3β1 (e.g., α3-null cells), or treated with an anti-α3β1 blocking antibody, revealed an essential role for this integrin in the formation of leading-edge lamellipodia and processive cell migration (Choma et al. 2004; deHart et al. 2003; Frank and Carter 2004). A pro-migratory role for α3β1 was also demonstrated in kidney collecting duct cells on LM-332 (Yazlovitskaya et al. 2015).

Interestingly, anti-migratory effects of α3β1 have also been described in keratinocytes. For example, treatment with an anti-α3β1 blocking antibody enhanced keratinocyte migration on fibronectin or collagen (Kim et al. 1992). This effect most likely stems from the ability of α3β1 to act as a trans-dominant inhibitor of other keratinocyte integrins that bind to non-LM ligands, including fibronectin and collagens (discussed more in Sect. 3.5.3) (Hodivala-Dilke et al. 1998). Another study showed that α3-null keratinocytes migrate better than wild-type keratinocytes, in this case due to a deficiency in the ability of the former cells to deposit and properly organize LM-332 over which they migrated (deHart et al. 2003). These important findings suggested that α3β1-dependent deposition, proteolytic processing and organization of LM-332 determine its influence on keratinocyte migration. Consistently, studies using recombinant LM molecules showed that processing of

LM-332, as well as presence of the γ2 chain C-terminus within the heterotrimeric molecule, has influence over the availability of the LG1-3 domains to bind α3β1 (Kunneken et al. 2004; Navdaev et al. 2008). Proteolytic cleavage of LM-332 as it is incorporated into the cutaneous BM in vivo can have substantial impact on its interactions with both its integrin receptors and other ECM components (Aumailley et al. 2003; Rousselle and Scoazec 2020) as we discuss more in Sect. 3.3. Finally, keratinocyte migration is influenced by the interplay between α3β1 and other keratinocyte integrins that bind different ligands in the mixed ECM, as we will discuss below in Sect. 3.5.3 and will revisit later in the context of wound healing (Sect. 5).

3.2 *Regulation of Signal Transduction by α3β1*

Like many integrins, α3β1-mediated cell adhesion can stimulate intracellular signaling pathways, often by activating or supporting receptor tyrosine kinases or other cell surface proteins, that regulate a wide variety of cellular processes. A comprehensive discussion of integrin signaling is beyond the scope of this chapter, but it has been reviewed extensively over the years (Clark and Brugge 1995; Cooper and Giancotti 2019; ffrench-Constant and Colognato 2004; Guo and Giancotti 2004; Hynes 1992; Ridley et al. 2003; Schwartz and Ginsberg 2002). Here, we will discuss a few select signal transduction pathways that have been linked to integrin α3β1 in different contexts.

3.2.1 FAK-Src Signaling Pathways

FAK and Src family kinases are among the most widely studied effectors of integrin signaling (Avizienyte and Frame 2005; Cary and Guan 1999; Cooper and Giancotti 2019; Mitra and Schlaepfer 2006; Playford and Schaller 2004). The FAK-Src signaling axis can be activated by α3β1-mediated adhesion in keratinocytes and cancer cells (Cagnet et al. 2014; Choma et al. 2007; Ramovs et al. 2021; Shang et al. 2001). Upon integrin-mediated adhesion, FAK phosphorylates itself on Y397, generating a high-affinity binding site for the SH2 domain of Src. FAK-bound Src can then phosphorylate additional residues on FAK (e.g., Y861, Y925), thereby generating binding sites for other adaptor/signaling proteins. In this way, FAK serves as an "activatable scaffold" that links integrin–ECM adhesions to downstream signaling effectors such as the Rac1 GTPase or the MAPKs, extracellular signal-regulated kinase-2 (ERK-2), c-Jun N-terminal kinase (JNK), or p38 (Brunton and Frame 2008; Playford and Schaller 2004; Schwartz and Ginsberg 2002).

α3β1-FAK-Src signaling is important in several epithelial cell types. In migrating keratinocytes, this pathway leads to the activation of Rac1 that drives the formation of stable, leading-edge lamellipodia (Choma et al. 2004, 2007). Another study showed that the Rac guanine nucleotide exchange factor, Tiam1, is required in

keratinocytes for α3β1-dependent Rac1 activation, LM-332 deposition and subsequent migration (Hamelers et al. 2005). In addition, α3β1-mediated adhesion to LM-332 promotes survival of immortalized keratinocytes through activation of FAK and ERK signaling (Manohar et al. 2004), and proliferation of epithelial cells through activation of MAPK signaling (Gonzales et al. 1999). In a mouse transgenic model of mammary basal-like carcinoma, genetic ablation of α3β1 in basal epithelial cells prevented tumor initiation, which is associated with reduced activation of FAK and downstream effector pathways involving Rac1/PAK1, MAPK, and JNK, indicating a critical role for these α3β1-mediated signaling events in mammary tumorigenesis (Cagnet et al. 2014).

3.2.2 Akt and STAT3 Signaling Pathways

Integrin α3β1 has also been linked to the activation of Akt, which in some cases may lie downstream of α3β1-mediated FAK-Src signaling. Studies in transformed keratinocytes and epidermis-specific α3 knockout mice subjected to chemically-induced skin carcinogenesis showed that α3β1 promotes early-stage tumor growth through FAK-Src activation and co-dependent activation of PI3K/Akt and STAT3 signaling pathways (Ramovs et al. 2021).

Studies using mice with deletion of α3 specifically in the ureteric bud of the developing kidney, and collecting duct cells derived from them, showed that α3β1-mediated cell adhesion to LM-332 promotes Akt activation in a manner that is independent of PI3K, but which involves suppression of PTEN, K63-linked polyubiquitination, and interaction of the integrin β subunit with the ubiquitin-modifying enzyme, TRAF6 (Yazlovitskaya et al. 2015). A subsequent study showed that simultaneous deletion of α3 and α6 caused severe inflammation and fibrosis in the collecting ducts, which was mild or absent from α3 or α6 knockout mice, indicating cooperative signaling between the three LM-binding integrins expressed in this model (i.e., α3β1, α6β1, and α6β4). Interestingly, collecting duct cells from α3/α6-null mice displayed increased activation of the NF-κB transcription factor, known to promote the production of pro-inflammatory cytokines and cadherin switching, indicating that the LM-binding integrins maintain homeostasis through suppression of NF-κB signaling (Yazlovitskaya et al. 2021).

3.2.3 Hippo and YAP/TAZ Signaling Pathways

The highly conserved Hippo signaling network integrates various extracellular inputs to control tissue development, homeostasis, and regeneration/repair, and its dysregulation contributes to a number of developmental defects and diseases (Varelas 2014), including cancer progression and metastasis (Moroishi et al. 2015; Lamar et al. 2012). The primary Hippo pathway effectors are two transcriptional co-activators, YAP (Yes-associated protein) and TAZ (transcriptional co-activator with a PDZ-binding domain), which are commonly referred to collectively as

YAP/TAZ due to functional overlap in their activation of target genes through interactions with TEAD/TEF transcription factors. In mammals, the Hippo pathway consists of a core MST1/2-LATS1/2 kinase cascade that when activated leads to phosphorylation of key serine residues on YAP/TAZ to promote their cytoplasmic sequestration and proteasomal degradation. Upon suppression of Hippo signaling, YAP/TAZ are transported to the nucleus to activate target genes that control cellular processes critical for tissue development and homeostasis, including proliferation, apoptosis, motility, and differentiation (Varelas 2014; Zhao et al. 2010). YAP/TAZ nuclear localization and activation are highly responsive to mechanical forces that are exerted through changes in ECM stiffness (Varelas 2014). Such mechanical signals can influence YAP/TAZ independently of the Hippo/LATS1/2 cascade (Dupont 2016; Dupont et al. 2011), indicating the existence of other upstream regulators. For example, in the epidermis YAP/TAZ phosphorylation can be controlled by Src family kinases (Li et al. 2016; Schlegelmilch et al. 2011), and mechanically-induced integrin-Src signaling is a key upstream activator of YAP/TAZ signaling (Thompson 2020). Furthermore, integrin–FAK–SRC signaling has been linked to YAP/TAZ activation in numerous epithelial cell types, as reviewed (Warren et al. 2018). As we discuss next, integrin α3β1 has been linked to the regulation of YAP/TAZ in several contexts, both through regulation of the Hippo pathway and independently of Hippo.

In the epidermis and oral epithelium, nuclear YAP/TAZ has been linked to stem/progenitor cell proliferation that is important for tissue homeostasis, regeneration, and wound healing (Elbediwy et al. 2016; Lee et al. 2014). Several studies have shown that certain β1 integrins, including α3β1, are important for YAP/TAZ regulation in the epidermis. Indeed, proliferation and wound healing phenotypes of mice with epidermis-specific YAP/TAZ deletion (Elbediwy et al. 2016) bear resemblance to phenotypes of mice with epidermis-specific deletion of the integrin β1 subunit (Brakebusch et al. 2000; Grose et al. 2002; Piwko-Czuchra et al. 2009; Raghavan et al. 2000), or of the integrin α3 subunit (Conti et al. 2003; Mitchell et al. 2009). Consistently, inhibition/suppression of β1 integrins with blocking antibodies or siRNA targeting *ITGB1* (gene which encodes the β1 integrin subunit), or inhibition of integrin signaling effectors FAK, Src, or PI3K, caused reduced YAP nuclear localization (Elbediwy et al. 2016). Another study in a mouse incisor model of continuous tissue renewal showed that α3β1 initiates a FAK-YAP-mTOR signaling axis that promotes and maintains stem cell expansion (Hu et al. 2017). Interestingly, α3β1-FAK activation of YAP in the oral epithelium was LATS-independent (Hu et al. 2017), and in a separate study epidermis-specific deletion of LATS1/2 did not alter keratinocyte fate (Varelas 2014), suggesting that in these tissues α3β1 may modulate YAP/TAZ signaling through non-Hippo effectors. A number of proteins that are secreted by cultured keratinocytes in an α3β1-dependent manner are encoded by known YAP/TAZ target genes, including *LAMC2* (encoding the LM γ2 chain) (Lin et al. 2015), *SERPINE1/PAI-1* (Liu et al. 2015), and *SPARC* (Wali et al. 2014), raising the possibility that α3β1-YAP/TAZ signaling controls a subset of the keratinocyte secretome (Longmate et al. 2021b).

In another context, α3β1 has been shown to negatively regulate YAP/TAZ through activation of the Hippo pathway. In prostate cancer cells, α3β1 signaling through Abl family kinases maintained the Hippo pathway to suppress YAP/TAZ expression and nuclear localization, thereby inhibiting cell invasion and anchorage-independent growth (Varzavand et al. 2016). Notably, this regulation distinguishes cancer-suppressive roles of α3β1 in prostate cancer cells from pro-tumorigenic/pro-metastatic roles that have been described for this integrin in breast cancer cells (Cagnet et al. 2014; Miskin et al. 2021; Mitchell et al. 2010), as we will discuss further in Sect. 6.

3.2.4 TGF-β Signaling Pathways

As reviewed elsewhere, there is extensive evidence of reciprocal crosstalk between transforming growth factor-β (TGF-β) and certain integrins, including α3β1 (Margadant and Sonnenberg 2010). Indeed, TGF-β can modulate integrin-mediated cell adhesion and migration by regulating the expression of integrins themselves, or of their ECM ligands or IAPs. In addition, some integrins can control the initial activation of TGF-β or its cell surface receptors, or modulate Smad-dependent or Smad-independent pathways through the regulation of TGF-β signaling effectors (Margadant and Sonnenberg 2010). There is strong evidence in some models of coordinated signaling through α3β1 and TGF-β. In hepatocellular carcinoma cells, TGF-β upregulates transcription of the *Itga3* gene through the Ets-1 transcription factor and induces α3β1-dependent invasion (Giannelli et al. 2002; Katabami et al. 2005). In immortalized or transformed keratinocytes, α3β1 augments the TGF-β-mediated induction of matrix metalloprotease-9 (MMP-9), and this regulation is acquired as part of the immortalized phenotype (Lamar et al. 2008a).

In a murine model of pulmonary fibrosis, the deletion of *Itga3* specifically in lung epithelial cells caused a blunted fibrotic response to TGF-β, including reductions in myofibroblast accumulation, collagen deposition, and expression of genes that promote epithelial-to-mesenchymal (EMT) (Kim et al. 2009a, b). In this model, the interaction of α3β1 with E-cadherin and TGF-β receptors was required for TGF-β to stimulate β-catenin phosphorylation and subsequent Smad2–β-catenin complex formation (which stimulates EMT), demonstrating a role for α3β1 in coordinating pro-fibrotic crosstalk between β-catenin and Smad signaling pathways (Kim et al. 2009a, b).

Coordinated signaling between α3β1 and TGF-β in cutaneous wounds is less clear, as different studies have reported disparate effects of manipulating these pathways on wound re-epithelialization. In wounds of full-thickness neonatal skin grafted onto nu/nu athymic mice, re-epithelialization was delayed in *Itga3*$^{-/-}$ grafts compared with wild-type grafts (Reynolds et al. 2008). Both α3-null keratinocytes and *Itga3*$^{-/-}$ grafts displayed elevated expression of Smad7 (Reynolds et al. 2008), which is a known inhibitor of TGF-β signaling through its ability to compete with Smad2 and Smad3 for association with the TGF-β receptor complex. Moreover, treatment with an anti-sense oligonucleotide to repress Smad7 enhanced migration

of α3-null keratinocytes and re-epithelialization of *Itga3*$^{-/-}$ wounds (Reynolds et al. 2008). These findings suggest that α3β1 can enhance responses of wound keratinocytes to TGF-β by down-regulating a pathway inhibitor (e.g., Smad7). However, TGF-β has also been shown to inhibit wound re-epithelialization (Margadant and Sonnenberg 2010), and other studies showed that epidermis-specific deletion of α3 slightly enhanced, rather than reduced, wound re-epithelialization (Margadant et al. 2009). It is possible that these disparate findings are due to salient differences between the in vivo models used, such as global versus epidermis-specific *Itga3* deletion, or analysis of wounds made in neonatal versus adult skin. In any case, they suggest that cooperative signaling between α3β1 and TGF-β during wound healing is highly dependent on context.

3.3 Regulation of Basement Membrane Assembly and Integrity by α3β1

All epithelial and endothelial cell layers are in contact with a BM that is rich in one or more LM isoforms that serve as adhesive ligands for α3β1 or other integrins. BMs also contain type IV collagen and other glycoproteins and proteoglycans, such as nidogen/entactin and perlecan, which together with LM comprise a specialized ECM that separates the epithelial/endothelial cell compartment from the adjacent connective tissue stroma (Aumailley 2021; Pozzi et al. 2017). Early studies in murine embryoid bodies that lack β1, and in epidermis-specific β1 knockout mice, revealed crucial roles for β1 integrins in the organization and stability of BMs (Aumailley et al. 2000; Raghavan et al. 2000). Among integrins, α3β1 has emerged as a particularly important regulator of BM assembly and integrity (Longmate and DiPersio 2017; Ramovs et al. 2017; Stipp 2010). Indeed, BM disorganization has been reported in multiple tissues/organs of α3 knockout mice or ILNEB patients with *ITGA3* mutations, including kidney, lung, and skin, and the loss of BM functional integrity is likely to be a root cause of the morphological defects observed in these tissues/organs (DiPersio et al. 1997; Has et al. 2012; Kreidberg et al. 1996; Longmate et al. 2014).

The mechanisms that underlie α3β1-dependent BM organization and integrity have been investigated most extensively in the skin. Absence of α3β1 from the epidermis of α3-null mice (DiPersio et al. 1997; Longmate et al. 2014) or from the skin of ILNEB patients (Has et al. 2012) results in a disorganized cutaneous BM, which eventually ruptures as evidenced by the distribution of LM-332 and other BM proteins to both the epidermal and dermal sides of the small blisters that form. Keratinocyte α3β1 has been shown to regulate the expression, proteolysis, and organization of LM-332 both in vivo and in vitro (deHart et al. 2003; Hamelers et al. 2005; Longmate et al. 2014), and it also promotes expression of the extracellular proteases MMP-9, MMP-3, and bone morphogenetic protein 1 (BMP1) (DiPersio et al. 2000a; Iyer et al. 2005; Lamar et al. 2008a; Longmate et al.

2021a, b). Of particular interest, BMP-1 can proteolyze the γ2 chain of LN-332 (Amano et al. 2000), and α3β1-dependent BMP-1 expression has been linked to α3β1-dependent processing of the LM γ2 chain in keratinocytes (Longmate et al. 2018).

Both the α3 and γ2 chains of LM-332 undergo proteolytic processing (Amano et al. 2000; Rousselle and Scoazec 2020; Sasaki et al. 2001; Tsubota et al. 2000). Although the in vivo relevance of LM-332 processing is not yet fully understood, it can influence keratinocyte motility and other behaviors (Goldfinger et al. 1998, 1999). LM-332 processing can also determine physical linkages with other matrix proteins that control its incorporation into the BM (Aumailley et al. 2003). For example, the matricellular protein fibulin-2 binds to LM-332 within the L4 module of the LM γ2 chain (Utani et al. 1997), and this module is required for stable incorporation of LM-332 into the developing BM (Gagnoux-Palacios et al. 2001). Studies in mouse models show that α3β1 promotes fibulin-2 expression in cultured keratinocytes, as well as in developing skin, healing wounds, and skin tumors in vivo (Longmate et al. 2014, 2021b; Missan et al. 2014). Interestingly, keratinocyte α3β1 also induces BMP-1, which mediates LM γ2 chain processing in the region where fibulin-2 binds (Longmate et al. 2014, 2018), an event that is associated with BM maturation (Aumailley et al. 2003; Sasaki et al. 2001). Together, these findings suggest a role for α3β1 in the induction of key proteins that contribute to the assembly, stability, and maturation of the epidermal BM during skin development and wound healing (Fig. 1). Thus, the regulation of BM assembly and integrity by α3β1 provides an opportunity for this integrin to alter its own extracellular cues and provide feedback to regulate its own epidermal functions. In later sections, we will revisit this role in the contexts of development (Sect. 4), wound healing (Sect. 5), and cancer (Sect. 6).

3.4 Modulation of Gene Expression Programs by α3β1

Integrin α3β1 is not essential in many epithelial cells for adhesion to the BM, as they usually express other LM-binding integrins (e.g., α6β1 or α6β4) that compensate in its absence (Margadant et al. 2010; Stipp 2010). For example, the genetic ablation of α3β1 from epidermal keratinocytes does not cause loss of adhesion to LM-332 either in cultured cells or in vivo, due to continued expression of α6β4 (Choma et al. 2004; deHart et al. 2003; DiPersio et al. 2000b). Indeed, as mentioned previously (Sect. 3.3), skin blisters that form in α3-null mice are caused by BM rupture rather than epidermal detachment from the BM (DiPersio et al. 1997). Interestingly, genetic ablation of α3β1 in immortalized keratinocytes in culture, or in tumor or wound keratinocytes in vivo, leads to altered expression of numerous genes that encode proteins with known roles in ECM remodeling, including ECM proteins (e.g., fibulin-2), extracellular proteases (e.g., MMPs, BMP-1), and growth factors/cytokines [e.g., CSF1, mitogen-regulated protein-3 (MRP-3)] (Iyer et al. 2005; Longmate et al. 2014, 2018, 2021a, b; Missan et al. 2014).

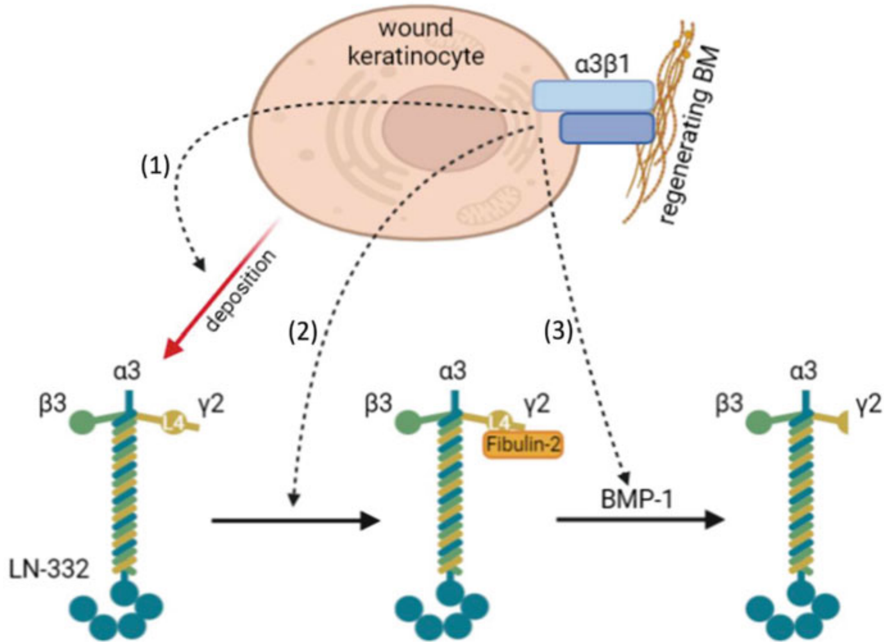

Fig. 1 Model depicting how integrin α3β1 on the surface of epidermal keratinocytes contributes to regeneration of the BM during wound healing. In vivo and cell culture studies have shown that α3β1 impacts the stable assembly of LN-332 into the BM at several different steps, including (1) LN-332 deposition, (2) expression of the LN-332 binding protein fibulin-2, and (3) proteolytic processing of the LN γ2 chain via induction of the protease BMP-1 (see text for discussion and supporting references). The α3, β3, and γ2 chains of the LN-332 trimer, along with the L4 module on the γ2 chain, are indicated. This illustration was created with BioRender.com, and adapted from Longmate and DiPersio (2014)

Collectively, the above findings suggest that an important consequence of α3β1-mediated adhesion and/or cytoskeletal regulation is the modulation of gene expression programs that help the cell carry out its tissue remodeling functions. In support of such a role, early studies in cultured keratinocytes identified α3β1-dependent regulation of individual genes involved in ECM remodeling, such as urinary-type plasminogen activator (uPA) (Ghosh et al. 2006) and MMP-9 (DiPersio et al. 2000a; Iyer et al. 2005). Later studies used gene microarrays to interrogate transcriptome changes caused by genetic deletion of the α3 gene, or RNA interference (RNAi)-mediated suppression of the α3 mRNA, in immortalized mouse keratinocytes or human breast cancer cell lines, respectively. While the repertoire of altered genes differed between these cell types/species, these studies revealed that α3β1 can globally regulate gene expression (Missan et al. 2014; Subbaram et al. 2014). α3β1-responsive genes included ECM proteins, extracellular proteases, growth factors, and cytokines that can allow epithelial cells to alter their tissue microenvironment through ECM remodeling or paracrine crosstalk with other cells in the

adjacent stroma. Remarkably, α3β1-dependent gene expression was not observed in freshly isolated, non-immortalized primary keratinocytes (DiPersio et al. 2000a; Lamar et al. 2008b; Missan et al. 2014), suggesting that that ability of α3β1 to modulate the transcriptome is acquired by "activated" wound or tumor keratinocytes, perhaps due to ligand availability, interactions with IAPs, or downstream signaling effectors. The extent to which the ability of α3β1 to modulate the transcriptome is shared by other integrins is not known. In any case, α3β1-mediated gene regulation serves as a paradigm to investigate how integrin-dependent changes in the transcriptome may control wound healing or drive tumor growth and progression. As we discuss next, published studies support roles for α3β1 in regulating gene expression at both the transcriptional and post-transcriptional levels.

3.4.1 Regulation of Post-Transcriptional mRNA Stability

It has been known for some time that α3β1 can control post-transcriptional mRNA stability to increase the expression of certain pro-tumorigenic/pro-angiogenic genes, which it does by controlling alternative mRNA processing events that determine transcript susceptibility to mRNA degradation pathways. In one example, genetic deletion of the *Itga3* gene in immortalized keratinocytes led to use of an alternative polyadenylation site in the *MMP9* gene, thereby generating an extended 3′-UTR that harbors several AU-rich elements (AREs) and destabilizes the mRNA transcript through ARE-mediated decay pathways (Missan et al. 2015) (Fig. 2). In a second example, stable RNAi-targeting of the *ITGA3* gene in MDA-MB-231 triple-negative breast cancer (TNBC) cells led to alternative splicing of the *PTGS2* mRNA that encodes cyclooxygenase-2 (Cox-2), thereby causing retention of an intron that harbors premature termination codons and targets the mRNA for nonsense-mediated

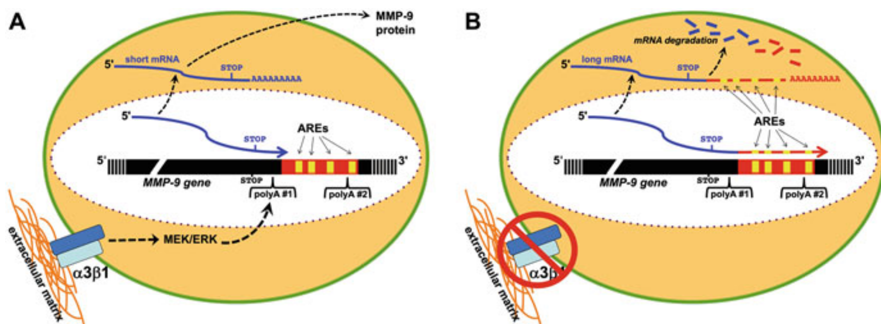

Fig. 2 Model depicting integrin α3β1-dependent alternative polyadenylation of the MMP-9 mRNA transcript. (**a**) α3β1-MEK/ERK signaling promotes use of the proximal polyadenylation site (polyA #1) within the *MMP9* gene to generate the shorter stable mRNA transcript. (**b**) In the absence of α3β1, polyadenylation defaults to the distal site (polyA #2) to generate the longer ARE-containing mRNA that is subject to degradation. This illustration was reproduced from Missan et al. (2015)

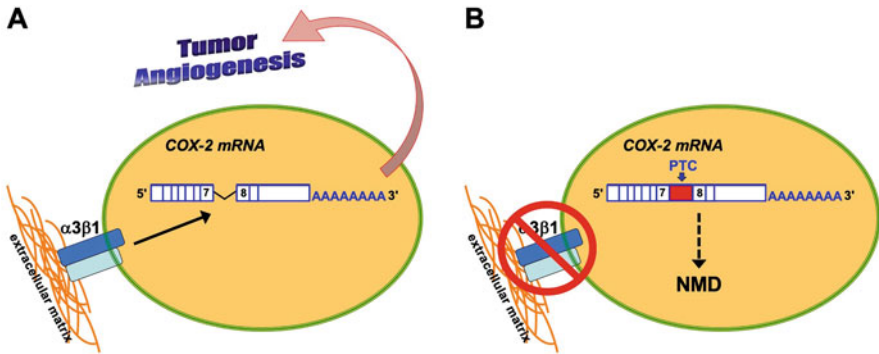

Fig. 3 Model depicting integrin α3β1-dependent splicing of the Cox-2 mRNA transcript. (**a**) α3β1 promotes normal splicing of exons 7 and 8 to generate an intact mRNA transcript that encodes Cox-2. (**b**) When α3β1 is suppressed, signals that maintain normal splicing are lost leading to retention of an intron that harbors a premature termination codon (red box), which targets the transcript for nonsense-mediated mRNA decay (NMD). This illustration was reproduced from Subbaram et al. (2014)

decay (NMD) (Subbaram et al. 2014) (Fig. 3). A common theme in both models is that α3β1 maintains target gene expression by preventing the inclusion of regulatory elements in the mRNA transcript that would otherwise target it for a mRNA degradation pathway. Transcriptome profiling of MDA-MB-231 cells using an Affymetrix human exon array revealed that suppression of α3β1 leads to alternative mRNA splicing or polyadenylation site usage for many other genes (Subbaram et al. 2010, 2014), suggesting a generally important role for α3β1 in post-transcriptional gene regulation.

3.4.2 Regulation of Gene Transcription

α3β1-dependent gene regulation can also occur at the transcriptional level. Indeed, the genome-wide array study mentioned above identified the transcription factor Brain-2 (Brn-2/Oct-7/N-Oct3/POU3F2) among the top 15 transcripts that were altered in MDA-MB-231 cells treated with *ITGA3*-targeting shRNA (Subbaram et al. 2014; Miskin et al. 2021). Although Brn-2 and some of its target genes are implicated in several types of cancer including melanoma, prostate cancer, and breast cancer (Fane et al. 2019; Bishop et al. 2017; Ishii et al. 2013), this was the first report that Brn-2 is regulated by an integrin. α3β1 increased Brn-2 expression at least partly through the induction of the Brn-2 transcriptional promoter, which required Akt signaling (Miskin et al. 2021). Importantly, α3β1-dependent Brn-2 expression promoted MDA-MB-231 cell invasion in vitro and metastatic lung colonization in vivo, and exogenous Brn-2 partially restored invasion to α3β1-deficient cells (Miskin et al. 2021). Although the study did not identify specific Brn-2-regulated genes that are α3β1-dependent in this model, the findings implicate

α3β1 in the transcriptional regulation of at least some Brn-2 target genes. Analysis of RNAseq data from patients with basal-like breast cancer revealed that high *BRN2* expression correlates with high *ITGA3* expression and poor survival, supporting a pro-cancer role for the α3β1-Brn-2 axis of transcriptional regulation (Miskin et al. 2021).

Interestingly, results of a separate study in MDA-MB-231 cells suggest that the ability of α3β1 to regulate the transcriptome may depend on a threshold level of the integrin's expression. In this study, incomplete suppression of cell surface α3β1 using siRNA caused >2-fold change (increase or decrease) in the expression of 883 genes. In contrast, the transcriptome was hardly changed following complete ablation of cell surface α3β1 using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) to inactivate the *ITGA3* gene, which caused >2-fold change in only 37 different genes (Kenney et al. 2021). These findings revealed an important distinction between using RNAi to partially suppress *ITGA3* expression (leaving residual α3β1 on the cell surface) or using CRISPR to completely ablate *ITGA3* expression (causing complete absence of α3β1). While the underpinnings of this discrepancy remain to be determined, these findings suggest that MDA-MB-231 cells acquire a compensatory mechanism to maintain gene expression when α3β1 cell surface levels drop below a certain threshold (Kenney et al. 2021). Such a threshold effect is reminiscent of the dose effects on tumor growth that have been reported for RGD-mimetic integrin inhibitors (Reynolds et al. 2009), or following genetic or pharmacological inhibition of FAK (Kostourou et al. 2013). Therapeutic implications of these findings are discussed later in Sect. 7.

3.5 Modes of Regulating α3β1 Function

The adhesion and signaling activities of integrin α3β1 can vary substantially between different cell types, or even within a cell type during different phases of a physiological or pathological process. In some cases, α3β1 levels on the cell surface are controlled through regulated expression of the α3 subunit (Ramovs et al. 2017; Stipp 2010). For example, TGFβ-mediated induction of the Ets-1 transcription factor can enhance *ITGA3* gene expression in some tumor cells (Kamoshida et al. 2012; Katabami et al. 2005). However, α3β1 adhesion and/or signaling functions often change dramatically without being linked to substantial changes in its expression, such as in wound/tumor keratinocytes and some cancer cells (Missan and DiPersio 2012; Ramovs et al. 2017). In this section, we will briefly discuss some of the documented mechanisms through which cells may regulate α3β1 functions that we covered in Sects. 3.1–3.4. These mechanisms include post-translational modification of α3β1 (e.g., glycosylation), dynamic interactions of α3β1 with IAPs, and functional interplay between α3β1 and other integrins. Later, we will revisit some of these mechanisms in the contexts of wound healing (Sect. 5) and cancer (Sect. 6).

3.5.1 Glycosylation

Post-translational glycosylation of α3β1 can have profound effects on its adhesion and signaling functions, and aberrant α3β1 glycosylation has been strongly linked to invasiveness of a variety of malignant cell types, as reviewed elsewhere (Ramovs et al. 2017). α3β1 harbors more than two dozen potential N-glycosylation sites, and interactions of galectin-3 with N-glycans on α3β1 can promote α3β1-Rac1 signaling that drives lamellipodia formation in epithelial cells (Saravanan et al. 2009). Hypoxia-induced changes to the glycosylation of α3β1 can modulate its translocation to the cell surface, altering the invasive potential of carcinoma cells (Ren et al. 2014). Glycosylation of α3β1 in cancer cells has been shown to regulate its interaction with the tetraspanin CD151 (see below), which in turn modulates cell motility, BM degradation and invasion (Ranjan et al. 2014). In addition, CD151 has been shown to promote specific glycosylation of α3β1 through direct contact, and reduced α3β1 glycosylation in CD151-depleted breast cancer cells was correlated with reduced migration toward LM-332 (Baldwin et al. 2008). While the precise mechanisms through which α3β1 glycosylation modulates cell migration and invasion remain unknown, its clinical relevance is supported by the identification of ILNEB patients with *ITGA3* gain-of-function or loss-of-function point mutations that alter N-glycosylation of the α3 subunit, which prevents heterodimerization with the β1 subunit and transport of mature α3β1 to the cell surface (Nicolaou et al. 2012; Yalcin et al. 2015).

3.5.2 Physical Interactions with Integrin-Associated Proteins

It has long been known that integrin adhesion and signaling functions can be modulated through their interactions with IAPs that cohabitate the cell surface (Brown 2002). Among integrins, α3β1 has a particularly high propensity for lateral associations with IAPs, including several members of the tetraspanin family (Berditchevski 2001; Hemler 2005) and uPAR (Ghosh et al. 2000, 2006; Wei et al. 2001; Zhang et al. 2003). While some such interactions occur at sites of cell–ECM adhesion, others occur at sites of cell–cell adhesion or from within specialized membrane microdomains (Chattopadhyay et al. 2003; Stipp 2010; Zhang et al. 2003), suggesting that in some cases α3β1 can signal independently of binding to its ECM ligands. Indeed, there is ample evidence to support that some α3β1 functions do not require LM-binding (Mitchell et al. 2010; Ramovs et al. 2021), as has been suggested for other integrins such as α6β4 (Ramovs et al. 2017). It stands to reason that cells may have distinct subcellular pools of α3β1 from within which it signals differently. Here we will briefly review interactions of α3β1 with the tetraspanin CD151 and uPAR, as these are among the best studied IAPs for this integrin.

Tetraspanin CD151

Tetraspanins are a large family of multi-transmembrane domain proteins that are found within tetraspanin-enriched microdomains (TEMs) on the cell surface (Hemler 2005). Some tetraspanins can associate with laminin-binding integrins within multiprotein complexes to regulate cell functions such as migration and stability of adherens junctions (Stipp 2010). Although integrin α3β1 can associate with several tetraspanin family members, its interaction with CD151 is direct and especially robust, and in some cells most or all of the α3β1 is complexed with CD151 (Stipp 2010; Yauch et al. 1998, 2000). CD151 is certainly the most widely investigated IAP for α3β1, as reviewed elsewhere (Ramovs et al. 2017; Stipp 2010). Association of α3β1 with CD151 occurs early in the integrin's biosynthesis (i.e., before transport to the cell surface) and has been linked directly or indirectly to a wide variety of epithelial or endothelial cell functions, including cell–ECM adhesion, cell–cell adhesion, cell migration, signal transduction, and intercellular communication (Baldwin et al. 2008; Chattopadhyay et al. 2003; Nishiuchi et al. 2005; Novitskaya et al. 2013; Sadej et al. 2009; Scales et al. 2013; Stipp 2010; Winterwood et al. 2006; Yanez-Mo et al. 1998). The association of α3β1 with CD151 has also been linked to the compartmentalization of TGF-β type I receptor and TGF-β1-induced activation of p38 signaling and experimental metastasis in breast cancer cells (Sadej et al. 2010).

Importantly, the functional consequences of the α3β1-CD151 complex appear to be highly dependent on context (Stipp 2010). For example in carcinoma cells, CD151 promotes α3β1 recycling on the cell surface to support rapid single cell motility (Winterwood et al. 2006). On the other hand, α3β1-CD151 stabilizes E-cadherin-based adherens junctions, which restrains collective carcinoma cell migration (Zevian et al. 2015). These and other studies suggest that the association of α3β1 with CD151 within TEMs at distinct subcellular locations can promote either pro-invasive functions (e.g., promoting migration from cell–ECM adhesion sites) or anti-invasive functions (e.g., stabilizing cell–cell adherens junctions), and the balance between these opposing activities may be determined by the composition of TEMs in a particular cancer cell type or at a particular stage of cancer progression (Ramovs et al. 2017; Stipp 2010).

The exact mechanisms whereby CD151 modulates α3β1 signaling are still not fully understood (Ramovs et al. 2017; Stipp 2010). Some identified points of regulation include glycosylation of α3β1 (Baldwin et al. 2008), cooperative signaling with growth factor receptors (Novitskaya et al. 2013), and the expression/recruitment of signaling effectors (Chattopadhyay et al. 2003). It is possible that much of this regulation occurs through ability of CD151 to influence the localization of α3β1 to sites within the cell where it encounters key signaling effectors (e.g., adaptor proteins, growth factor receptors, kinases). Consistently, Ramovs and coworkers showed that in transformed keratinocytes the activation of Akt and STAT3 signaling by α3β1 required its interaction with CD151 within cell–cell contacts but was independent of binding to LM-332, whereas activation of FAK-Src signaling by α3β1 was independent of CD151 (Ramovs et al. 2021). This dichotomy is a compelling example of two spatially separated α3β1 signaling

pathways that each promote tumorigenesis from within distinct subcellular compartments, and it highlights that subcellular localization of α3β1, determined by its interactions with different ligands or IAPs, can compartmentalize its signaling functions.

Patients with *CD151* or *ITGA3* gene mutations both display renal pathologies, BM disorganization in the kidney and skin, and skin blistering (Has and Fischer 2019; Has et al. 2012; Karamatic Crew et al. 2004; Vahidnezhad et al. 2018), supporting an essential role for the α3β1-CD151 complex in vivo. There are also some phenotypic similarities in CD151 knockout mice and α3 knockout mice, such as reduced susceptibility to skin tumorigenesis (Sachs et al. 2012b, 2014), and kidney defects with disorganized glomerular BM (Baleato et al. 2008; Kreidberg et al. 1996; Sachs et al. 2006). However, some phenotypes of α3 knockout mice are milder or absent in CD151 knockout mice, indicating that some α3β1 functions are less dependent on CD151. For example, α3-null mice die within hours after birth, while CD151-null mice are viable and healthy (Wright et al. 2004). Glomerular BM disorganization in CD151-null mice is less severe than it is in mice with podocyte-specific ablation of α3β1 (Baleato et al. 2008; Sachs et al. 2006). Moreover, while BM is disorganized in healing wounds of both CD151-null mice and epidermis-specific α3 knockout mice (Cowin et al. 2006; Longmate et al. 2014), wound re-epithelialization is reduced in CD151-null mice (Cowin et al. 2006) but slightly enhanced in epidermis-specific α3 knockout mice (Margadant et al. 2009). In addition, as already mentioned, some signaling functions of α3β1 do not require interaction with CD151 (Ramovs et al. 2021), and CD151 can influence some cell functions (e.g., migration) independently of α3β1 (Ramovs et al. 2017). Therefore, while the interaction of CD151 is unquestionably critical for the regulation of many α3β1 functions, it does not appear to be obligate. Throughout this chapter, we will consider how CD151 may contribute to the role of α3β1 in different physiological or pathological processes where evidence supports its involvement.

Urokinase-type plasminogen activator receptor

A number of studies have investigated the interaction of integrin α3β1 with uPAR, the receptor for uPA (Ghosh et al. 2000, 2006, 2010; Wei et al. 2001; Zhang et al. 2003), as reviewed elsewhere (Ramovs et al. 2017). In cancer cells, uPAR has been linked to α3β1-dependent signaling pathways involving Src, ERK1/2, and Rho family GTPases that regulate cell motility, protease expression, and invasive phenotypes (Ghosh et al. 2006, 2010; Shi et al. 2011). In a study of oral squamous cell carcinoma (OSCC) cells, it was shown that LM-332-α3β1-uPAR engagement leads to Src-mediated phosphorylation of p130CAS, which in turn promotes motility through activation of Cdc42, but not Rac1 (Shi et al. 2011). Interestingly, the latter study identified a mechanism of α3β1-dependent epithelial cell motility that is distinct from that described in immortalized keratinocytes, wherein α3β1 adhesion to LM-332 activates FAK-Src signaling, which required Rac1 (Choma et al. 2004, 2007). While it is not clear if these distinct pathways of α3β1-dependent cell motility stem from differences between cutaneous and oral keratinocytes, or are due to overexpression of uPAR in OSCC cells (Shi et al. 2011), they indicate that α3β1

signaling may be modulated through changes in its association with uPAR. There is also evidence that uPAR-mediated cell adhesion to vitronectin can trigger integrin signaling by altering surface tension of the cell membrane in the absence of integrin binding to an ECM ligand (Ferraris et al. 2014), which could explain why some α3β1 signaling functions do not require LM-binding (Ramovs et al. 2017). Finally, α3β1-uPAR and α3β1-CD151 associations may occur concurrently in some cases (Rao Malla et al. 2013), adding another level of potential complexity to the regulation of α3β1 function through IAPs.

3.5.3 Functional Interactions with Other Integrins

Most cells express a number of different integrins, which affords opportunity for functional cooperation or crosstalk between them as a cell responds to a mixed ECM environment. Integrin-to-integrin crosstalk has been well documented and reviewed extensively, and the impact that it may have on the interpretation of studies wherein individual integrins are inhibited, suppressed, or knocked out is widely appreciated (Calderwood et al. 2004; Diaz-Gonzalez et al. 1996; Gonzalez et al. 2010). Nevertheless, interplay between different integrins remains underexplored, especially in the context of physiological or pathological tissue remodeling. There are several clear examples of integrin α3β1 cooperating with, suppressing, or being suppressed by other integrins to modulate the cell's adhesion or signaling response to a complex ECM environment. A few examples are described below.

In an example of coordinated adhesive function between two keratinocyte integrins that bind to different ECM ligands, α3β1 binding to LM-332 was shown to promote RhoGTP activation that allows subsequent α2β1 binding to collagen (Nguyen et al. 2001). In an example of cooperativity between two keratinocyte integrins that bind to the same ECM ligand, α3β1 and α6β4 were shown to cooperate in the stabilization of LM-332-based adhesions through their mutual interaction with the tetraspanin CD151 (Te Molder et al. 2019). In another study, α3β1 and α6β4 were shown to coexist in a multiprotein complex that includes syndecans and epidermal growth factor receptors that may cooperate to activate downstream signaling pathways (Wang et al. 2015).

Early studies in keratinocytes showed that either inhibition of α3β1 with a function-blocking antibody, or absence of α3β1 due to α3 knockout, caused an increase in cell adhesion to fibronectin or collagen, indicating that α3β1 cross-suppresses the adhesion functions of other keratinocyte integrins (Hodivala-Dilke et al. 1998; Kim et al. 1992). This regulation is reminiscent of "trans-dominant inhibition," which was originally described to occur when ligand-bound αIIbβ3 suppresses the function of a target integrin such as α5β1 (Diaz-Gonzalez et al. 1996) and has since been ascribed to other instances of integrin-to-integrin crosstalk (Gonzalez et al. 2010).

Finally, in one of the clearest in vivo examples of trans-dominant inhibition, integrin α9β1 on wound keratinocytes was shown to cross-suppress α3β1-dependent paracrine signaling that stimulates wound angiogenesis (Longmate et al. 2017). This

study utilized mice with epidermis-specific deletion of α3β1, α9β1, or both integrins, as well as keratinocyte lines engineered to express these same integrin combinations. In cultured keratinocytes, α9β1 suppressed the ability of α3β1 to signal through FAK-Src and drive MMP-9 expression and paracrine stimulation of endothelial cells. Moreover, gene microarrays of cells with different combinations of α9β1 and α3β1 revealed that many α3β1-dependent genes, including some pro-angiogenic genes, were suppressed by α9β1 (Longmate et al. 2017). Wounds of epidermis-specific α9 knockout mice showed a delay in blood vessel regression coupled with reduced apoptosis of endothelial cells, indicating that epidermal α9β1 cross-suppresses α3β1 pro-angiogenic functions at later stages of wound healing to achieve vascular normalization (Longmate et al. 2017). In a separate study using immortalized human keratinocytes, α9β1 was shown to suppress ERK1/2 signaling pathways (Hight-Warburton et al. 2021), which are stimulated by α3β1 (Manohar et al. 2004), consistent with a role for α9β1 in the trans-dominant inhibition of α3β1. Although α3β1 and α9β1 are both upregulated at the start of wound healing (Hertle et al. 1991; Singh et al. 2004), the timely coordination of their activities is likely to be determined by the temporally regulated appearance of their ECM ligands during wound healing. Interestingly, α9β1 cross-suppression of α3β1 did not occur in skin tumors (Longmate et al. 2017), most likely due to downregulation of α9β1 in tumor cells through epigenetic silencing or gene copy loss of *ITGA9* (Varney et al. 2021). Presumably, there is a strong selection for loss of this regulation during skin tumorigenesis and development of squamous cell carcinoma (SCC), to maintain pro-angiogenic/pro-invasive functions of α3β1 in these cells.

4 Roles for Integrin α3β1 in Organ Development and Function

The developmental importance of α3β1 is evident from the pleiotropic pathologies of ILNEB patients with inherited mutations in the *ITGA3* gene (Has et al. 2012; He et al. 2016; Kinyo et al. 2021; Liu et al. 2021b; Nicolaou et al. 2012; Yalcin et al. 2015). Tragically, these patients usually survive for only a short time after birth (~2–19 months of age), with lung involvement being the primarily contributor to the lethal course of the disease. However, some patients experience slow disease progression with delayed or no pulmonary involvement, surviving into adolescence or adulthood (Kinyo et al. 2021; Liu et al. 2021b). Mild variants of ILNEB with incomplete kidney and/or lung involvement have also been described in patients with single or compound heterozygous *ITGA3* mutations (Cohen-Barak et al. 2019; Colombo et al. 2016; Okamoto et al. 2021). Although the specific mutations differ between cases, most lead to amino acid substitutions or mRNA splicing deficiencies that alter α3 subunit conformation and/or glycosylation and prevent biosynthesis and cell surface expression of a functional α3β1 heterodimer. Notably, some aspects of ILNEB bear resemblance to phenotypes reported more than a decade previously in

α3β1-deficient mice with global knockout of *Itga3*, particularly in the skin and kidney (DiPersio et al. 1997; Kreidberg et al. 1996). Although the respiratory and renal aspects of this syndrome predominate, it was the relatively mild skin fragility that provided initial clues to the diagnosis (Has et al. 2012), due to its similarity with the unique blistering phenotype involving rupture of the BM at the epidermal–dermal junction that had been described in neonatal α3 knockout mice (DiPersio et al. 1997). The original global knockout of the *Itga3* gene in mice led to perinatal lethality (Kreidberg et al. 1996), consistent with the short lifespan of most ILNEB patients (Has et al. 2012; Nicolaou et al. 2012). Moreover, ILNEB patients display areas of disorganized BM and compromised barrier function in kidney, lung, and skin, which mirror phenotypes of α3 knockout mice (DiPersio et al. 1997; Kreidberg et al. 1996). Indeed, much of what we know about the developmental roles of α3β1 stems from what we have learned from murine models of global or tissue-specific α3 knockout, or from cells derived from these mice. Since the α3 subunit pairs exclusively with the β1 subunit (Hynes 2002a; Tsuji 2004), α3β1 is the only integrin that is affected directly by engineered or naturally occurring *ITGA3* gene mutations, making it easy to attribute phenotypes of mice (or patients) with α3 gene mutations to the absence of, or defects in, integrin α3β1, in contrast with gene mutations in integrin subunits that pair with multiple partners, such as αv, α6 and most β subunits (Hynes 2002a).

Branched-structured organs are present in all mammals, as branching allows an organ to remain compact in size while enhancing its functional surface area. Factors regulating branching morphogenesis during tissue development or regeneration are numerous and complex; however, one important mode of regulation involves cell–ECM interactions mediated by integrins, as reviewed elsewhere (Pozzi and Zent 2011). As mentioned above, global α3-null mice die perinatally and display developmental defects in multiple tissues, many of which are associated with BM disorganization and/or impaired branching morphogenesis (Anton et al. 1999; DiPersio et al. 1997; Kreidberg 2000; Kreidberg et al. 1996; Tsuji 2004). Indeed, α3β1 appears to be a predominate integrin in mediating the development of branched organs, and it seems likely that this role stems largely from its ability to regulate BM assembly and gene expression programs that impact branching morphogenesis. Due to early lethality, use of mice with global α3 knockout is limited to studies of developmental defects. However, constitutive or inducible deletion of α3 in specific tissues often allows mice to survive into adulthood, which has allowed investigators to explore roles for α3β1 in postnatal processes such as cutaneous wound healing, mammary gland function, and cancer (Longmate et al. 2014, 2017, 2018, 2021a, b, Margadant et al. 2009; Mitchell et al. 2009; Ramovs et al. 2021; Raymond et al. 2011; Sachs et al. 2012b). In the following subsections, we review how murine models of global or conditional α3 knockout have so far revealed important roles for integrin α3β1 in the morphogenesis of several organ systems, namely the kidney, lung, salivary gland, mammary gland, vasculature, and skin.

4.1 Kidney

The collecting duct system of the kidney, into which the nephrons drain, develops from the ureteric bud, which undergoes branching morphogenesis followed by tubular elongation to form the mature papilla. Global α3-null mice display major developmental defects in branching of the collecting ducts and organization of the glomerular BM (Kreidberg et al. 1996). Defects in BM organization were also reported in the kidneys of ILNEB patients (Has et al. 2012). Analysis of the kidneys from one ILNEB patient revealed unilateral hypodysplasia with perturbation of Wnt, BMP, and TGF pathways, along with upregulation of early embryonic genes and downregulation of kidney differentiation markers, implicating defective renal differentiation (Shukrun et al. 2014). While α3-deletion specifically in the ureteric bud did not result in a collecting duct branching defect, it did cause a papillary outgrowth defect characterized by failed tubular elongation that was linked to aberrant Wnt signaling and failure to appropriately modulate cell survival (Liu et al. 2009). A similar phenotype was observed in LM α5-null embryos (Liu et al. 2009), presumably reflecting a requirement for α3β1 binding to α5 chain-containing LMs (e.g., LN-511) for appropriate kidney development. A subsequent study in compound knockout mouse showed that the combined deletion of all three LM-binding integrins (i.e., α3β1, α6β1, and α6β4) from the developing ureteric bud results in a mild kidney phenotype that progresses postnatally to a severe inflammation and fibrosis around the collecting ducts, eventual leading to lethality at around 1 year of age (Yazlovitskaya et al. 2021). Since this phenotype is more severe than those of mice deficient in either α3β1 (mild phenotype into adulthood) or α6 integrins (no phenotype), these findings indicated a cooperative role for LM-binding integrins in the kidney collecting ducts (Yazlovitskaya et al. 2021).

Some kidney phenotypes observed in α3β1-deficient mice or ILNEB patients may be due to loss of the α3β1-CD151 complex. Indeed, α3 knockout mice (either global or podocyte-specific) and CD151 knockout mice share kidney defects, including a disorganized glomerular BM (Kreidberg et al. 1996; Sachs et al. 2006). One study showed that CD151 is a critical modifier of α3β1-mediated podocyte adhesion to the glomerular BM, and that blood pressure is an important factor in the initiation and progression of nephropathy in CD151 knockout mice (Sachs et al. 2012a). Moreover, CD151 knockout mice recapitulate many of the kidney pathologies seen in human patients that lack α3β1 or CD151, including BM disorganization (Has et al. 2012; Karamatic Crew et al. 2004; Sachs et al. 2006). However, there is good evidence that the genetic background dictates the presence or severity of kidney pathologies seen in CD151-null mice, suggesting the existence of key modifier genes (Hemler et al. 2013).

4.2 Lung

Bronchi, the main airways of the lungs, branch off into smaller passageways called bronchioles that terminate into tiny sacs called alveoli. Lungs from α3-null mice lack appropriately branched bronchioles, with large bronchi extending into lung periphery (Kreidberg et al. 1996). Genetic deletion of both the α3 and α6 subunits together (i.e., *Itga3*$^{-/-}$:*Itga6*$^{-/-}$ mice) resulted in more severe defects in lung development, likely reflecting cooperative roles for α3β1 and α6 integrins (De Arcangelis et al. 1999). Lung defects in biopsies from ILNEB patients included simplified air spaces or thickened alveolar septa with reactive type II pneumocytes (Has et al. 2012; Nicolaou et al. 2012). However, defects in bronchial development that were observed in α3-null mice (Kreidberg et al. 1996) were not reported in ILNEB patients with *ITGA3* loss-of-function mutations, suggesting that respiratory failure that occurred in these patients was secondary to other perturbations (Has et al. 2012). Importantly, BM disorganization was described in the lungs of ILNEB patients (Has et al. 2012).

4.3 Salivary Gland

The submandibular salivary gland (SMG) is a highly branched structure composed of secretory acinar cells and non-secretory ductal epithelial cells. SMGs isolated from integrin α3-null mice during late embryogenesis displayed branching abnormalities associated with defects in differentiation, apical-basal polarity, and irregularities within the BM, indicating an essential role for α3β1 in SMG development (Menko et al. 2001). α3-null SMGs also displayed altered Ras-Erk signaling and reduced levels of the Rho family GTPases, RhoA and Cdc42 (Menko et al. 2001), and Cre-induced deletion of α3β1 from a pro-acinar ductal cell line (derived from the SMG of a mouse with floxed *Itga3* alleles) recapitulated the downregulation of Cdc42 (Thiemann et al. 2019). Interestingly, combined ablation of integrins α3β1 and α6β1 (i.e., *Itga3*$^{-/-}$:*Itga6*$^{-/-}$ mice) caused more severe defects in SMG development than did deletion of either integrin alone (Georges-Labouesse et al. 1996; Menko et al. 2001), and also phenocopied SMGs from mice lacking the LM α5 chain (Rebustini et al. 2007). These findings likely reflect cooperative roles for the binding of these two integrins to LM-511 in the BM of the developing SMG.

4.4 Mammary Gland

The mammary gland is a highly branched structure consisting of ducts and milk-secreting alveoli. The mammary epithelium of these structures is made up of milk-producing luminal cells surrounded by basal myoepithelial cells, which have

contractile function and are in contact with their BM through the LM-binding integrins α3β1, α6β1, and α6β4 (Raymond et al. 2012). Early studies in which rudiments of developing mammary gland from *Itga3*$^{-/-}$:*Itga6*$^{-/-}$ embryos were transplanted into syngeneic hosts demonstrated that α3β1, α6β1, and α6β4 are not required for ductal morphogenesis/branching or ability to synthesize β-casein, indicating that the major laminin-binding integrins are not essential for gland development or function (Klinowska et al. 2001). However, later studies identified important in vivo roles for β1 integrins in the adult mammary epithelium. Indeed, genetic deletion of the β1 integrin subunit from basal cells of the adult mammary gland resulted in defective ductal branching morphogenesis and failure to maintain a functional stem cell population during pregnancy (Taddei et al. 2008), while deletion of β1 from the luminal cells did not cause branching defects but resulted in disorganized alveoli with reduced FAK activation and decreased cell proliferation (Li et al. 2005; Naylor et al. 2005). When a Cre-Lox approach was used to delete α3 specifically in basal myoepithelial cells of the adult mammary gland, ablation of α3β1 did not alter structural integrity or functional differentiation of the mammary epithelium (Raymond et al. 2011). However, absence of α3β1 did result in reduced milk ejection due to impaired contractility of myoepithelial cells that was caused by reduced FAK signaling and an altered Rho/Rac balance, indicating an essential role for α3β1 in mammary gland function (Raymond et al. 2011). Results of another study in which Cre-Lox was used to delete the *Itga3* and/or *Itga6* genes in mammary basal cells revealed their roles in maintaining the mammary stem cell niche (Romagnoli et al. 2019). Indeed, the combined deletion of α3β1 and α6-integrins caused increased myosin II activity and p53-induced growth arrest, while deletion of either *Itga3* or *Itga6* alone revealed partial functional redundancy among these LM-binding integrins (Romagnoli et al. 2019).

4.5 Vasculature

Integrin α3β1 is expressed on endothelial cells where its association with tetraspanin CD151 at endothelial cell–cell junctions has been linked to the regulation of cell motility (Yanez-Mo et al. 1998), and its binding to the LM α4 chain has been shown to regulate endothelial cell function and promote blood vessel development (Gonzalez et al. 2002). Formation of the α3β1-CD151 complex may also be a prerequisite for pro-angiogenic signaling pathways that are activated by CD151 (Liu et al. 2011). However, roles for α3β1 in vasculogenesis and angiogenesis are complex, and they appear to depend largely on its functions from within distinct tissue compartments. Vasculogenesis appears largely normal in α3-null embryos, although their kidneys display capillary branching defects (Kreidberg et al. 1996). On the other hand, α3β1 expression on endothelial cells has been linked to the repression of pathological angiogenesis, through different mechanisms. In one study, α3β1-mediated endothelial cell adhesion to type IV collagen inhibited pro-angiogenic Cox-2 signaling (Boosani et al. 2007). In another study, the same

group showed that endothelial cell-specific deletion of α3 led to increased tumor angiogenesis and hypoxia-induced retinal angiogenesis, and they provided evidence that endothelial α3β1 suppresses pathological angiogenesis through modulation of vascular endothelial growth factor (VEGF) and its receptor, Flk-1 (da Silva et al. 2010). In a distinct mechanism, anti-angiogenic effects of α3β1 were attributed to binding TIMP-2 on the endothelial cell surface (Seo et al. 2003). Interestingly, these anti-angiogenic effects of α3β1 from within endothelial cells may be counter-balanced by its pro-angiogenic functions from within the epithelial compartment of wounds or tumors, where it may promote Cox-2 signaling (Mitchell et al. 2010) or the production of secreted proteins such as MRP-3 and MMP-9 that stimulate endothelial cells through paracrine signaling (Longmate et al. 2017; Mitchell et al. 2009). Later in Sect. 7, we will discuss how these opposing roles of α3β1 as both promoter and suppressor of angiogenesis from within different cell types may impact the development of therapeutic strategies to target this integrin.

4.6 Skin

In vivo functions of integrin α3β1 have been most extensively studied in the epidermis. Here we will focus on its roles in skin development and maintenance, then in the following section we will discuss its roles in cutaneous wound healing. The stratified epidermis of the skin is a continuously regenerating tissue wherein loss of dead keratinocytes from the outermost layer is balanced by proliferation in the basal layer (Fuchs 2008). In normal resting epidermis, the expression of α3β1 (and other integrins) is restricted to the basal keratinocytes attached to the BM that separates the epidermis from the underlying dermis. As differentiating keratinocytes detach from the BM and move upwards into the suprabasal layers of the epidermis, integrins are downregulated (Watt 2002). Genetic studies in mice have shown that ablation of all β1 integrins from the epidermis (through deletion of the *Itgb1* gene), leads to impaired proliferation, defects in hair follicles and sebaceous glands, disruption of the BM, and epidermal blistering (Brakebusch et al. 2000; Raghavan et al. 2000). In contrast, genetic deletion of α3β1 or any other individual β1 integrin (through deletion of individual α subunit genes) leads to a subset of the β1-null phenotypes in each case, indicating that different integrins have distinct, albeit overlapping functions in the epidermis (reviewed in Longmate and DiPersio 2014). Remarkably, the effects of deleting either all β1 integrins or any of several individual integrins (α3β1, α2β1, α9β1, α6β4, or αvβ5) on epidermal stratification and differentiation were mild or absent (Brakebusch et al. 2000; DiPersio et al. 1997, 2000b; Dowling et al. 1996; Huang et al. 2000; Raghavan et al. 2000; Singh et al. 2009; van der Neut et al. 1996; Zweers et al. 2007), indicating that that this process is not dependent on any particular integrin(s).

LM-332 is the main adhesive ligand in the BM of resting epidermis, and its effects on keratinocyte behavior are mediated primarily through the integrins α3β1 and α6β4 (Litjens et al. 2006; Ramovs et al. 2017). α6β4 is an essential component of

hemidesmosomes that mediate stable epidermal adhesion and must be dissolved for keratinocyte migration during wound healing (Litjens et al. 2006). α3β1-mediated adhesions are associated with the actin cytoskeleton (Carter et al. 1990a). Since α3β1 and α6β4 differ in their cytoskeletal interactions and signaling activities, their combined effects on keratinocyte function occur mainly through convergence of functions rather than through redundant functions, as reviewed (Longmate and DiPersio 2014; Margadant et al. 2010). Epidermal stratification during skin development was largely normal in mice with prenatal deletion of either LM-332 or both integrins α3β1 and α6β4, indicating that LM-332 and its integrin receptors are not essential for epidermal morphogenesis prior to birth (DiPersio et al. 2000b; Dowling et al. 1996; Meng et al. 2003; Pesch et al. 2017; Ryan et al. 1999). However, early studies in mice with global knockout of the *Lama3* gene (which encodes the LM α3 chain) revealed abnormalities in the survival and late-stage differentiation of epidermal cells (Ryan et al. 1999), and a recent study using a model of inducible *Lama3* gene deletion in basal keratinocytes of adult mice showed that the progressive loss of LM-332 alters the keratinocyte genetic program and disrupts epidermal homeostasis (Tayem et al. 2021). Taken together, these findings indicate important post-developmental roles for LM-332 and/or for other LM-332-binding receptors in epidermal homeostasis.

Human patients with homozygous mutations in the genes that encode individual chains of LM-332, or that encode either subunit of the hemidesmosome integrin, α6β4, have been linked to different forms of the inherited human blistering disorder, junctional epidermolysis bullosa (JEB) (Kivirikko et al. 1995; Pulkkinen et al. 1994a, b, 1997; Takizawa et al. 1997). Skin blistering in most forms of JEB is caused by detachment of basal keratinocytes from the underlying BM, and it is recapitulated in mice with homozygous null mutations in α6, β4, or the LM γ2 chain (Dowling et al. 1996; Georges-Labouesse et al. 1996; Meng et al. 2003; van der Neut et al. 1996). As mentioned previously, integrin α3-null neonatal mice display disorganized BM that is accompanied by relatively small skin blisters (DiPersio et al. 1997). However, distinct from blisters that form in α6β4-deficient or LM-332-deficient mice, blisters in α3β1-deficient mice show distribution of LM-332 (and other BM proteins) to both dermal and epidermal surfaces, indicating rupture within the plane of the BM rather than detachment *per se* of keratinocytes from LM-332 (DiPersio et al. 1997; Longmate et al. 2014). This unique distribution of LM-332 to the epidermal sides of blisters in α3 knockout mice is due to retention of α6β4-mediated attachment, since LM-332 is detected only at the dermal side of blisters in mice that lack both α3β1 and α6β4 (DiPersio et al. 2000b). Skin blisters that form in ILNEB patients with *ITGA3* mutations also show distribution of LM-332 to both epidermal and dermal sides of blisters (Has et al. 2012), distinguishing this form of JEB from other forms in which the epidermis detaches from the underlying BM (Has and Fischer 2019).

Thus, while binding of integrin α6β4 to LM-332 within hemidesmosomes is clearly essential for adhesion at the epidermal–dermal junction, α3β1 contributes to the maintenance of this junction through its roles in BM integrity. Indeed, regions of highly disorganized BM occur between the blisters of newborn α3-null mice,

which presumably predisposes the skin to blistering (DiPersio et al. 1997). Subsequent studies in viable epidermis-specific α3 knockout mice revealed that adult mice largely recover from perinatal blistering, displaying only occasional, minor microblisters (Longmate et al. 2014; Margadant et al. 2009). However, blistering due to BM failure was observed in re-epithelialized wounds of adult epidermal-specific α3 knockout mice, as we will discuss in the next section.

Blistering in both neonatal α3-null skin and wounds of epidermis-specific α3 knockout mice was linked to reduced levels of fibulin-2 (Longmate et al. 2014). Moreover, genetic deletion of fibulin-2 (in the presence of α3β1) was sufficient to generate blisters in neonatal skin, indicating a necessary role for this matricellular protein in maintaining junctional integrity (Longmate et al. 2014). As already covered in Sect. 3.3, fibulin-2 binds to LM-332 within the L4 module of the LM γ2 chain (Utani et al. 1997), which is required for the stable incorporation of LM-332 into the BM (Gagnoux-Palacios et al. 2001); thus, α3β1 may contribute to stability of the nascent BM as it is assembled by inducing fibulin-2 expression (see Fig. 1). Interestingly, both perinatal blisters and wound blisters eventually resolved in α3-null skin/epidermis, and fibulin-2 was upregulated in these regions of the skin, suggesting that blistering triggers a compensatory upregulation of fibulin-2 that is independent of α3β1 and may contribute to successful blister resolution in these mice (Longmate et al. 2014).

α3β1 binding to LM-332 activates FAK-Src-Rac1 signaling in cultured keratinocytes (Choma et al. 2004, 2007), and FAK, Rac1, and α3β1 have each been linked to maintenance of the epidermal stem cell compartment within the hair follicle bulge (Sachs et al. 2012b). Interestingly, epidermis-specific knockout studies showed that neither α3β1 nor FAK is essential for wound re-epithelialization (Essayem et al. 2005; Margadant et al. 2009; McLean et al. 2004; Mitchell et al. 2009). However, both α3β1 and FAK contribute to the development of skin tumors (McLean et al. 2001, 2004; Sachs et al. 2012b), suggesting that an α3β1-FAK-Src-Rac1 signaling axis might control stem cell expansion that contributes to skin tumorigenesis.

Finally, as already discussed in Sect. 3.5.2, mice or patients with *CD151* or *ITGA3* gene mutations both display disorganization of the cutaneous BM and mild skin blisters (Has and Fischer 2019; Has et al. 2012; Karamatic Crew et al. 2004; Vahidnezhad et al. 2018), suggesting that some α3β1 functions involve its interaction with CD151. However, re-epithelialization differences in wounds of mice that lack epidermal CD151 or α3β1 indicate that some α3β1 functions are independent of CD151 (Cowin et al. 2006; Margadant et al. 2009).

5 Roles for Integrin α3β1 in Cutaneous Wound Healing

During wound healing, the migration and proliferation of keratinocytes are required for prompt re-epithelialization, important to restore barrier function of the epidermis and minimize risk of infection. Keratinocytes also actively modulate the wound

microenvironment through the secretion of ECM proteins, proteases, cytokines, and growth factors that can promote tissue remodeling and, in a paracrine fashion, stimulate other cells in the wound stroma to support angiogenesis, the inflammatory response, and wound contraction (Ghahary and Ghaffari 2007; Piipponen et al. 2020; Werner et al. 2007). Keratinocytes of the wound epidermis express a number of different integrins that work cooperatively to effect proper wound healing (Koivisto et al. 2014). In this section, we will revisit some of the previously discussed α3β1 functions in the context of wound healing. First, we will cover what might be considered "classical" roles of α3β1 in the regulation of functions intrinsic to keratinocytes that ensure proper wound re-epithelialization, including survival, proliferation, migration, and local BM regeneration. Next, we will address more recently appreciated roles of α3β1 in controlling gene expression programs of keratinocytes that determine their secretome, thereby allowing these cells to modify the wound microenvironment through paracrine crosstalk to stromal cell types and ECM remodeling.

5.1 Regulation of Cell-Intrinsic Functions of Wound Keratinocytes

Many studies of keratinocyte integrins, including α3β1, have focused on "classical" roles that integrins are known to play in the regulation of cell-intrinsic processes such as migration, proliferation, survival, and local matrix assembly. Given the diversity of integrins expressed in wound epidermis, there is much potential for either cooperative or opposing interactions between them, with cumulative effects on keratinocyte function (Koivisto et al. 2014; Longmate and DiPersio 2014). In addition, the variety of ligands present in the wound ECM in vivo may allow for compensation between integrins with overlapping roles (Margadant et al. 2010). Redundant integrin function in the context of wound healing makes sense evolutionarily, given the importance of rapidly restoring epidermal barrier function.

The global importance of integrins in cell adhesion and motility suggests that a key function of integrins in healing wounds is to promote epidermal migration. Indeed, ablation in the epidermis of all β1 integrins severely impaired wound re-epithelialization (Grose et al. 2002), and numerous cell culture studies have shown that individual integrins can mediate keratinocyte migration on their respective ECM ligands (for example, Carter et al. 1990a, b; Choma et al. 2004; Frank and Carter 2004; Grose et al. 2002; Pilcher et al. 1997; Sehgal et al. 2006). Surprisingly, perhaps due to greater complexity of ECM in vivo, deleting individual integrins in mice had only mild or no effects on wound re-epithelialization (Grenache et al. 2007; Margadant et al. 2009, 2010; Singh et al. 2009; Zweers et al. 2007). Integrin α3β1 is no exception to this discordance. Indeed, α3β1 promotes the polarization and migration of cultured keratinocytes on LM-332 through FAK/Src-mediated activation of Rac1 (Choma et al. 2004, 2007). Moreover, knockout of Tiam1 caused

impaired wound re-epithelialization due to loss of Tiam1-mediated Rac activation that occurs downstream of α3β1 (Hamelers et al. 2005). Surprisingly, however, a study using epidermis-specific α3 knockout mice showed that α3β1 is not only dispensable for wound re-epithelialization in vivo, but that its absence caused a slight increase in the re-epithelialization rate (Margadant et al. 2009). In yet another study, wound re-epithelialization was impeded in skin grafts isolated from neonatal α3-null mice (i.e., with global deletion of α3β1) (Reynolds et al. 2008), indicating that α3β1 was required either in wound epidermis of young mice or in other wound cell types present in the full-thickness grafts. Taken together, these apparently discordant findings suggest that the role of α3β1 in epidermal migration is complex and may be sensitive to composition of the ECM and/or developmental stage.

Epidermal homeostasis is maintained by a population of resident stem cells in the basal layer that gives rise to committed progenitor cells, called transit-amplifying cells, which replenish the differentiated keratinocytes that are eventually shed from the outer layer of the skin (Fuchs 2008; Owens and Watt 2003; Watt 2002). While this replenishment is thought to occur mainly in the interfollicular epidermis, stem cells residing in the hair follicle bulge contribute substantially to the proliferation of keratinocytes for wound re-epithelialization (Ito et al. 2005; Mascre et al. 2012). The expression of β1 integrins is higher in epidermal stem cells, where they are thought to help control the balance between renewal and terminal differentiation (Fuchs 2008; Janes and Watt 2006; Jones et al. 1995; Jones and Watt 1993; Watt 2002; Zhu et al. 1999), and it is likely that integrins control a shift in this balance to support epidermal regeneration during wound healing. While there is not extensive evidence that integrin α3β1 plays an essential role in this process, some studies have suggested its involvement in the maintenance of a stem cell compartment within the hair follicle (Conti et al. 2003; Sachs et al. 2012b). However, at least two groups have shown that α3β1 is not required for efficient regeneration of the epidermis during wound healing (Margadant et al. 2009; Mitchell et al. 2009), indicating that it is not essential for keratinocyte proliferation in this process.

In a cutaneous wound, keratinocytes lose contact with damaged BM and encounter the “provisional” ECM, which is the fibrin- and fibronectin-rich ECM that appears immediately upon wounding due to blood coagulation following vascular damage (Clark et al. 1982). Under these conditions the keratinocytes must maintain survival signals to prevent them from undergoing anoikis, a form of apoptosis that can be induced by reduced or altered adhesion to matrix (Frisch and Screaton 2001). Integrin α3β1 has been shown to promote the survival of cultured keratinocytes through signaling pathways that involve FAK and ERK, suggesting a possible role in promoting survival of wound keratinocytes in response to new LM-332 that they deposit into the wound bed (Manohar et al. 2004; Nguyen et al. 2000). One early study demonstrated that the interaction of α3β1 with LM-332 promotes communication of adjoining keratinocytes through gap junctions (Lampe et al. 1998), which could conceivably promote collective survival within the epidermis. However, while mice lacking both LM-332-binding integrins α3β1 and α6β4 displayed apoptotic basal keratinocytes over blistered regions of the epidermis, apoptosis was not enhanced in regions of attached epidermis (DiPersio et al. 2000a). This observation

indicates that LM-332-mediated adhesion is not essential for keratinocyte survival in unwounded epidermis and suggests that attachment through other integrins is sufficient to protect keratinocytes from undergoing anoikis in vivo.

5.2 Regulation of Basement Membrane Regeneration

The cutaneous BM provides a physical separation between the epidermis and dermis as well as extracellular cues to keratinocytes that regulate their survival, differentiation, polarization, and migration. As already discussed in Sect. 3.3, integrin α3β1 is essential in the epidermis for BM assembly and integrity during skin development (DiPersio et al. 1997), and this role is recapitulated during wound healing as the BM is regenerated beneath the neo-epidermis (Longmate et al. 2014). This critical role for α3β1 is supported through both in vitro and in vivo studies. Indeed, absence of α3β1 from cultured keratinocytes leads to defects in the organization of LM-332 that these cells deposit (deHart et al. 2003), as well as delayed processing of the LM γ2 chain (Longmate et al. 2014). Some of these changes could be partly due to altered spreading of α3β1-deficient cells in culture. However, healing wounds of adult mice that lack α3β1 in the epidermis also show defects in LM γ2 chain processing and disorganization of the BM that is associated with blistering of the newly regenerated epidermis, phenocopying the formation of skin blisters in neonatal α3-null mice (Longmate et al. 2014). Thus, the developmental role for α3β1 in maintaining BM integrity is recapitulated in adult wound healing. Like neonatal blisters, blisters that form over healed wounds recover after a second round of wound re-epithelialization (Longmate et al. 2014).

As discussed earlier in Sect. 4.6, the importance of α3β1-dependent fibulin-2 expression in skin development appears to be recapitulated during adult wound healing. Indeed, epidermal blistering in neonatal α3-null mice was linked to reduced fibulin-2 levels, and genetic deletion of fibulin-2 was sufficient to cause neonatal skin blisters (Longmate et al. 2014). As fibulin-2 binds to the N-terminus of the LM γ2 chain (Utani et al. 1997), and this interaction appears necessary for stable incorporation of LM-332 into the BM (Gagnoux-Palacios et al. 2001), a role for α3β1-dependent fibulin-2 expression as the BM assembles in re-epithelialized wounds seems likely (Longmate et al. 2014).

Wound keratinocytes express extracellular proteases that can effect changes in the ECM or release matrix-bound growth factors that drive wound healing, and mis-regulation of protease expression or function contributes to the pathology of chronic wounds (Longmate and DiPersio 2014). Therefore, the ability of α3β1 to regulate the expression of proteases such as MMP-9, MMP-3, and BMP-1 is important during wound healing (DiPersio et al. 2000a; Longmate et al. 2018). In particular, α3β1-dependent expression of BMP-1 was linked to BMP-1-mediated processing of LM γ2 chain (DiPersio et al. 2000a; Longmate et al. 2018), the significance of which is discussed below.

Regulated proteolysis of LM-332 is likely important for BM architecture as it can modulate its linkages with other ECM proteins, including fibulin-2. As mentioned previously (Sect. 3.3), an unprocessed LM γ2 chain with an intact L4 module is required for stable assembly of LM-332 into the developing BM (Gagnoux-Palacios et al. 2001). Notably, the L4 module of LM γ2 contains two binding sites for fibulin-2, as well as other ECM components (Utani et al. 1997), suggesting that these ECM linkages are required for the stable incorporation of LM-332 into the matrix. Subsequent proteolysis of the LM γ2 chain leads to loss of the L4 module and its ECM linkages (Sasaki et al. 2001), and cleavage of the L4 module is a hallmark of BM maturation (Aumailley et al. 2003; Sasaki et al. 2001). Interestingly, epidermal α3β1 has been shown to promote LM γ2 chain processing in keratinocytes cultured under conditions of high calcium and during wound healing in vivo (Longmate et al. 2014). The ability of α3β1 to promote LM γ2 processing relies, at least in part, on α3β1-dependent expression of the metalloprotease, BMP-1 (Longmate et al. 2018), which can cleave LM γ2 in skin and in cultured keratinocytes (Amano et al. 2000; Muir et al. 2016; Veitch et al. 2003). While the importance of this LM-332 processing event is not understood completely, it has been demonstrated that the full-length γ2 chain (e.g., within premature LM-332) promotes stronger cell adhesion rather than migration (Gagnoux-Palacios et al. 2001), probably through enhanced ECM linkages mentioned above. Conversely, the processed γ2 chain (e.g., within mature LM-332) may promote keratinocyte migration, since most ECM linkages are lost (Sasaki et al. 2001). Thus, these ECM linkages required in nascent (e.g., assembling) BM appear dispensable in mature (e.g., fully developed) BM. It stands to reason that proteolytic processing of LM γ2 in mature BM may support keratinocyte differentiation and/or prepare skin for the next insult by poising the epidermis in a "migration-ready" state (Longmate et al. 2014).

In summary, integrin α3β1 has a dual role in BM assembly that involves both deposition and proteolytic processing of matrix proteins. After a cutaneous wound is incurred, α3β1 promotes BM stability through induction of fibulin-2 (Longmate et al. 2014), followed by BM maturation through processing of LM γ2 via induction of BMP-1 (Longmate et al. 2018) (see Fig. 1). Interestingly, as mentioned previously in Sect. 3.5.3, the epidermal integrin α9β1 opposes the inductive effects of α3β1 on expression of fibulin-2 and BMP-1, and LM γ2 processing (Longmate et al. 2018). The temporal regulation of this α9β1 suppressive function suggests that it acts as a "brake" to BM regeneration functions of α3β1 (Longmate et al. 2017).

5.3 Regulation of the Keratinocyte Secretome

It is well known that the epidermis can send paracrine signals to distal cells that contribute to wound healing, such as vascular endothelial cells and stromal cells. These signals can occur as physical changes to the ECM (e.g., through proteolysis that alters its mechanical properties or releases/activates latent growth factors) or through secretion of diffusible growth factors or cytokines. While most studies of

keratinocyte α3β1 have focused on how it regulates cell-intrinsic processes and local BM assembly, it is now clear that epidermal α3β1 can also have far-reaching effects on the wound microenvironment, including other cellular compartments of the wound. Such "paracrine" roles of α3β1 are due largely to its ability to regulate the repertoire of proteins that is secreted by keratinocytes, or the keratinocyte secretome (Longmate 2020). Indeed, studies in keratinocyte cell lines have shown that α3β1 regulates the production of many secreted proteases, growth factors, cytokines, and ECM/matricellular proteins with established roles in modulating the microenvironment (Table 1) (Longmate et al. 2021b). The impact of the α3β1-regulated keratinocyte secretome on remodeling of the stromal ECM and crosstalk to other cell types of the wound (e.g., endothelial cells, immune cells, fibroblasts) are beginning to be uncovered and are discussed below. Later in Sect. 6.1.1, we will revisit this topic in the context of tumorigenesis.

5.3.1 Modification of the Stromal Extracellular Matrix

MMPs and other extracellular proteases contribute to changes in the ECM that are critical for all stages of wound healing, from early removal of damaged ECM to late-stage remodeling of scar tissue (Gill and Parks 2008). In addition to its role in local BM regeneration during wound healing (Sect. 5.2), keratinocyte α3β1 is likely to have long-range effects on ECM remodeling within the wound through its ability to regulate the expression and secretion of proteases such as MMP-9, MMP-3, uPA, BMP-1 (Ghosh et al. 2000; Iyer et al. 2005; Longmate et al. 2021a), and others in the α3β1-dependent secretome (Table 1) (Longmate et al. 2021b). Indeed, these proteases not only degrade ECM, but some are also linked to proteolytic release of cell- and matrix-bound growth factors that promote wound healing (Gill and Parks 2008; Page-McCaw et al. 2007). For example, MMP-9 can degrade ECM to release reservoirs of ECM-associated growth factors (e.g., VEGF) that can promote wound angiogenesis (Bergers et al. 2000; McCawley and Matrisian 2001).

5.3.2 Paracrine Crosstalk to Stromal Cells

Proper wound healing involves a complex network of communication between various cell types that reside in the different tissue compartments of skin. Epidermal keratinocytes produce growth factors and cytokines that can diffuse to other wound compartments (Singer and Clark 1999), allowing them to crosstalk in a paracrine manner with other distal cell types in the wound bed. Recent evidence indicates that some of these paracrine signals are regulated by integrin α3β1 (Longmate and DiPersio 2014).

A role for keratinocyte α3β1 in the paracrine stimulation of endothelial cells to promote wound angiogenesis has been established. Mice with epidermis-specific α3 deletion showed reduced wound angiogenesis, and cultured α3-null keratinocytes showed reduced secretion of factors that stimulate endothelial cell migration in vitro

Table 1 List of α3β1-regulated proteins of the keratinocyte secretome identified using Mass Spectrometry (MS)-based proteomics. MS was performed on serum-free medium conditioned by immortalized wild-type or α3-null mouse keratinocytes, as described (Longmate et al. 2021b). Third column shows fold-change up or down in α3β1-expressing cells, compared with cells lacking α3β1. Supporting references are listed in fourth column. This partial data set was published in Longmate et al. (2021b) and is reproduced here with permission. The full proteomic data set is available on The ProteomeXchange Consortium (dataset identifier PXD018425)

Gene symbol	Protein description	Fold-change	References
Upregulated in α3β1-expressing keratinocytes			
ECM or matricellular			
Fbln2[a,b]	Fibulin-2	4.5	Longmate et al. (2014, 2021b), Missan et al. (2014)
Lamb1	Laminin subunit β1	2.0	
Mmrn2	Multimerin-2	3.8	
Plaur	Urokinase plasminogen activator surface receptor (uPAR)	2.2	
Sdc4	Syndecan-4	3.5	
Sparc[a,b]	SPARC	6.9	Missan et al. (2014)
Growth factors & cytokines			
Csf1[a]	Macrophage colony-stimulating factor 1	5.0	Longmate et al. (2021b)
Csf2	Granulocyte-macrophage colony-stimulating factor	2.0	
Csf3	Granulocyte colony-stimulating factor	3.0	
Cxcl2	C-X-C motif chemokine 2	2.3	
Cxcl3	C-X-C motif chemokine 3	2.6	
Cxcl5	C-X-C motif chemokine 5	3.8	
Il1a[a]	Interleukin-1α	2.9	Zheng et al. (2019)
Prl2a1	Prolactin-2A1	4.5	
Prl2c2[b]	Prolactin-2C2/MRP-1/PLF-1	6.1	Missan et al. (2014)
Prl2c3[a,b]	Prolactin-2C3/MRP-3/PLF-3	20.8	Mitchell et al. (2009), Missan et al. (2014)
Prl6a1	Prolactin-6A1	3.8	
Vegfc	Vascular endothelial growth factor C (VEGFC)	9.4	
Proteases & their inhibitors			
Adams1	ADAM with thrombospondin motifs 1	2.0	
Bmp1[a]	Bone morphogenetic protein 1	1.9	Longmate et al. (2018, 2021a)
Mcpt8	Mast cell protease 8	6.0	
Mmp3[a]	MMP3/Stromelysin-1	3.2	Longmate et al. (2021a)
Mmp10	MMP10/Stromelysin-2	2.0	
Mmp13	MMP13/Collagenase 3	2.9	
Plat	Tissue-type plasminogen activator (tPA)	1.9	

(continued)

Table 1 (continued)

Gene symbol	Protein description	Fold-change	References
Plau[a]	Urokinase-type plasminogen activator (uPA)	3.4	Ghosh et al. (2000)
Prss23	Serine protease 23	1.9	
Prss27	Serine protease 27	6.9	
Serpine1	Plasminogen activator inhibitor 1 (PAI-1)	1.9	
Serpinb2[b]	Plasminogen activator inhibitor 2 (PAI-2)	2.0	Missan et al. (2014)
Spink5	Protein Spink5	2.4	
Timp1	Metalloproteinase inhibitor 1 (TIMP1)	3.3	
Timp2	Metalloproteinase inhibitor 2 (TIMP2)	3.4	
Downregulated in α3β1-expressing keratinocytes			
Ccl8	C-C motif chemokine 8	2.4	
Ccn2	Connective tissue growth factor (CTGF)	2.5	
Fbln5	Fibulin-5	2.6	
Lama2	Laminin subunit α2	2.3	
Lamb2	Laminin subunit β2	2.0	

[a]Proteins that have been confirmed in other studies to be α3β1-dependent in cultured keratinocytes and/or in vivo (skin tumors or wound epidermis)
[b]Proteins for which the corresponding gene has been shown in other studies to be α3β1-dependent

(Mitchell et al. 2009). This α3β1-dependent stimulation of endothelial cells occurred at least partly through induction of the pro-angiogenic growth factor, MRP-3 (Mitchell et al. 2009). As mentioned previously, the epidermal integrin α9β1 suppressed the ability of α3β1 to promote paracrine stimulation of endothelial cells in vitro and wound angiogenesis in vivo, and that this cross-suppression of α3β1 occurred later in wound healing to promote vascular regression (Longmate et al. 2017). Interestingly, LM-332 (the major ligand for α3β1) is upregulated in early wounds while cellular fibronectin (a ligand for α9β1) is upregulated later in wound healing, suggesting that integrin–ECM interactions control the timing of this interplay such that α9β1 suppresses α3β1 at later stages to “normalize” or reduce the over-exuberant wound vasculature (Longmate et al. 2017). Together, these studies identify a coordinated interplay between different epidermal integrins that controls keratinocyte-to-endothelial cell crosstalk to regulate wound angiogenesis.

A role for keratinocyte α3β1 in the paracrine stimulation of fibroblasts/myofibroblasts has also been demonstrated. Co-culture studies have shown that many fibroblast genes are regulated by keratinocyte-derived secreted factors (Nowinski et al. 2004). Several studies support a role for keratinocyte α3β1 in regulating crosstalk to dermal fibroblasts. In a mouse model of lung fibrosis, ablation of α3β1 in lung epithelial cells resulted in decreased β-catenin/Smad signaling, accompanied by reduced accumulation of lung myofibroblasts (Kim et al. 2009b).

More recently, a study using α3 knockout mice and α3-null cells showed that α3β1-dependent secretion of IL-1α by keratinocytes regulates the differentiation state of myofibroblasts during wound healing (Zheng et al. 2019), consistent with an earlier study showing that expression of a β1 integrin transgene in the epidermis promoted the secretion of IL-1α (Hobbs and Watt 2003). Interestingly, a subsequent study showed that integrin α4β1 is required on fibroblasts for their Nrf2-mediated signaling response to IL-1α produced by keratinocytes, which induces Cox-2 in fibroblasts and controls their differentiation state (Zheng et al. 2021). Thus, α3β1-regulated paracrine signaling from the epidermis to fibroblasts/myofibroblasts may influence wound contraction, and we speculate that changes in such crosstalk could contribute to fibrosis and hypertrophic scar formation (DiPersio et al. 2016).

Although α3β1-dependent crosstalk from keratinocytes to immune cells that is mediated by keratinocyte α3β1 has not been explored extensively, there is preliminary evidence to support such crosstalk. In one study, treatment of epithelial cells with an antibody against α3β1 inhibited the induction of macrophage chemoattractant protein 1 (MCP-1) and other interleukins involved in immune cell homing (Lubin et al. 2003). In a study of skin tumorigenesis that is discussed in detail below in Sect. 6.1.1, epidermal deletion of α3β1 resulted in reduced expression of colony-stimulating factor-1 (CSF-1), which was accompanied by a reduced number of tumor-associated macrophages (Longmate et al. 2021b). Consistent with the above findings, the α3β1-dependent secretome contains several factors known to regulate immune cells (Longmate et al. 2021b) (Table 1), suggesting that α3β1-dependent paracrine signals from keratinocytes may modulate the presence of immune cells in the microenvironments of wounds and tumors.

6 Roles for Integrin α3β1 in Cancer

Integrin α3β1 has been implicated in the development and progression of many types of cancer, including breast cancer, SCC, gastric cancer, glioma, and melanoma, making it a potential therapeutic target (Ramovs et al. 2017; Stipp 2010; Subbaram and DiPersio 2011; Tsuji 2004). Numerous preclinical studies using genetic or orthotopic graft models have identified roles for α3β1 in the initiation and progression of cutaneous tumors and certain subtypes of breast cancer (Cagnet et al. 2014; Longmate et al. 2021b; Mitchell et al. 2010; Ramovs et al. 2021; Sachs et al. 2012b; Zhou et al. 2014), and increased expression of α3β1 and/or its LM ligands are markers of poor prognosis in breast cancer and SCCs (Longmate et al. 2021a; Ramovs et al. 2017; Zhou et al. 2014). It is well known that similar cellular and molecular mechanisms are active in healing wounds and tumors/cancerous tissues; however, dysregulation of these mechanisms in cancer leads to unrestrained cell proliferation, invasion, and metastasis (Schafer and Werner 2008). These observations are consistent with the long-held concept that a tumor has characteristics of a perpetual wound that does not heal (Dvorak 1986). Many cancer-promoting functions of α3β1 bear similarities to its pro-wound healing functions (e.g., ECM

remodeling, control of the epithelial secretome, induction of angiogenesis). However, in contrast with the tight spatial and temporal regulation of α3β1 function that occurs during normal wound healing, these functions are not properly controlled in the context of a developing tumor. It stands to reason that a better understanding of how α3β1 is regulated during wound healing (discussed in Sect. 5) may lead to new strategies to target α3β1 as a therapeutic intervention in cancer.

Interestingly, α3β1 has also been reported to have suppressive effects on tumorigenesis and metastasis in some cancers, indicating that its roles in cancer are quite complex (Ramovs et al. 2017). Whether α3β1 is supportive or suppressive of malignant progression or metastasis can depend on the type or subtype of cancer (Cagnet et al. 2014; Miskin et al. 2021; Mitchell et al. 2010; Ramovs et al. 2019; Varzavand et al. 2013, 2016; Zhou et al. 2014). Moreover, α3β1 can have distinct effects at different stages of progression within a tumor/cancer type (Longmate et al. 2021b; Sachs et al. 2012b). As we will discuss, these opposing roles of α3β1 between cancers, or switches within cancers, may reflect differences/switches in the regulation of its function determined by the availability of key molecules with which the integrin interacts, including ECM ligands, IAPs (such as CD151) on the cell surface, or intracellular effectors of α3β1 signaling pathways.

In this section, we will review the roles that α3β1 expressed by tumor cells has been shown to play in promoting tumorigenesis, malignant progression, and metastasis (Sect. 6.1). Our discussion incudes key intracellular signaling pathways through which α3β1 has been shown to regulate cellular processes that drive tumor growth and invasion. We will also expand on the recently discovered role for α3β1 in controlling the keratinocyte secretome (discussed previously in the context of wound healing), which allows tumor keratinocytes to modify the tumor microenvironment (TME) through remodeling of the ECM and paracrine stimulation of stromal cells. Next, we will discuss suppressive roles in tumorigenesis or metastasis that have been reported for α3β1 in certain types of cancer, or at specific stages within a cancer's progression (Sect. 6.2). Finally, we will discuss roles that α3β1 plays from within non-tumor cell types of the TME that support tumor growth and progression, such as endothelial cells of the tumor vasculature and cancer-associated fibroblasts (Sect. 6.3).

6.1 Cancer-Supportive Roles for α3β1 in Tumor Cells

6.1.1 Pro-tumorigenic Roles

Regulation of tumor cell-intrinsic functions

Much of what we know about pro-tumorigenic roles for α3β1 were discovered in studies using the two-step skin tumorigenesis model, which is used widely to investigate stepwise cancer development and recapitulates many important features of human carcinogenesis (Abel et al. 2009). In this model, a single treatment with 7,12-dimethylbenz[a]-anthracene (DMBA) is followed by repeated treatment over

weeks with 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce benign skin tumors, a proportion of which progress to SCC (Abel et al. 2009). Studies from two different groups using this model showed that Cre-mediated deletion of floxed *Itga3* alleles specifically in the epidermis caused reduced tumor incidence and size, revealing that α3β1 is essential for skin tumorigenesis (Longmate et al. 2017; Sachs et al. 2012b). A subsequent study using a model of tamoxifen-inducible, epidermis-specific *Itga3* deletion showed that ablation of α3β1 from epidermal cells of growing tumors caused rapid tumor regression, revealing that α3β1 is also essential to maintain tumor growth (Longmate et al. 2021b).

Consistent with the above studies in the skin tumorigenesis model, studies with α3-null transformed keratinocytes showed that α3β1 promoted in vivo growth in a subcutaneous injection model (Lamar et al. 2008b). α3β1 also promoted the expression of pro-tumorigenic genes in immortalized keratinocytes (e.g., *Mmp9*, *Fbln2*, and others) (DiPersio et al. 2000a; Missan et al. 2014). Interestingly, the ability of α3β1 to induce most pro-tumorigenic genes was acquired by immortalized/transformed keratinocytes, since it was generally not observed in non-immortalized primary keratinocytes (DiPersio et al. 2000a; Lamar and DiPersio 2011; Lamar et al. 2008a; Missan et al. 2014). However, it remains unclear whether this acquisition of α3β1-dependent gene expression by immortalized cells is a *de novo* switch that occurs within progenitor cells or reflects the selective outgrowth of progenitor cells that already possess this pathway.

In previous sections, we discussed how the epidermal integrin α9β1 suppresses pro-angiogenic signaling functions of α3β1 at later stages of wound healing, in order to effect vascular normalization (Longmate et al. 2017). Interestingly, α9β1 is downregulated in cultured keratinocytes, as well as in epidermal tumors that form in the two-step murine model (Varney et al. 2021). Moreover, restoring α9β1 expression in transformed mouse keratinocytes suppressed their ability to form subcutaneous tumors in vivo (Varney et al. 2021). These observations are significant, as loss of α9β1 from tumor keratinocytes is predicted to relieve the cross-suppression of α3β1 that occurs during wound healing. Presumably, there is a strong selection for loss of this regulation during tumorigenesis in order to maintain pro-angiogenic/pro-tumorigenic α3β1 functions (Varney et al. 2021). Consistent with this hypothesis, analysis of transcriptome and methylome data from published human SCC data sets showed that *ITGA3* mRNA is increased while *ITGA9* mRNA is reduced, and that loss of *ITGA9* expression is driven by epigenetic silencing and/or reduced copy number of the *ITGA9* locus (Varney et al. 2021). Importantly, the extent to which this tumor-suppressive role of integrin α9β1 extends to other tumor types is not yet known. Indeed, α9β1 has been shown to promote malignant growth from within some cancer cells, and to have pro-angiogenic functions from within endothelial cells (Gupta et al. 2013; Vlahakis et al. 2007).

α3β1-mediated activation of FAK-Src signaling (discussed previously in Sect. 3.2.1) is likely to be important for the tumor cell-intrinsic functions that drive tumor growth and progression (Choma et al. 2007; Shang et al. 2001). Indeed, FAK and Src are both elevated in skin, breast, and other cancer types where they contribute to both initial tumor growth and malignant tumor progression (Brunton and Frame 2008;

McLean et al. 2004; Playford and Schaller 2004), and α3β1 is required for efficient FAK activation during the initiation phase of the two-step tumorigenesis model (Ramovs et al. 2021). Similarly, reduced malignant growth following deletion of α3β1 from mammary tumor cells was attributed to reduced activation of FAK and downstream Rac1/PAK1, MAPK, and JNK pathways (Cagnet et al. 2014).

A recent study from the Sonnenberg group nicely illustrates how spatial separation of distinct, pro-tumorigenic α3β1 signaling pathways is achieved within epidermal tumor cells during the initiation phase of DMBA/TPA-induced skin tumorigenesis through interactions of α3β1 with either LM-332 or the tetraspanin CD151 (Ramovs et al. 2021). In this study, the authors used a model of Cre-mediated α3 deletion from the epidermis, together with an in vitro spheroid model of transformed keratinocyte growth, to tease apart spatially distinct signaling functions of α3β1 in basal versus suprabasal cells. In basal keratinocytes, α3β1 was engaged with LM-332 in the ECM and activated FAK-Src signaling (Ramovs et al. 2021), consistent with previous literature (Cagnet et al. 2014; Choma et al. 2007). In suprabasal keratinocytes, α3β1 was co-localized to cell–cell adherens junctions with CD151, from where it activated Akt and STAT3 signaling to support cell survival. Moreover, rescue with an α3 point mutant that does not bind LM-332 was able to restore Akt and STAT3 signaling in α3-knockout spheroids, demonstrating that this CD151-dependent signaling axis was independent of α3β1 binding to LM-332. The authors suggested that the latter pathway maintains survival of suprabasal keratinocytes that had lost contact with BM, thereby delaying epidermal turnover and supporting eventual tumor formation. However, tumor outgrowth involved CD151-independent, α3β1-mediated activation of FAK-Src that promotes basal keratinocyte proliferation. This study highlights the important point that interactions of α3β1 with different binding partners (e.g., CD151 versus LM-332) may be key in determining its subcellular localization and signaling functions. Therapeutic implications of this concept will be discussed later in Sect. 7.

Regulation of the tumor cell secretome

Tumor cells secrete MMPs and other proteases that can promote tumor growth and progression through ECM proteolysis, or proteolytic release of ECM-bound growth factors (Bonnans et al. 2014; Page-McCaw et al. 2007). Tumor cells also secrete factors that mediate paracrine stimulation of stromal cells that support cancer progression (Marcucci et al. 2014; Yuan et al. 2016; Zhang and Liu 2013). Integrins are well positioned to control the tumor cell secretome and promote a tumor-supportive TME. As described earlier in the context of wound healing (Sect. 5.3), α3β1 has emerged as an important regulator of the keratinocyte secretome (Longmate 2020), thereby allowing these cells to modulate their microenvironment. Although it seems likely that this role for α3β1 extends to other epithelial cells and tissues, it has so far been best described in keratinocytes during normal or pathological processes that involve skin remodeling such as wound healing (Longmate et al. 2014; Mitchell et al. 2009), epidermal blistering disorders (He et al. 2018; Longmate et al. 2014), and epidermal tumorigenesis (Longmate et al. 2021b;

Ramovs et al. 2020). Below, we will expand our discussion of the α3β1-regulated secretome before delving into this role in the context of cancer.

α3β1-mediated modulation of the tissue microenvironment can occur via the secretion of extracellular proteases or matrix proteins that alter composition or mechanical properties of the ECM, or of cytokines and growth factors that mediate paracrine crosstalk to other cell types in the adjacent dermis. Mechanistically, the regulation of secreted factors by α3β1 may occur at the level of gene expression or protein secretion. Recent studies using mass spectrometry (MS)-based proteomics on conditioned medium from cultured keratinocytes have allowed for large-scale analyses of the α3β1-dependent secretome under different conditions. One such study profiled secreted proteins in keratinocytes isolated from ILNEB patients with inherited *ITGA3* gene mutations. MS analysis revealed that these α3β1-deficient cells have an altered secretome that changes the microenvironment, including enhanced deposition of fibronectin and upregulation of fibronectin-binding integrins (He et al. 2018). Other studies in keratinocytes and in vivo wounds of mice have demonstrated clear roles for α3β1 in modulating components of the keratinocyte secretome (Longmate et al. 2014, 2017; Mitchell et al. 2009).

Recently, a similar role has been described for α3β1 in mouse models of skin tumorigenesis. In one study, α3β1 in hair bulge stem cells was shown to modulate connective tissue growth factor (CCN2) expression to promote skin tumorigenesis (Ramovs et al. 2020). In another study, genetic ablation of *Itga3* from tumor keratinocytes during the tumor growth phase caused rapid tumor regression with concomitant changes in the tumor stroma, including increased stromal cell apoptosis, indicating that α3β1 is essential on tumor cells to maintain growth and promote a supportive secretome (Longmate et al. 2021b). Comparative MS analysis of the secretome between wild-type and α3-null keratinocytes confirmed substantial α3β1-dependent expression of numerous proteins predicted to promote a pro-tumorigenic microenvironment (Table 1). Decreased expression of two such factors, fibulin-2 (a matricellular protein) and CSF-1 (a paracrine stimulator of macrophages) was confirmed during the tumor regression that followed Cre-Lox-mediated deletion of α3β1 from tumor cells (Longmate et al. 2021b). This role for α3β1 in promoting a pro-tumorigenic secretome is likely to extend to other cancers. For example, α3β1 in TNBC cells promotes MMP-9 expression (Morini et al. 2000) while it represses the expression of the *RELN* gene, which encodes an extracellular glycoprotein, Reelin, known to inhibit cell invasion (Ndoye et al. 2021).

Finally, it is worth mentioning that integrins have been implicated in pro-tumorigenic effects of tumor-derived exosomes, which are emerging as an important mode of intercellular communication through horizontal transfer of proteins, mRNA transcripts, and miRNAs (Liu et al. 2021a). While it seems possible that some integrins may regulate the generation of tumor-derived exosomes, or the cargo within them, we are not currently aware of any such role for α3β1. However, it is well established that integrins themselves, including α3β1, or their mRNAs can be found in exosome cargo (Carney et al. 2017; Paolillo and Schinelli 2017; Xiao et al. 2019), suggesting that their horizontal transfer may be a mode of intercellular communication (Fedele et al. 2015; Singh et al. 2016), or a determinant of

organ-targeted metastasis as they fuse with target cells in distal tissues to form a pre-metastatic homing niche (Hoshino et al. 2015).

6.1.2 Pro-invasive/Pro-metastatic Roles

The cell adhesion and tractional forces that integrins provide are fundamentally important for cancer cells to invade the local stroma adjacent to the primary tumor and metastasize to distal organs (Brakebusch et al. 2002; Hamidi and Ivaska 2018; Missan and DiPersio 2012). Numerous studies support a pro-invasive/pro-metastatic role for integrin α3β1 in a variety of cancer cell types, including melanoma, SCC, breast cancer, and glioma (Kawataki et al. 2007; Subbaram and DiPersio 2011). The ability of α3β1 to promote carcinoma cell invasion certainly involves its ability to bind to the LM ligands that these cells deposit into the pericellular ECM as they move into and through the tumor stroma (Rousselle and Scoazec 2020). Pro-invasive properties of α3β1 also stem from its ability to regulate the expression of genes that encode extracellular proteases, ECM proteins, or growth factors with known roles in invasion (Missan and DiPersio 2012). For example, α3β1 can promote the expression of MMP-9 in gastric cancer cells (Saito et al. 2010), immortalized keratinocytes/SCC cells (DiPersio et al. 2000a; Lamar et al. 2008b; Longmate et al. 2021a), and breast cancer cells (Morini et al. 2000), and of uPA in oral keratinocytes (Ghosh et al. 2006). Importantly, a recent study demonstrated that α3β1-mediated invasion of TNBC cells can be separated from the ability of α3β1 to modulate the transcriptome, suggesting that at least some pro-invasive α3β1 functions do not require its ability to alter gene expression, at least on a global scale (Kenney et al. 2021).

There is good evidence that pro-invasive functions of α3β1 can be modulated through its interactions with CD151 (Stipp 2010), although the effect depends on the mode of cell migration/invasion. For example, RNAi-mediated suppression of CD151 in the A431 epidermoid carcinoma cell line leads to loss of the α3β1-CD151 complex at cell–cell adherens junctions and promotes α3β1-dependent, cohesive migration/invasion of the epithelial sheet (Zevian et al. 2015). On the other hand, silencing CD151 in the same cells reduced single cell migration on LM-332 due to impaired internalization of α3β1 (Winterwood et al. 2006). Taken together, these results indicate that CD151 regulates the balance of pro-junctional and pro-migratory α3β1 functions in these cells, as the α3β1-CD151 complex promotes the migration of single cells (Winterwood et al. 2006) but restrains the collective migration of intact epithelial sheets (Zevian et al. 2015).

α3β1 also has clear pro-metastatic roles in breast and other types of cancer (Stipp 2010; Subbaram and DiPersio 2011; Tsuji 2004). A number of studies using in vivo mouse models of spontaneous or experimental metastasis have shown that expression of α3β1 on tumor cells increases their metastasis to lungs, lymph nodes, and the peritoneum (Kenney et al. 2021; Miskin et al. 2021; Takatsuki et al. 2004; Wang et al. 2004; Zhou et al. 2014). In a lung cancer model, disruption of the α3β1-CD151

complex was shown to impair the metastasis-promoting effects of CD151 (Peng et al. 2020).

6.2 Cancer-Suppressive Roles for α3β1 in Tumor Cells

Despite its established roles in promoting tumorigenesis and malignant progression in some models, there are also clear examples of cancer types or subtypes wherein α3β1 suppresses tumor growth or reduces metastatic potential (Ramovs et al. 2017; Stipp 2010). As mentioned earlier in Sect. 3.2.3, α3β1 signals through Abl family kinases in prostate cancer cells to maintain the Hippo pathway, thereby suppressing YAP/TAZ-induced gene expression that supports cell invasion and anchorage-independent growth (Varzavand et al. 2016). A suppressive role for α3β1 has also been described in HER2-driven breast cancer, where downregulation of α3β1 in either a murine model or human breast carcinoma cells caused enhanced tumor progression and invasiveness (Ramovs et al. 2019). This suppressive role was not observed in TNBC cells, indicating that cancer-promoting and cancer-suppressive roles of α3β1 in breast cancer can be subtype-specific (Ramovs et al. 2019).

Remarkably, α3β1 can also exert opposing functions within the same type of cancer during its development and progression, switching from a tumor-promoting integrin at early stages to a cancer-suppressive integrin at later stages. This phenomenon is perhaps best illustrated by the collective findings of different groups that have used epidermis-specific α3 knockout models combined with chemically-induced epidermal tumorigenesis. As described above (Sect. 6.1.1), studies in these models revealed a clear requirement for α3β1 in initial tumor formation (Sachs et al. 2012b) and maintenance of tumor growth (Longmate et al. 2021b). However, when an aggressive carcinogenesis protocol of weekly DMBA applications was applied to this same genetic model, fewer tumors again formed in α3 knockout epidermis but they showed a significantly higher malignancy grade indicating an increased rate of progression to undifferentiated, invasive carcinoma (Sachs et al. 2012b).

The ability of α3β1 to switch from tumor-promoting to cancer-suppressive roles in the two-step skin carcinogenesis model, combined with the observation that pro-tumorigenic functions of α3β1 are acquired upon keratinocyte immortalization (DiPersio et al. 2000a; Lamar et al. 2008b; Missan et al. 2014), indicates that α3β1 functions are dynamically regulated throughout the development and progression of cutaneous SCC. There is compelling circumstantial evidence that regulation of the α3β1-CD151 interaction is important for at least some of these functional switches (Stipp 2010). Indeed, deletion of either CD151 or α3β1 leads to reduced tumor incidence and growth, while CD151 deletion has no effect on progression (Li et al. 2013; Longmate et al. 2017; Sachs et al. 2012b, 2014), suggesting that they may become functionally uncoupled later in tumor progression. As we described in Sect. 6.1.1, the possibility of such uncoupling is evident at the onset of skin tumorigenesis, during which α3β1-CD151 mediates STAT3 and Akt signaling in suprabasal cells

that promotes their survival, while CD151 is not required for α3β1-LM-332-mediated activation of FAK-Src signaling in basal cells that promotes their proliferation (Ramovs et al. 2021). TGF-β may also play a role in α3β1 functional switches, as effects of TGF-β signaling are well known to change during tumor/cancer progression, and functional coupling of α3β1 and TGF-β in epidermal keratinocytes appears highly dependent on context (see Sect. 3.2.4) (Lamar et al. 2008a; Margadant and Sonnenberg 2010; Reynolds et al. 2008). Further investigation is important to determine the underlying mechanisms of how α3β1 switches from pro-tumorigenic to anti-invasive, and to determine whether such switches occur in other cancers where this integrin has pro-tumorigenic functions, since there are obvious implications for the development of anti-α3β1 cancer therapies as we will discuss below in Sect. 7.

6.3 Roles for α3β1 in Cancer-Associated Stromal Cells

It is widely accepted that tumor growth and malignant progression require a permissive TME that includes non-tumor stromal cells with tumor-supportive roles (Yuan et al. 2016; Zhang and Liu 2013). Cancer-associated fibroblasts (CAFs) provide a compelling example of a stromal cell type from within which α3β1 function has been shown to have pro-tumorigenic functions. Using CRISPR to delete α3 from immortalized CAFs that were isolated from pancreatic duct adenocarcinoma, Cavaco and coworkers identified an essential role for α3β1 in the induction and maintenance of the differentiated CAF phenotype (Cavaco et al. 2018). α3β1 was shown to promote the deposition by CAFs of LM-332 into the TME, which promotes and sustains CAF differentiation and supports the invasion of both CAFs and carcinoma cells (Cavaco et al. 2018), thus expanding the pro-tumorigenic roles of α3β1 beyond the tumor cell compartment. In another example, α3β1 on vascular endothelial cells may contribute to the extravasation of circulating tumor cells by helping to stabilize their adhesion to the vascular endothelium (Glinskii et al. 2014). However, endothelial α3β1 appears to be a negative regulator of tumor angiogenesis, as endothelial-specific α3 knockout caused increased tumor angiogenesis (da Silva et al. 2010), and α3β1 was reported to inhibit pro-angiogenic Cox-2 signaling in endothelial cells (Boosani et al. 2007). Importantly, this suppressive role of α3β1 from within endothelial cells is contrary to its pro-angiogenic role from within epidermal keratinocytes that occurs through paracrine signaling (Longmate et al. 2017; Mitchell et al. 2009). Thus, the potential effects of blocking/deleting α3β1 in endothelial cells should be considered when designing anti-α3β1 therapeutic strategies to inhibit tumor growth, which might be most effective if they can be targeted specifically to α3β1 that is expressed on tumor cells and/or CAFs (discussed below in Sect. 7).

7 Prospects for Integrin α3β1 as a Potential Therapeutic Target

As signaling receptors that are easily accessible on the cell surface and regulate both cell-mediated changes to the tissue microenvironment and cellular responses to such changes, integrins are generally attractive targets for therapeutic drugs or nanomaterials to treat wounds or cancer (Schnittert et al. 2018). Given that integrin α3β1 on epithelial cells regulates key cell-intrinsic functions and promotes a stroma-supportive secretome, targeting this integrin could have the pleiotropic effect of impacting both wound/tumor cells and stromal cells. Therefore, α3β1 is a particularly attractive therapeutic target to promote wound healing or treat chronic wounds (Longmate and DiPersio 2014), or to treat certain cancers (Ramovs et al. 2017; Stipp 2010; Subbaram and DiPersio 2011). In this section, we will discuss the potential value of α3β1 as a drug target or mediator of drug delivery, as well as the challenges associated with α3β1-targeting strategies that arise from its biology discussed in the previous sections.

7.1 α3β1 as a Potential Target for Cancer Therapies

Integrins have important roles at all stages of cancer and are attractive therapeutic targets for anti-cancer therapies, as has been reviewed extensively (Brakebusch et al. 2002; Cooper and Giancotti 2019; Desgrosellier and Cheresh 2010; Hamidi and Ivaska 2018; Missan and DiPersio 2012; Schnittert et al. 2018; Winograd-Katz et al. 2014). Integrin signaling has also been shown to mediate resistance to conventional radiation therapies and chemotherapies, suggesting that integrin targeting may be most effective in the adjuvant setting (Dickreuter and Cordes 2017). Most clinical efforts have targeted endothelial cell integrins (e.g., αvβ3 or αvβ5) to suppress tumor angiogenesis, while strategies to inhibit integrins expressed on tumor cells are underdeveloped (Desgrosellier and Cheresh 2010; Goodman and Picard 2012). Moreover, clinical efforts have focused on the relatively few integrins that bind to RGD-containing ligands and are therefore targeted by RGD-mimetics that antagonize this function (Desgrosellier and Cheresh 2010; Goodman and Picard 2012; Stupp and Ruegg 2007; Weller et al. 2016), leaving those integrins that bind non-RGD ligands, including α3β1, untested in the clinic.

The potential value of integrin α3β1 as a therapeutic target to inhibit tumorigenesis or metastasis has long been appreciated (Ramovs et al. 2017; Stipp 2010; Subbaram and DiPersio 2011; Tsuji 2004). However, the complex and often opposing roles that α3β1 plays in cancer discussed in the previous section raise some formidable challenges to its development as an anti-cancer target. Indeed, as we will discuss here, effective α3β1-targeting strategies are likely to require combinatorial targeting of different α3β1 functions that occur from within distinct subcellular compartments, possibly with stage-specific precision. Other considerations include

the possible need to achieve optimal levels of α3β1 inhibition in tumor cells, as well as cell type-specific targeting of α3β1 within tumors.

A seemingly straightforward approach is to interfere with α3β1 binding to ECM ligands or IAPs necessary for its pro-tumorigenic functions. The general concept of therapeutically blocking integrin–ligand interactions is already established with the integrin-blocking agent Cilengitide, an RGD-mimetic that showed some efficacy in clinical trials of recurrent glioblastoma multiforme (Kochar et al. 2018; Reardon et al. 2008). While there is no such antagonist for α3β1, it is intuitive that pharmaceutical agents could be used to block essential interactions of α3β1 with LM-332 or CD151. As one example, a 17-mer peptide (α325) that is homologous to the uPAR-binding site on α3β1 and disrupts the α3β1-uPAR complex (Wei et al. 2001) has been shown to inhibit uPA induction and invasive behavior of immortalized oral keratinocytes (Ghosh et al. 2006). Moreover, α3β1-binding motifs within both LM-332 and CD151 have been identified and might be exploitable in this regard (Berditchevski et al. 2001; Kazarov et al. 2002; Kunneken et al. 2004; Shang et al. 2001; Zhang et al. 1999), and synthetic peptide inhibitors and snake venom disintegrins that antagonize α3β1-mediated adhesion to LMs have been reported (Eble et al. 2003; Kusuma et al. 2012; Sroka et al. 2006). Monoclonal antibodies against α3β1 have also shown therapeutic potential in preclinical models (Arias-Pinilla et al. 2020; Ke et al. 2020; Li et al. 2014).

A different approach to blocking pro-tumorigenic α3β1 functions is to exploit the ability of α9β1 to cross-suppress α3β1. As we discussed in earlier sections, the tissue remodeling and gene regulatory functions of α3β1 that promote ECM remodeling and angiogenesis are transient in normal wound healing, being suppressed by α9β1 at later stages (Longmate et al. 2017). However, such α3β1 functions appear chronic in skin tumors, wherein α9β1 is downregulated presumably to relieve suppression of these functions (Varney et al. 2021). If the molecular mechanisms and signaling pathways through which α9β1 suppresses α3β1 during wound healing can be identified, then it might be possible to exploit this knowledge to develop anti-α3β1 strategies to be applied at early stages of cancer development.

A significant challenge of targeting α3β1 that could arise in the context of progressing disease is highlighted by the recent findings of Ramovs and coworkers (Ramovs et al. 2021). As described above in Sect. 6.1, this group identified dual pro-tumorigenic signaling functions of epidermal α3β1 that are regulated by distinct and spatially separable interactions with its different binding partners, CD151 or LM-332. While the extent to which compartmentalization of multiple α3β1 functions extends to other tumor types remains to be seen, this phenomenon reveals a limitation of simple blocking strategies since both interactions may need to be inhibited simultaneously. In fact, it is conceivable that such complications contribute to the modest efficacy of RGD-mimetics such as Cilengitide (Lasinska and Mackiewicz 2019). Indeed, many RGD-binding integrins can also bind to IAPs through non-RGD interactions (Brown 2002), suggesting that some downstream signaling may not be blocked by RGD-mimetics.

Another potential challenge arises from the observation in preclinical models that cancer progression is influenced differently by the presence or absence of α3β1 at

distinct stages. Indeed, as discussed already α3β1 has a pro-tumorigenic role at early stages of skin tumor development, but its absence at later stages leads to accelerated carcinoma progression (Ramovs et al. 2019; Sachs et al. 2012b). This switch in the role of α3β1 suggests that combinatorial approaches may be required to target drivers of progression that persist following the inhibition of α3β1. In addition, different breast cancer types/subtypes may respond in opposite ways to inhibition of α3β1, given that it promotes invasion/metastasis in TNBC models (Cagnet et al. 2014; Miskin et al. 2021; Mitchell et al. 2010), but suppresses invasion/metastasis in models of HER2-driven breast cancer (Ramovs et al. 2019) and prostate cancer (Varzavand et al. 2016). Finally, the observation that partial versus complete inhibition of α3β1 function may produce different effects on the transcriptome of TNBC cells (Kenney et al. 2021) suggests that dose may be an important consideration, as has been demonstrated for RGD-mimetic drugs that block other integrins (Reynolds et al. 2009).

It may also be necessary to develop strategies that target α3β1 specifically in tumor/cancer cells (Subbaram and DiPersio 2011) and/or CAFs (Cavaco et al. 2018) since inhibiting α3β1 on endothelial cells may enhance tumor angiogenesis (da Silva et al. 2010), and inhibiting α3β1 on cells outside of the tumor could impair its functions in the homeostasis or repair of normal tissues (Longmate et al. 2014; Mitchell et al. 2009).

Finally, it may be possible to exploit the expression of α3β1 on tumor cells (or CAFs) as a target for nanoparticle-mediated drug delivery systems. Indeed, in recent years, the field of cancer nanomedicine has seen a burgeoning development of diagnostic and therapeutic strategies that utilize integrin-targeted nanomaterials (e.g., polymeric nanoconstructs, liposomes, inorganic nanoparticles), as has been reviewed extensively (Arosio et al. 2017; Duro-Castano et al. 2017; Wang et al. 2010; Wu et al. 2019). In this approach the surface of a nanomaterial is decorated with the biological ligand for target integrins on cancer cells or the tumor vasculature to direct the actions of the nanomaterial to those cells. Rationally designed strategies include stable or cleavable conjugation of cytotoxic drugs/genetic agents/imaging agents to peptidic or peptidomimetic ligands for integrins to achieve tumor-targeted delivery (Arosio et al. 2017; Duro-Castano et al. 2017). Although most studies have focused on targeting RGD-binding integrins, researchers from Kit Lam's group have developed a 9-amino-acid cyclic peptide, LXY30, that shows considerable promise as an α3β1-targeting ligand. Indeed, LXY30 targets α3β1 on non-small cell lung cancer cells and ovarian cancer cells. It also provides a platform for using biosensor surface-immobilized LXY30 to specifically detect tumor-derived exosomes that carry α3β1 as cargo (Carney et al. 2017; Xiao et al. 2016, 2019). Ongoing work from this group supports LXY30 as a promising reagent in the context of a "smart supramolecular peptide" that can assemble into nanoparticles, laying the groundwork to develop imaging agents or nanomedicines that are targeted to cancer cells expressing α3β1 (Zhang et al. 2021). However, α3β1 is also expressed in most/all normal epithelial cells and several other cell types, so it is likely that this approach will require adaption toward the targeting of α3β1 specifically on tumor cells.

7.2 *α3β1 as a Potential Target for Wound Therapies*

Abnormal integrin expression or function is likely to be a major contributor to wound pathologies involving over-exuberant healing and fibrosis (e.g., hypertrophic scars) or diminished healing (e.g., chronic wounds), which supports a rationale for exploiting keratinocyte integrins as therapeutic targets (Koivisto et al. 2014; Longmate and DiPersio 2014). As we discussed extensively in Sects. 5 and 6, there are many parallels between wound healing and cancer, and tumors are often regarded as wounds that do not heal (Dvorak 1986; Schafer and Werner 2008). Since α3β1 governs the production by wound keratinocytes of pro-angiogenic growth factors and ECM-degrading proteases, disruption in the timely regulation of these functions may contribute to the persistence of chronic wounds, as it does in tumors (Longmate et al. 2021a, b; Mitchell et al. 2009). It stands to reason that α3β1-targeting therapies are relevant to the treatment of chronic wounds for many of the same reasons that they are relevant to the treatment of cancer. In addition, potential utility of α3β1 as a therapeutic target to diminish hypertrophic scarring warrants investigation, given its recently described role in controlling IL-1α-mediated paracrine signaling from keratinocytes that regulates the differentiation state of dermal fibroblasts/myofibroblasts (Zheng et al. 2019). In this case, the promotion of α3β1-dependent secretion of IL-1α by keratinocytes may stimulate Cox-2-dependent PGE2 production in fibroblasts, in turn leading to reduced myofibroblast function.

Many of the possible strategies that we discussed in Sect. 7.1 to target α3β1 in cancer may also be applicable to the development of wound healing therapeutics. Indeed, it seems likely that peptidomimetics or other agents developed to manipulate α3β1 functions in cancer might be adaptable for utility in wound healing therapies to promote wound healing or suppress over-exuberant wound healing, as appropriate. Similarly, strategies based on mimicking the ability of α9β1 to cross-suppress α3β1 might be useful for treatment in cases where over-exuberant α3β1 activity is found to contribute to the pathology of such wounds.

Nanomedicine approaches for treating wound pathologies are being actively pursued, as reviewed elsewhere (Bellu et al. 2021; Cui et al. 2021). An area of intense investigation has been the presentation of ECM-derived ligands, or ligand mimetics, in the context of polymeric biomaterial scaffolds or self-assembling nanomaterials that engage specific integrins and induce cellular responses that support wound healing (e.g., cell migration, proliferation, differentiation) (Dhavalikar et al. 2020; Kang et al. 2021), although there has been less focus on targeting the LM-binding integrins. The use of nanoparticles to target specific integrins in the context of wound healing is less well developed than in the context of cancer nanomedicines, but the latter might eventually be adaptable toward the treatment of some wound pathologies. Recently, encouraging results were obtained using a peptidomimetic with high binding affinity for integrin α5β1 that was nanopatterned onto hydrogels at discrete spacings, allowing for precise control of adhesive site organization. The study showed than an optimal nano-spacing of the α5β1 ligand most efficiently induced the collective migration of keratinocyte sheets

(Di Russo et al. 2021), which has relevance to the mode of epidermal migration during wound re-epithelialization. It will be interesting to develop similar approaches to assess effects of engaging α3β1 and other keratinocyte integrins, either individually or in groups.

The development of α3β1-targeted wound therapies has its challenges. One challenge is the likely need for multi-combinatorial strategies that will target other integrins, in addition to α3β1, given the high potential for overlapping or compensatory functions among the several integrins expressed in wound epidermis (Koivisto et al. 2014; Watt 2002). Another challenge is that the best integrin–ligand interactions on which to base such strategies may not yet be clear, as some ECM ligands in the wound microenvironment remain to be identified for some integrins, and ligands other than LM-332 and -511 may exist for α3β1. Finally, as described in Sect. 7.1 for anti-cancer strategies, it may be necessary to target α3β1 specifically in wound keratinocytes to avoid pro-angiogenic effects of inhibiting α3β1 on endothelial cells (da Silva et al. 2010).

In summary, the field has made good progress in identifying functions of α3β1 in wound keratinocytes, and in understanding how these functions are coordinated with those of other epidermal integrins to effect normal wound healing. However, before we attempt to translate this knowledge into the development of therapeutic approaches to treat wound healing deficiencies or pathologies, further work is necessary to determine how the mis-regulation of α3β1 function may contribute to these pathologies.

8 Conclusions and Future Perspectives

Beyond its "classical" roles in controlling cell adhesion and migration, ongoing investigations continue to uncover critical post-adhesion roles for integrin α3β1 that govern normal and pathological tissue remodeling. An emerging role is the ability of α3β1 to direct functionally appropriate gene expression programs in response to ECM or other extracellular cues by triggering key intracellular signaling pathways, which it often does in collaboration with IAPs or other signaling receptors. Since many α3β1-responsive genes contribute to the keratinocyte secretome, an important consequence of this regulation is the production of proteins and enzymes that are required to remodel ECM or generate a mature BM, or that have paracrine effects on other cells in wounds and tumors. Continued use of preclinical animal models, combined with the knowledge that we gain from clinical and bioinformatic studies of human pathologies, should provide a better understanding of the mechanistic underpinnings of α3β1-regulated gene expression that will allow for its therapeutic targeting.

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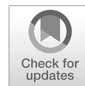
Part II
Fibrosis and Cancer

Integrins: Key Targets in Tissue Fibrosis and Tumor Stroma



Devin Veerman and Jai Prakash

Abstract Fibrosis, the hallmark of the excessive deposition of extracellular matrix (ECM), is the response of the body to tissue injury. The tissue matrix remodeling during fibrogenesis is mediated via specific cell-ECM interactions, which is regulated by integrins, a class of ECM-binding receptors. Integrins relay signals from the extracellular space to the inside and vice-versa, thereby controlling the phenotypic characteristics of fibroblasts which are the key cell type in fibrogenesis. Fibrosis is not limited to organs but is also found in many solid tumors, where it acts as a barrier for penetration of chemotherapy as well as of immune cells contributing to immunosuppression. Cancer-associated fibroblasts (CAFs) in tumors are the major producers of ECM components and play a major role in ECM remodeling. By interacting with integrins they regulate the tumor stiffness and ECM production. Recent work on heterogeneity of CAFs has demonstrated pro- and anti-tumoral effects of various CAF subsets in different tumor types. The role of integrins in CAF subsets is a subject of current investigations. In different fibrotic diseases, integrins play a major role in the onset and progression of the disease and are considered potential therapeutic targets, while their therapeutic role in tumor fibrosis is not yet well established. In this chapter, we will briefly introduce integrins and their

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interactions with ECM proteins as well as their signaling pathways. In the context of fibrosis, we will discuss the role of various integrins with a focus on fibroblasts.

1 Integrins

Integrins are a family of transmembrane glycoprotein receptors expressed on the surface of different cell types. Integrins exist in the form of heterodimers consisting of two subunits, namely an α-subunit and a β-subunit. 18α and 8β subunits have been discovered that can form heterodimers with various combinations of α and β subunits. In total, 24 heterodimers have been identified as 'functional' integrins. Both the α-subunit and the β-subunit have a short C-tail that remains intracellularly, while the N-terminal functions as the receptor or, in case of extracellular matrix (ECM)-binding, as a physical support when it is holding on to the ECM (Rüegg and Alghisi 2010). One integrin heterodimer can bind to multiple ligands and a single ligand can interact with different integrin receptors. Specific binding to a ligand enables cells to connect with the ECM which controls various cellular functions such as cell invasion and motility. Interestingly, a physical connection between the cells and the ECM allows bidirectional sensing of signals i.e. "inside-out signaling" and "outside-in signaling" (Shen et al. 2013). Indeed, integrins can also relay signals via the cytoskeleton that can tune cell-behavior, like migration, proliferation, and differentiation. Within the microenvironment, the specific expression of integrins and ligands determines the cell response to its surrounding environment (Seguin et al. 2015).

During pathological events, such as fibrosis and cancer, integrins can sense the physical environment of the accumulated ECM. For example, high stiffness can alter cell behavior and lead to ECM remodeling (Seguin et al. 2015). Integrins are also able to participate in cell-cell adhesion by binding to counter receptors, like 'A Disintegrins And Metalloproteinases' or ADAMs. Another example is Intercellular Adhesion Molecules (ICAMs) which are overexpressed on inflamed endothelium and can bind to integrin αLβ2 expressed on T cells. With the adhesion to ICAM-1 on endothelium, integrin αLβ2 plays a crucial role in T cell activation and migration (Walling and Kim 2018).

Integrins can be categorized according to their ECM-binding properties, as summarized in Table 1. Integrins that belong to the β1-containing collagen-receptors, include α1β1, α2β1, α10β1, and α11β1. Laminin receptors include integrins α3β1, α6β1, α7β1, and α6β4. These integrins facilitate cell attachment to the basement membrane. Integrins that bind to the RGD peptide sequence (arginine-glycine-aspartic acid) found in various ECM proteins such as fibronectin, include α5β1, α8β1, αvβ3, αvβ5, αvβ6, αvβ8, and αIIbβ3. Furthermore, other fibronectin-binding integrins independent of binding to the RGD sequence, include α4β1, α9β1, and α4β7. These integrins bind to different peptide-sequences including REDV, and EILDV (Desgrosellier and Cheresh 2010) and in particular α4β1 and α4β7 play a role in cell-cell adhesion. The last group consists of leukocyte-binding

Table 1 Current known integrin receptors and their corresponding ligands

Integrin type	Integrin receptor family
α1β1, α2β1, α10β1, α11β1	Collagen-binding integrins
α5β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, αIIbβ3	RGD-binding integrins (fibronectin, vitronectin, fibrinogen, and thrombospondin)
α4β1, α9β1, α4β7	Fibronectin-binding integrins (non-RGD)
α3β1, α6β1, α7β1, α6β4	Laminin-binding integrins
αDβ2, αLβ2, αMβ2, αXβ2, αEβ2, α4β1, α4β7	Leukocyte-binding integrins

integrins αDβ2, αLβ2, αMβ2, αxβ2, and αEβ2. These integrins can bind, as mentioned before, to ICAMs and plasma proteins (Koivisto et al. 2014).

1.1 Integrin Signaling

Integrins mediate bi-directional signaling which is often referred to as "outside-in" and "inside-out"-signaling. This bi-directional signaling is schematically depicted in Fig. 1. In the "inside-out" signaling, the integrin activity is regulated by molecular interactions with their cytosolic domain. During the cell activation, for instance by a

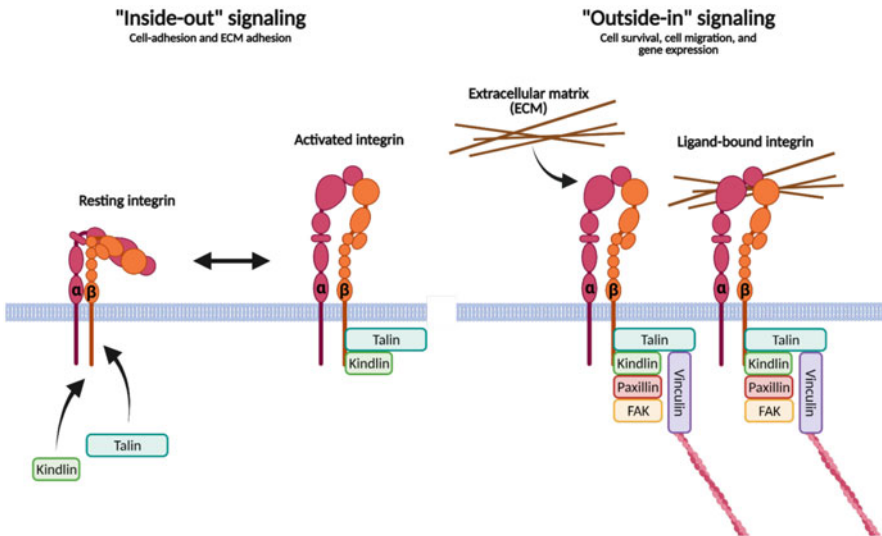

Fig. 1 Simplified schematic representation of integrin-mediated bidirectional signaling. In "Inside-out" signaling, adaptor proteins talin and kindlin bind to the β-subunit of the resting integrin. As a result, both the α- and β subunits unfold and bind to various ECM ligands. In "Outside-in" signaling, ECM molecules bind to the integrins which mediate signal through the focal adhesion complex and actin filaments of the cytoskeleton. Created with BioRender.com

cytokine, a signal is triggered from inside to the cell surface (Shen et al. 2013). The integrin activation involves the binding of the adaptor proteins talin and kindlin to the cytoplasmic tails of integrin β-subunits. This binding induces separation of the cytoplasmic domains of α and β subunits, which leads to a large conformational change in the extracellular domains via unbending and extension, allowing them to bind to the ECM ligands. Integrins realign themselves to form clusters in the cell membrane and get activated to a high avidity state in which these molecules recruit adaptor and signaling molecules to form so-called focal adhesions (Desgrosellier and Cheresh 2010). Noteworthy, the distribution of the focal adhesions is dependent on the dimension of their environment, for example, the composition varies dramatically in a three-dimensional (3D) environment, as compared to a two-dimensional (2D) environment (Berrier and Yamada 2007). Furthermore, the affinity and activation of integrins can also be driven by intracellular signaling molecules, like the GTPase RAP1A (Zöller 2009). Furthermore, there are also growth factor receptors and oncogenes that are responsible for integrin affinity and activity (Desgrosellier and Cheresh 2010).

In "outside-in" signaling, the ECM ligand binding and force application to integrins cause transmission of signals to the cytoskeleton (Fig. 1). Integrins do not have kinase activity; however, they do recruit and activate kinases. Examples include focal adhesion kinases (FAK), scaffold molecules such as P130CRK-associated substrate (also known as p130CAS or BCAR1), and Src family kinase (SFKs) (Desgrosellier and Cheresh 2010). Other than activating these kinases, integrins can also bind to proteins, such as talin, paxillin, and vinculin which then form the cluster of these proteins at the β cytoplasmic chain (Desgrosellier and Cheresh 2010). Besides that, cell migration and survival are controlled by the integrin-linked kinase (ILK)-PINCH-parvin (IPP complex). The latter-named complex has a key role in signaling and scaffolding functions that ultimately enables integrin-mediated effects on cell migration and survival (Legate et al. 2006). Thus, cell adhesion and migration in the ECM are facilitated by these focal adhesions.

2 Integrins in Tissue and Tumor Fibrosis

Fibrosis is the hallmark of excessive deposition of ECM in an organ in response to a chronic injury. The accumulation of ECM proteins disrupts the normal architecture of a tissue and can lead to organ malfunction. Fibrosis can virtually occur in any organ including lungs, liver, kidneys, heart, and skin and is associated with high morbidity and mortality (Distler et al. 2019; Henderson et al. 2020; Wynn 2008). It is estimated that fibrosis is responsible for up to 45% of the total deaths in the modern world and this number is predicted to increase in the coming years (Friedman et al. 2013; Henderson et al. 2020; Wynn 2008). The initiation of organ fibrosis is highly distinct and is mainly depending on the sites of injury and organ-specific risk factors. An initial injury leads to a cascade of reparative processes in the injured tissue to repair and restore organ integrity. As a result of the injury, immune cells infiltrate

into the tissue inducing early inflammation which then causes the release of pro-fibrotic mediators. These mediators trigger the activation and proliferation of myofibroblasts, the key cell type in fibrogenesis. Myofibroblasts are highly contractile and release abundant ECM proteins which induce stiffness of the tissue. These cells can originate from different cell sources such as resident fibroblasts, pericytes, endothelial cells, bone marrow-derived fibrocytes, and epithelial cells. During normal wound healing, myofibroblasts undergo apoptosis after the tissue repair and thereafter the wound healing process ends. However, in the fibrotic tissue myofibroblasts continue proliferating as an uncontrolled process due to the continuation of chronic injury or self-sustaining activation processes.

In recent years, research has been focusing on fibroblast heterogeneity. Fibroblast heterogeneity can be understood in terms of cell lineage, phenotype, and response to signals from its microenvironment, as described by Mascharak et al. (2020). To date, fibroblast heterogeneity between organs and even within organs remains largely unexplored due to the lack of specific markers found on fibroblasts (Muhl et al. 2020). Therefore, one can understand that the impact of fibroblast heterogeneity on integrin expression, was not considered in the "early" studies discussed in this chapter.

Interactions between myofibroblasts and ECM are facilitated by integrins. Integrins support further deposition of ECM and mediate cross-communication between inflammatory cells, fibroblasts, and parenchymal cells. The latter describes integrins as an important link in tissue fibrosis (Fausther and Dranoff 2014). In this section, we will mainly focus on integrins expressed in fibroblasts and, in certain cases, parenchymal cells. Integrin subunits in four types of tissue fibrosis are discussed, namely pulmonary fibrosis, liver fibrosis, cardiac fibrosis, and renal fibrosis. Finally, the role of integrins and integrin subunits in tumor fibrosis will be discussed. The findings are summarized in Table 2.

2.1 Integrins in Pulmonary Fibrosis

Chronic lung disease or pulmonary fibrosis is, as in other fibrotic diseases, depicted by an excessive deposition of ECM. Replacement of functional lung tissue with fibrotic lung tissue is associated with a loss of normal lung function, causing inefficient oxygen uptake which could have implications on various other organs of the body (Sarkar et al. 2017). Currently, three main types of pulmonary fibrosis are described in literature. These three types include: diffuse interstitial fibrosis, diffuse fibrosing alveolitis, and idiopathic pulmonary fibrosis (IPF) (Todd et al. 2012). Among these three types, IPF is considered as the most severe and the most common type of pulmonary fibrosis. In the next paragraphs, the role of integrins and their subunits is described in pulmonary fibrosis.

Table 2 Integrins and integrin subunits in pulmonary, liver, cardiac, renal, and tumor fibrosis

Integrin or integrin subunit	Type of fibrosis	Main role in fibrosis	Cell type	Model	Selected references
β1	Liver fibrosis	Recreation of normal hepatocyte architecture after injury	Hepatocytes	Primary murine hepatocytes, human chronic hepatitis C patient samples	Masuzaki et al. (2021), Nejjari et al. (2001)
	Cardiac fibrosis	Normal cardiac function	Cardiomyocytes	WT and β1-deficient coronary artery ligation-induced myocardial infarction in mice	Krishnamurthy et al. (2006)
	Cardiac fibrosis	Activation of latent TGF-β	Cardiac fibroblasts	TAC-induced cardiac hypertrophy in mice, human heart failure patient samples	Takawale et al. (2017)
	Cardiac fibrosis	Activation of latent TGF-β	Principal cell-depleted renal collection duct cells	PKD1-deficient mice, β1-deficient mice	Lee et al. (2015)
		Normal cardiac function (substitute renal by cardiac)	Cardiomyocytes	TAC-induced cardiac hypertrophy in mice	Keller et al. (2001)
	Renal fibrosis	Normal renal function	Renal principal cells	β1-deficient mice	Mamuya et al. (2017)
β3	Cardiac fibrosis	Survival of cardiomyocytes	Endothelial cells, cardiomyocytes	LAD ligation-induced myocardial infarction in mice, TAC-induced cardiac hypertrophy in mice	Sun et al. (2003), Suryakumar et al. (2010)
	Cardiac fibrosis	Migration of cardiac fibroblasts	Cardiac fibroblasts	TAC-induced cardiac hypertrophy in mice	Balasubramanian et al. (2012)
αv	Cardiac fibrosis	Activation of latent TGF-β	Cardiac fibroblasts	CTX-induced cardiac fibrosis in mice,	Murray et al. (2017)
	Renal fibrosis		Kidney epithelial cells	Human kidney epithelial cells, UUO-induced renal fibrosis in mice	Henderson et al. (2013), Wallace et al. (2008)

αvβ1	Pulmonary fibrosis	Activation of latent TGF-β	Lung fibroblasts	Bleomycin-induced pulmonary fibrosis in mice	Reed et al. (2015)
	Liver fibrosis		Hepatic stellate cells	Primary human hepatic stellate cells (HSCs), CCl_4-induced liver fibrosis in mice	Han et al. (2021), Reed et al. (2015)
	Renal fibrosis		Renal fibroblasts	UUO-induced renal fibrosis in mice	Chang et al. (2017)
αvβ3	Pulmonary fibrosis	Activation of latent TGF-β, fibroblast contraction	Lung fibroblasts	Primary human lung fibroblasts, bleomycin-induced pulmonary fibrosis in mice	Fiore et al. (2018)
	Liver fibrosis		Hepatic stellate cells	Human HSCs, CCl_4-induced liver fibrosis in mice, bile duct ligation-induced biliary fibrosis in mice	Shao et al. (2020), Zhou et al. (2004)
	Cardiac fibrosis		Cardiac fibroblasts	DOX-induced myocardial fibrosis in mice, ischemic cardiac injury-induced cardiac fibrosis in mice	Sui and Hou (2021), Yokota et al. (2020)
	Renal fibrosis		Podocytes	Contrast-induced nephropathy in mice	Hayek et al. (2020)
	Tumor fibrosis	Tumor invasion	Cancer-associated fibroblasts	Primary human fibroblasts from colon tumors, CT26 cancer cells, 3D spheroids	Attieh et al. (2017)
αvβ5	Pulmonary fibrosis	Activation of latent TGF-β	Lung fibroblasts	Primary human lung fibroblasts, bleomycin-induced pulmonary fibrosis in mice	Scotton et al. (2009)
	Cardiac fibrosis			Spontaneously hypertensive rats	Perrucci et al. (2018)
αvβ6	Pulmonary fibrosis	Activation of latent TGF-β	Lung epithelial cells	Human bronchial epithelial cells, β6-deficient mice, human IPF patient samples	Kimura et al. (2019), Munger et al. (1999)
	Liver fibrosis		Hepatic stellate cells	Bile duct ligation-induced cirrhosis in mice, TAA- induced cirrhosis in mice, human chronic hepatitis C patient samples	Popov et al. (2008)

(continued)

Table 2 (continued)

Integrin or integrin subunit	Type of fibrosis	Main role in fibrosis	Cell type	Model	Selected references
	Renal fibrosis		Kidney epithelial cells	Alport mice, human kidney disease patient samples	Hahm et al. (2007)
αvβ8	Pulmonary fibrosis	Activation of latent TGF-β	Lung fibroblasts	Human IPF patient samples	Araya et al. (2007)
α1β1	Tumor fibrosis	Fibroblast differentiation, neovascularization, connective tissue organization	Cancer-associated fibroblasts, cancer cells	α1-deficient mice, CT26 colon adenocarcinoma mice autografts, human colorectal adenocarcinoma patient samples	(Boudjadi et al. 2013; Rodriguez et al. 2009)
α2	Pulmonary fibrosis	Induction of pulmonary fibrosis	Lung fibroblasts	Murine fibroblasts, bleomycin-induced pulmonary fibrosis in mice	Agarwal et al. (2020)
	Pulmonary fibrosis	Protection from pulmonary fibrosis	Lung epithelial cells	Human alveolar epithelial cells	Agarwal et al. (2020)
	Cardiac fibrosis	Protection from cardiac fibrosis	Cardiac fibroblasts	Primary murine cardiac fibroblasts	Hong et al. (2017)
	Renal fibrosis	Induction of renal fibrosis	Mesangial cells	Adriamycin or 5/6 nephrectomy-injured WT and α2-deficient mice, WT and α2-deficient Alport mice	Borza et al. (2012), Rubel et al. (2014)
	Tumor fibrosis	Anti-tumoral and pro-tumoral effects	Cancer-associated fibroblasts	Various breast cancer mouse models	Levental et al. (2009), Zeltz and Gullberg (2016)
α3	Pulmonary fibrosis	Mediating EMT via TGF-β signaling	Lung epithelial cells	Bleomycin-induced pulmonary fibrosis in mice	Kim et al. (2009a)
	Renal fibrosis		Renal proximal tubular epithelial cells	WT and E-cadherin-deficient UUO and IR-induced renal fibrosis in mice	Zheng et al. (2016)
	Tumor fibrosis	CAF differentiation, maintaining CAF phenotype	Cancer-associated fibroblasts	Primary human CAFs, 3D spheroids, human PDAC patient samples	Cavaco et al. (2018)

α5	Pulmonary fibrosis	Inducing CTGF expression by binding to Pref-1	Lung fibroblasts	Human lung fibroblasts, OVA-induced airway fibrosis in mice, human asthma patient samples	Cheng et al. (2021)
	Cardiac fibrosis	Inducing cardiac fibrosis	Cardiac fibroblasts	Human atrial fibrillation patient samples	Zhao et al. (2013)
	Tumor fibrosis	Migration, proliferation, adhesion, and contraction	Cancer-associated fibroblasts	Primary human PSCs, 3D PANC-1 PSCs heterospheroids, PDAC mice (xenograft and PDX), human PDAC patient samples	Kuninty et al. (2019)
		Cancer cell migration in the ECM	Cancer cells	3D-collagen matrices of A549 cells, WI-38 and PANC-1, OUS-11 cells	Miyazaki et al. (2019)
α5β1	Liver fibrosis	Activation, proliferation, and migration of HSCs	Hepatic stellate cells	Rat HSCs, TAA-induced chronic hepatic fibrosis in rats	(Du et al. 2020)
α8	Liver fibrosis	Activation of HSCs	Hepatic stellate cells	NASH mice, bile duct ligation-induced fibrosis in mice, CCl_4-induced liver fibrosis in mice, human liver fibrosis patient samples	Nishimichi et al. (2021)
	Renal fibrosis	Maintaining integrity of the glomerular capillary tuft	Mesangial cells	DOCA-treated and UUO-induced renal fibrosis in WT and α8-deficient mice	Hartner et al. (2002), Marek et al. (2016)
α11	Pulmonary fibrosis	Mediating fibroblast-ECM interactions	Lung fibroblasts	Human IPF patient samples	Bansal et al. (2017)
	Liver fibrosis		Hepatic stellate cells	CCl_4-induced liver fibrosis in mice, human cirrhotic liver patient samples	Bansal et al. (2017)
	Cardiac fibrosis		Cardiac fibroblasts	Human cardiac fibroblasts, STZ-induced diabetic rats and mice,	Talior-Volodarsky et al. (2012)
	Renal fibrosis	Stimulating myofibroblast differentiation, ECM accumulation	Renal fibroblasts	UUO-induced renal fibrosis in mice	Bansal et al. (2017)
	Tumor fibrosis	Promoting fibrillar collagen reorganization and tissue stiffness	Cancer-associated fibroblasts	NSCLC WT and α11-deficient mice, human tumor tissue samples	Zeltz et al. (2019), Zhu et al. (2007)

2.1.1 Role of Integrin αv Subunit in Pulmonary Fibrosis

In pulmonary fibrosis, the integrin αv family has a pivotal role in the onset and the progression of the fibrotic pathway (Slack et al. 2021; Tan et al. 2019). A major role of αv integrins, together with their β subunits, is to activate latent TGF-β. Latent TGF-β is complex that consists of TGF-β and a protein which is known as latency-associated peptide (LAP). LAP contains an RGD sequence that can bind to αv integrins and can therefore facilitate the binding of TGF-β to the TGFβ receptor, as proposed by Munger et al. (Munger et al. 1999). TGF-β has a key role in the progression of fibrosis in multiple organs (Henderson et al. 2013). In addition, the αv subunit can bind to various ECM molecules, such as fibronectin and vitronectin.

The role of integrin αvβ1 in the activation of the latent TGF-β was investigated in mice by Reed et al. (Reed et al. 2015). One of the main findings was that in bleomycin-induced pulmonary fibrosis, integrin αvβ1 was overexpressed on myofibroblasts. Using selective inhibitor C8, Reed and colleagues showed attenuation of bleomycin-induced pulmonary fibrosis (Reed et al. 2015).

Another family member of the αv family that has a role in pulmonary fibrosis, is the integrin αvβ3 (Fiore et al. 2018; Nanri et al. 2020; Wan et al. 2019). Fiore et al. demonstrated that integrin αvβ3 drives progressive fibrosis through stiffening of the fibrotic niche, which was performed using in vitro and in vivo studies (Fiore et al. 2018). Integrin subunit β3, which pairs with αv, has been shown to be upregulated in LPS-treated human MRC-5 lung fibroblasts and is, therefore, involved in pulmonary fibrosis. Authors showed that the inhibition of αvβ3 using an inhibitor cilengitide prohibited LPS-induced pulmonary fibrosis in mice (Wan et al. 2019). Recently, Nanri et al. showed a crosstalk between TGF-β and periostin, an ECM protein, via integrins αvβ3 and αvβ5 in four strains of fibroblasts collected from IPF-containing human lungs. They also demonstrated that an αvβ3 inhibitor attenuated pulmonary fibrosis in vitro and in vivo, confirming the role of αvβ3 in pulmonary fibrosis (Nanri et al. 2020). The role of integrin αvβ5 in pulmonary fibrosis was also shown earlier by (Scotton et al. 2009). Scotton et al. demonstrated that coagulation factor X (FXa)-induced fibroblast to myofibroblast differentiation is mediated via a TGF-β-dependent Smad2 pathway and that blockade of αvβ5 integrin inhibited this response, suggesting a key role of αvβ5 integrin in mediating fibroblast differentiation in IPF (Scotton et al. 2009).

Integrin αvβ6 has been extensively studied in pulmonary fibrosis (Kimura et al. 2019; Liu et al. 2020; Lukey et al. 2020; Munger et al. 1999). Munger et al. investigated the activation of latent TGF-β by integrin αvβ6 and found that integrin β6-transfected cells showed induced TGF-β activity. Moreover, in animal studies using β6-deficient mice, it was found that these mice were protected against pulmonary fibrosis. It is worth mentioning that Munger and colleagues were the first to propose a mechanism on the activation of latent TGF-β through integrin αvβ6 (Munger et al. 1999). Kimura and colleagues tested their new cystine knot peptide, knottin, for its recognition of integrin αvβ6. The peptide recognized αvβ6 and showed increased signal in IPF patients as compared to healthy individuals (Kimura

et al. 2019). In another study, Lukey et al. performed PET/CT imaging in IPF patients using an αvβ6-selective ligand and showed an induced expression of αvβ6 integrin in these patients (Lukey et al. 2020). Recently, a study showed that integrin αvβ6 mediates epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells induced by lipopolysaccharides (LPS) via TGF-β1-Smad2/3 signaling pathway and proposed integrin αvβ6 as a potential therapeutic target (Liu et al. 2020).

Another member of αv family which has been implied in the progression of pulmonary fibrosis is αvβ8 (Araya et al. 2007; Cui et al. 2021; Kitamura et al. 2011). Araya et al. showed that the expression of integrin αvβ8 was upregulated in lung fibroblasts derived from human COPD patients and revealed that the severity of COPD, and small airway wall thickening correlated with the upregulated expression of integrin αvβ8 (Araya et al. 2007). In addition, a study by Kitamura et al. demonstrated that dendritic cell trafficking, mediated by lung fibroblasts, is dependent on αvβ8-mediated activation of TGF-β in pulmonary fibrosis (Kitamura et al. 2011). More recently, Cui et al. investigated genes that are involved in the development of IPF. By comparing the expression profiles of IPF patients and healthy individuals, the researchers could identify a significant increase in integrin subunit β8 levels and its upregulated expression was paired with integrin subunit αv (Cui et al. 2021). These studies indicate that integrin αvβ8 has a crucial role in regulating a fibrotic and an immunogenic response in pulmonary fibrosis.

2.1.2 Role of Integrin Subunits α2 and α11 in Pulmonary Fibrosis

Other integrins that have demonstrated to have a role in pulmonary fibrosis include collagen-binding integrins α2β1 (Agarwal et al. 2020) and α11β1 (Bansal et al. 2017). Agarwal et al. studied the effect of collagen type I signaling on TGF-β-mediated activation of murine fibroblasts and demonstrated that fibroblasts cultured on type I collagen and on a fibrotic decellularized lung matrix, had an augmented activation mediated by integrin α2β1. Furthermore, they showed that bleomycin-induced pulmonary fibrotic Col1a2-CreERT mice with fibroblast-specific deletion of integrin subunit α2, which was achieved through tamoxifen administration, were protected from fibrosis. Yet, it was not mentioned what subtype of fibroblasts were affected by the deletion of integrin subunit α2. On the contrary, blocking α2 in alveolar epithelial cells (AECs) led to an increased fibrotic response indicating that the role of integrin subunit α2 in fibrosis varies between cell types (Agarwal et al. 2020). The other collagen-binding integrin to be discussed in pulmonary fibrosis, is integrin α11β1. We analyzed the integrin subunit α11 expression in patients with IPF and demonstrated an upregulation of its expression both at the protein and the gene levels, which suggested α11 as a potential target in pulmonary fibrosis (Bansal et al. 2017).

2.1.3 Role of Integrin Subunit α3 in Pulmonary Fibrosis

Integrin subunit α3, that pairs with integrin subunit β1 and binds to laminins, appears to have a pro-fibrotic role in the lungs as well (Kim et al. 2009a, 2009b). Kim et al. demonstrated the role of integrin α3β1 in mediating EMT via TGF-β1 signaling, which is known to be a key feature in fibrosis (Kim et al. 2009b). K. Kim and colleagues generated lung epithelial cell-specific α3-deficient mice and subjected them to bleomycin injury to induce pulmonary fibrosis. The study verified that α3 was required for the initiation of EMT (Kim et al. 2009a).

2.1.4 Role of Integrin α5 Subunit in Pulmonary Fibrosis

There are many other integrins which have been studied for their potential role in lung fibrosis. Cheng et al. investigated the role of preadipocyte factor-1 (Pref-1) in airway fibrosis. Pref-1 overexpression was discovered in lung tissues of chronic obstructive asthma patients and mice. By investigating its mechanism in vitro, the researchers suggested that Pref-1 binds to integrin α5β1, a receptor for fibronectin, which induces connective tissue growth factor (CTGF) expression that plays an important role in airway fibrosis (Cheng et al. 2021).

Altogether, the αv family of integrins has shown to be a key player in the onset and progression of pulmonary fibrosis. Overexpression of integrin subunit α3 has been implied in mediating EMT. The integrin subunit α2 has a cell-dependent role in pulmonary fibrosis, α5 and α11 appear to be upregulated during pulmonary fibrosis suggesting a progressive role in pulmonary fibrosis.

2.2 *Integrins in Liver Fibrosis*

Chronic liver injuries are the foundation of developing liver fibrosis. Liver fibrosis can ultimately lead to liver cirrhosis (i.e., scarring of the liver which is caused by long-term liver damage) or even hepatocellular carcinoma (HCC). Both diseases can lead to death if left untreated (Brenner 2013; Kisseleva and Brenner 2008; Sun and Kisseleva 2015). In the next paragraphs, specific integrins and their interactions in fibrotic livers will be discussed.

2.2.1 Role of Integrin Subunit β1 in Liver Fibrosis

The specific role of integrin subunit β1 expressed on hepatocytes in liver fibrosis has been studied by many researchers (Guo et al. 2019; Masuzaki et al. 2021; Nejjari et al. 2001; Voutilainen et al. 2021). In the early 2000s, Nejjari and colleagues analyzed integrin expression in 94 patients with chronic hepatitis C and revealed an

increased integrin subunit β1 expression in 88.2% of the cases (Nejjari et al. 2001). In another study, inhibition of β1 subunit ameliorated liver fibrosis in non-alcoholic steatohepatitis (NASH) mice (Guo et al. 2019). Furthermore, Voutilainen and colleagues investigated liver allograft expression of fibrogenic genes. In the group in which fibrosis but no inflammation was present, integrin subunit β1 was significantly upregulated (Voutilainen et al. 2021). In contrast to the latter studies, a recent study by Masuzaki et al. suggested that integrin subunit β1 is a key determinant of liver architecture and has a critical role as a regulator of TGF-β secretion. They showed that deletion of integrin subunit β1 in adult hepatocytes prevented recreation of normal hepatocyte architecture after liver injury, which resulted in fibrosis (Masuzaki et al. 2021). Therefore, to understand the role of integrin subunit β1 in liver fibrosis, further investigations are needed.

2.2.2 Role of Integrin Subunit αv in Liver Fibrosis

One of the most prominent and well-studied integrin subunits in liver fibrosis is αv. The αv subunit plays a prominent role in the onset and progression of liver fibrosis. The role of integrin αvβ1 in the activation of the latent TGF-β was investigated in mice by Reed et al. (2015). It appeared that in CCl_4-induced liver fibrosis, integrin αvβ1 was overexpressed on myofibroblasts. Here, the selective inhibitor C8 was used to attenuate liver fibrosis in vivo (Reed et al. 2015). Furthermore, αvβ1 has been found to be highly abundant in primary human hepatic stellate cells (HSCs) and, interestingly, integrin αvβ1 is able to regulate TGF-β signaling independent of activating latent TGF-β. Thus, this suggests a pivotal role for integrin αvβ1 in the onset and progression of fibrosis (Han et al. 2021).

Considering the number of studies that showed upregulation of integrin αvβ3 in hepatic stellate cells during NASH-induced liver fibrosis, upregulation of this integrin subunit is probably the hallmark of this type of liver fibrosis (Hiroyama et al. 2020; Shao et al. 2020; Zhou et al. 2004). Earlier, Zhou et al. extracted HSCs from normal human livers and by using various culture conditions with different ECM protein-coated well plates, they showed an increase in integrin αvβ3 expression when the cells were activated (Zhou et al. 2004). Zhou and colleagues were the first to show that integrin αvβ3 regulates the fate of HSCs. More recently, Shao et al. induced fibrosis in CD1 male mice by CCl_4 treatment in one group and bile duct ligation-induced biliary fibrosis in the other group. Using PET/CT imaging using an RGD-sequence-containing tracer molecule named [^{18}F]-Alfatide, they demonstrated that integrin αvβ3 was overexpressed in fibrotic mice (Shao et al. 2020). Other studies also showed the upregulation of αvβ3 integrin in PET/CT imaging using tracer molecule ^{18}F-FPP-RGD_2 in NASH rats (Hiroyama et al. 2020) and correlated integrin αvβ3 expression with the onset of fibrosis in NASH mice (Rokugawa et al. 2018).

Next to αvβ3, the role of integrin αvβ6 has also been described in many studies (Popov et al. 2008; Schuppan et al. 2018; Zagory et al. 2019; Zhao-Bin He et al. 2020). In early studies conducted by Popov et al., integrin αvβ6 expression was

investigated in biliary and portal liver fibrosis. Livers from cirrhotic rats and patients with chronic hepatitis C and various end-stage liver diseases showed high levels of integrin αvβ6 compared to normal livers. Moreover, severity of fibrosis correlated with an increased expression of subunit β6 mRNA levels (Popov et al. 2008). Furthermore, Schuppan et al. discussed the role of integrin αvβ6 in NASH. Blocking of integrin αvβ6 resulted in the attenuation of fibrogenesis via the inhibition of TGF-β1 activation (Schuppan et al. 2018). Another interesting study demonstrated the role of prominin-1 in the progression of biliary atresia through subunit β6 integrin in functional *Prom1* knockout mice (Zagory et al. 2019). More recently, He et al. showed the induced expression of the αvβ6 integrin in hepatitis B virus (HBV)-associated cirrhosis and the increased expression correlated with the severity of the disease (Zhao-Bin He et al. 2020).

2.2.3 Role of Integrin Subunit α5 in Liver Fibrosis

The role of α5β1 in liver fibrosis has also been described (Du et al. 2020; Milliano and Luxon 2003; Yaqoob et al. 2020). An early study conducted by Milliano and Luxon, demonstrated the activation of HSCs by fibronectin-coated beads and colocalization of these beads with α5 and β1 antibodies. This study indicates that, by binding to fibronectin, integrin α5β1 could activate HSCs and, therefore, possibly drive fibrosis (Milliano and Luxon 2003). More recently, Yaqoob et al. investigated the role of TGF-β and synectin, a PDZ-domain-containing adaptor protein, in liver fibrosis. From in vitro studies, Yaqoob and coworkers concluded that integrin α5β1 was overexpressed in activated HSCs when recombinant IGFBP-3, a protein secreted upon GIPC stimulation, was added to the culture (Yaqoob et al. 2020). Du and colleagues investigated the role of SPOCK1 (SPARC (osteonectin), cwcv, and kazal-like domain proteoglycan 1) in liver fibrosis. SPOCK1 appeared to activate the α5β1/PI3K/Akt signaling pathway that regulated HSC activation, proliferation, and migration. In addition, HSC-specific knockdown of SPOCK1 impaired liver fibrosis, demonstrating once more that integrin α5β1 has a pivotal role in the onset and progression of liver fibrosis (Du et al. 2020).

2.2.4 Role of Integrin Subunit α8 in Liver Fibrosis

In recent years, there is an increasing number of studies showing the role of integrin subunit α8, another RGD-binding integrin, in liver fibrosis (Cai et al. 2020; Nishimichi et al. 2021; Ogawa et al. 2018; Yang et al. 2021). Ogawa et al. showed an upregulation of integrin subunit α8 in fibrotic mice and pinpointed integrin subunit α8 as a marker for myofibroblasts in liver fibrosis (Ogawa et al. 2018). Furthermore, Cai and colleagues studied the effects of a certain microRNA on integrin subunit α8. Along with the non-alcoholic fatty liver disease (NAFLD) patient sample analysis, they also performed experiments in which inhibition of microRNA-125b-5p resulted in integrin subunit α8 upregulation, leading to liver

fibrosis in NAFLD (Cai et al. 2020). In addition, Nishimichi et al. studied the role of different integrins that could activate HSCs and found that activated HSCs showed a significant upregulation of integrin subunit α8 (Nishimichi et al. 2021). Furthermore, in three different murine fibrosis models treated with an anti-α8 neutralizing antibody, various markers for fibrosis were downregulated. Moreover, ITGA8 gene expression was significantly elevated in patient with liver fibrosis indicating the significance of α8 in liver fibrosis (Nishimichi et al. 2021). Furthermore, the role of α8 subunit as a key modulator of LOXL1, a molecule responsible for crosslinking and stabilizing the ECM, in fibrosis was demonstrated by Yang and colleagues. In vitro studies revealed that by silencing the ITGA8 gene in cultured fibroblast, the level of LOXL1 was decreased and the fibroblast remained inactivated. Thus, the integrin subunit α8 was suggested to mediate a pro-fibrotic response via LOXL1 (Yang et al. 2021).

2.2.5 Role of Integrin Subunit α11 in Liver Fibrosis

The role of integrin subunit α11 in liver fibrosis has only been described by us and Martin et al. (Bansal et al. 2017; Martin et al. 2016). Martin et al. showed upregulation of integrin subunit α11 in primary activated HSCs and verified that Yes-associated protein 1 (YAP-1) signaling, a key mediator of pro-fibrotic signaling, is reduced when α11 is not present (Martin et al. 2016). Furthermore, we showed an upregulation of integrin α11β1 in fibrotic livers of the CCl_4-induced mouse models which was verified at both protein and gene expression levels. Immunohistological analysis in human cirrhotic liver samples showed high expression of integrin subunit α11 which was co-localized with α-SMA, a marker for activated HSCs. Furthermore, patient transcriptome data analysis revealed a significant upregulation of the ITGA11 gene in severe fibrosis as compared to mild fibrosis (Bansal et al. 2017).

Thus, integrins of the αv-family, which include αvβ1, αvβ3, and αvβ6, have been shown to be upregulated during various liver diseases. Furthermore, an indirect role for integrin subunit α5, which appeared to increase the activation and the proliferation of HSCs, and the use of integrin α8 as a marker for liver fibrosis have been described. The integrin subunit β1 is upregulated in certain liver diseases but was also shown to prevent fibrosis, indicating a bidirectional role of β1 which needs further investigation. Integrin subunit α11 appeared to be upregulated during liver cirrhosis and correlated with severity of fibrosis. Yet, more studies are needed to verify these observations, especially at protein and cell levels in vivo.

2.3 Integrins in Cardiac Fibrosis

Cardiac fibrosis or scarring of the heart upon injury is prone to occur after myocardial infarction but can also reside from non-infarction-related diseases (Bharati and Lev 1995; Disertori et al. 2017; Jellis et al. 2010; McCalister et al. 2015; Talman and

Ruskoaho 2016). In general, cardiac fibrosis can lead to several major impairments, including problems regarding systolic and diastolic function as well as an increased stiffness of the ventricles which could retard contraction-relaxation of the heart. This complication is usually found in the perimysial space of the heart (Kong et al. 2014). Cardiac fibrosis comes in various types including (1) replacement fibrosis, (2) endomyocardial fibrosis, (3) infiltrative interstitial fibrosis, and (4) reactive interstitial fibrosis (Mewton et al. 2011). In the next paragraphs, the role and interaction of specific integrins in cardiac fibrosis will be discussed.

2.3.1 Role of Integrin Subunits β1 and β3 in Cardiac Fibrosis

Both integrin subunits β1 and β3 have been well investigated in cardiac fibrosis (Balasubramanian et al. 2012; Ge et al. 2020; Harikrishnan et al. 2019; Keller et al. 2001; Krishnamurthy et al. 2006; Saraswati et al. 2020; Shai et al. 2002; Sun et al. 2018; Sun et al. 2003; Takawale et al. 2017; Wilson et al. 2011). Both β-subunits pair with the integrin subunit αv. Binding of αv to subunit β1 and β3 seem to have biphasic role that seems to depend on cell type. In an early study, the role of integrin subunits β3 and β1 was investigated during tissue remodeling after myocardial infarction. Both subunits were upregulated after myocardial infarction. The β3 subunit was upregulated in endothelial cells and smooth muscle cells, which are known to be the sites of inflammation and fibrosis. Furthermore, integrin β1A subunit was primarily localized in fibroblasts while β1D was found in cardiomyocytes. However, Sun and colleagues did observe a transition from β1D to β1A in cardiomyocytes subunit upon exposure to TNF-α (Sun et al. 2003). The role of β3 in cardiac fibrosis was investigated by Balasubramanian et al. and they showed that integrin subunit β3-deficient pressure-overloaded C57BL/6 mice had significant lower ECM-production as compared to the wild-type mice. Furthermore, it was found that integrin-mediated Pyk2 signaling in cardiac fibroblasts plays a pivotal role in pressure overload-induced cardiac fibrosis (Balasubramanian et al. 2012). On the other hand, the role of the integrin β3 subunit expressed on cardiomyocytes was found to be cardioprotective during pressure-overload hypertrophy, which is known to cause cardiac fibrosis (Suryakumar et al. 2010).

Few have described the anti-fibrotic role of integrin subunit β1. Keller et al. revealed that reduction of β1A, using inhibitor Tac-β1A, in cardiomyocytes was associated with hypertrophic changes, decreased basal contractility, and relaxation of the heart in mice which could eventually lead to cardiac fibrosis (Keller et al. 2001). Furthermore, Shai and coworkers demonstrated that specifically inactivating the gene encoding for integrin subunit β1 in cardiac myocytes in $\beta1^{Flox/Flox}$/$MLC2v^{+/Cre}$ mice, resulted in the development of postnatal cardiac fibrosis and subsequently heart failure by the age of 6 months (Shai et al. 2002). Krishnamurthy and colleagues investigated the role of integrin subunit β1 in left ventricular remodeling after myocardial infarction. The study revealed that in integrin subunit β1-deficient mice, impairment of cardiac function after myocardial infarction was present. Yet,

Krishnamurthy and coworkers obtained their data using smaller infarct models (Krishnamurthy et al. 2006).

In contrast to the latter described studies, integrin subunit β1 also seems to have pro-fibrotic role in cardiac fibrosis. Takawale et al. investigated the role of tissue inhibitor of metalloproteinase-1 (TIMP1) on fibroblasts in cardiac fibrosis. TIMP1 is often used as a marker for cardiac fibrosis, and it was found that, using two in vivo models of myocardial fibrosis, TIMP1-deficient mice had significantly reduced fibrosis. Furthermore, studies using TGF-β activated adult cardiac fibroblast isolated from pressure-overloaded mice revealed that TIMP1 induces cardiac fibrosis via an interaction between integrin subunit β1 and CD63 (Takawale et al. 2017). Sun et al. showed that right ventricular hypertension, induced by pulmonary artery banding in Sprague-Dawley rats, resulted in an upregulation of β1 expression in cardiac fibroblasts. This upregulation of integrin subunit β1A was said to colocalize with myofibroblasts. Yet, immunofluorescent data was not presented. In addition, Sun and coworkers demonstrated that in vitro inhibition of integrin subunit β1 in cardiac fibroblasts, derived from various regions of the heart, suppressed mechanical stretch-mediated collagen type I production (Sun et al. 2018). In a more recent study, Harikrishnan et al. researched the crosstalk between discoidin domain receptor 2 (DDR2) and integrin subunit β1. Crosstalk between both collagen-receptors increased collagen type I expression in angiotensin II-stimulated fibroblasts. Upon increased collagen type I expression, fibroblasts differentiated to myofibroblasts (Harikrishnan et al. 2019). In favor of the pro-fibrotic role of β1 in fibroblasts, Saraswati et al. demonstrated that small proline-rich repeat 3 (SPRR3), a mechanosensitive protein, facilitated the activation of PDGFRβ by the β1 subunit in cardiac fibroblasts which resulted in increased proliferation and ECM synthesis in pressure-induced heart failure C57Bl/6 mice. However, comparing integrin subunit β1 expression in wild-type and *Sprr3*$^{-/-}$ fibroblasts did not show any statistically significant difference (Saraswati et al. 2020).

2.3.2 Role of Integrin Subunit αv in Cardiac Fibrosis

Like other types of fibrosis, the αv family also plays a key role in cardiac fibrosis as shown by many studies (Bouvet et al. 2020; Lin et al. 2021; Murray et al. 2017; Perrucci et al. 2018; Sui and Hou 2021; Yokota et al. 2020). Murray et al. showed that selective depletion of integrin subunit αv in PDGFRβ+ cells (most likely pericyte- and fibroblast-derived myofibroblasts) and inhibition of integrin subunit αv using small molecule αv integrin inhibitor CWHM 12, reduced activation of latent TGF-β and, therefore, attenuated cardiac fibrosis in mice. This indicated the role for αv in the progression of cardiac fibrosis (Murray et al. 2017). More recently, Bouvet et al. proposed αv as a key regulator in post-myocardial infarction in mice, as antibodies against integrin subunit αv reduced cardiac fibrosis post-myocardial infarction (Bouvet et al. 2020). Anti-integrin subunit αv antibody therapy and inhibitors of integrin αvβ3 and αvβ5 were also evaluated by others. Studies showed that inhibition of these integrins reduced cardiac fibrosis in hypertensive rat-derived

cardiac fibroblasts and doxorubicin-induced fibrotic rat models (Perrucci et al. 2018; Sui and Hou 2021). In a recent study, Yokota et al. investigated collagen type V which appeared to regulate the size of scars after ischemic injury. By subjecting C57BL/6 mice to ischemic cardiac injury, they developed a mouse model in which they could examine the role of collagen type V which is usually poorly expressed in non-injured hearts. Results revealed that collagen type V regulated the scar size through integrins αvβ3 and αvβ5 (Yokota et al. 2020).

2.3.3 Role of Integrin Subunits α2 and α11 in Cardiac Fibrosis

Loss of integrin α2β1, which is an integrin that is often expressed on healthy cardiac fibroblasts (Gullberg et al. 1992), could have a role in the development of cardiac fibrosis, as studied by Hong et al. (Hong et al. 2017). By mimicking the in vivo 3D structure of ECM of post-myocardial infarction patient-derived myofibroblasts, they found that low expression of integrin α2β1 increased the proliferation and differentiation of myofibroblasts (Hong et al. 2017). Regarding other α subunits that have a role in the onset and progression of cardiac fibrosis, integrin subunit α11 has been investigated by a few (Civitarese et al. 2016; Romaine et al. 2018; Talior-Volodarsky et al. 2012). Talior-Volodarsky et al. investigated whether differentiation to myofibroblasts is mediated by the adhesion of cardiac fibroblasts to glycated collagens in streptozotocin-induced diabetic cardiomyopathy rat models. In diabetic rats, an increased expression of α11 was observed (Talior-Volodarsky et al. 2012). Furthermore, Civitarese et al. showed that deletion of integrin α11β1 attenuated cardiac fibrosis in the diabetic mouse model, indicating that expression of integrin α11β1 has a pro-fibrotic effect in cardiac fibrosis as a result of diabetes (Civitarese et al. 2016). In addition, Romaine and colleagues showed that mice overexpressing integrin subunit α11 developed left ventricular concentric hypertrophy with an increased deposition of collagen as compared to wild-type mice (Romaine et al. 2018).

2.3.4 Role of Integrin Subunit α5 in Cardiac Fibrosis

Integrin subunit α5 appears to have a role in ischemic heart disease which is a consequence of cardiac fibrosis (Li et al. 2021; Steenbergen and Frangogiannis 2012; Zhao et al. 2013). Zhao et al. demonstrated that integrin subunit α5 is upregulated during atrial fibrillation, a condition that is associated with congestive heart failure and often coexists with ischemic heart disease (Everett and Olgin 2007; Volpe and Gallo 2020; Zhao et al. 2013). Li et al. showed that microRNA miR-30d, secreted in extracellular vesicles by cardiomyocytes that directly targeted integrin subunit α5 expressed on cardiac fibroblasts, improved cardiac function and reduced cardiac fibrosis. Furthermore, attenuated apoptosis of cardiomyocytes in both rats and mice with ischemic heart failure was observed. These results signify the importance of integrin subunit α5 in cardiac fibrosis (Li et al. 2021).

Altogether, most studies suggest that overexpression of integrin subunit αv has a pro-fibrotic effect in cardiac fibrosis. Some contrasting studies, however, show that certain αv integrins might have a beneficial effect in cardiac fibrosis. Integrin subunits β1 and β3 are involved in the progression of cardiac fibrosis. Yet, integrin subunit β1 expression appears to be important for normal cardiac function of cardiomyocytes. Integrin subunits α11 and α5 have been shown for their pro-fibrotic role as well and have shown to be upregulated during certain cardiac disease that could lead to cardiac fibrosis.

2.4 Integrins in Renal Fibrosis

Renal fibrosis is usually the end-stage of both chronic and progressive nephropathies (Humphreys 2018). Progressive renal fibrosis is irreversible and causes significant loss of functional renal cells. Loss of renal cells leads to kidney dysfunction and kidney failure (Humphreys 2018). Permanent hemodialysis or kidney transplantation is the only viable intervention (Remuzzi et al. 2006). In the next paragraphs, the role of integrins and their subunits will be described for renal fibrosis.

2.4.1 Role of Integrin Subunit β1 in Renal Fibrosis

Integrin subunit β1 seems to have a biphasic role which depends on whether the kidney is diseased or not (Lee et al. 2015; Mamuya et al. 2017). Mamuya et al. showed that the loss of integrin subunit β1 on healthy principal cells in the renal collecting duct was associated with developing peritubular and interstitial fibrosis (Mamuya et al. 2017). On the other hand, inactivation of integrin subunit β1 in the collecting duct inhibited renal cystogenesis and fibrosis in autosomal dominant polycystic kidney disease (ADPKD) in mice (Lee et al. 2015).

2.4.2 Role of Integrin Subunit αv in Renal Fibrosis

Few studies have shown an upregulation of αv expression in combination with different β subunits in renal fibrosis (Chang et al. 2017; Hayek et al. 2020; Henderson and Sheppard 2013; Wallace et al. 2008). Wallace et al. showed that binding of periostin to integrins αvβ3 and αvβ5 can lead to an activation of integrin-linked kinase (ILK), a component of the focal adhesion plaque, in cystic kidney cells derived from patients with autosomal dominant polycystic disease. Furthermore, stimulated by periostin, the overexpression of the αv integrins could activate latent TGF-β which can result in an increased production of ECM molecules (Wallace et al. 2008). Henderson and colleagues showed upregulation of αv integrins in myofibroblast in the unilateral ureteric obstruction (UUO) murine model (Henderson et al. 2013). Furthermore, renal fibroblasts were shown to overexpress αvβ1 as the

main αv integrin used by these cells to directly bind to latency-associated peptide (LAP) of TGF-β1. Although inhibition of either αvβ1 or αvβ3 reduced the activation of latent TGF-β, the effects of αvβ3 inhibition seemed to be an in vitro artifact. Therapeutic delivery of αvβ1 inhibitor C8 reduced UUO-induced renal fibrosis in vivo, highlighting αvβ1 integrin as a promising target for developing anti-fibrotic therapy (Chang et al. 2017). More recently, Hayek and coworkers investigated the role of soluble urokinase plasminogen activator receptor (suPAR), a signaling glycoprotein, in the activation of αvβ3 integrin during the pathogenesis of acute kidney disease. In murine models that overexpressed the suPAR protein, it was shown that continuous exposure of suPAR leads to the activation of the αvβ3 integrin on podocytes, a cell type involved in kidney fibrosis (Hayek et al. 2020).

In renal fibrosis, the upregulation of integrin αvβ6 is described in certain studies (Hahm et al. 2007; Xiong et al. 2019; Zhu et al. 2020). Hahm et al. demonstrated the upregulation of the αvβ6 integrin in human kidney epithelial cells in diabetes mellitus, glomerulonephritis, and IgA nephropathy can eventually lead to renal fibrosis. Blocking integrin αvβ6 resulted in a decrease of activated TGF-β and, therefore, a reduction of activated fibroblasts as well as in the reduction in deposited interstitial collagen matrix (Hahm et al. 2007). Xiong and colleagues demonstrated the effect of a selective class IIa histone deacetylase (HDAC) inhibitor that decreased the expression of αvβ6, which in turn reduced the progression of renal fibrosis. This indicates a pivotal role for αvβ6 in renal fibrosis (Xiong et al. 2019). Recently, Zhu and coworkers showed that Tenascin-C, an ECM glycoprotein binding to αvβ6, plays a crucial role in the progression of kidney fibrosis. This study demonstrated that Tenascin-C was overexpressed in patients and that after blockade of integrin αvβ6, Tenascin-C could not fulfill its function which resulted in repression of the EMT (Zhu et al. 2020).

2.4.3 Role of Integrin Subunits α2 and α11 in Renal Fibrosis

Integrin α2 appears to have a pro-fibrotic role in renal fibrosis (Borza et al. 2012; Rubel et al. 2014). Using integrin subunit α2-deficient mice, Borza and colleagues showed that these mice developed significantly less glomerular sclerosis as compared to the wild-type. Furthermore, the pharmacological inhibition of the α2β1 integrin in the wild-type mice resulted in decreased collagen synthesis by mesangial cells (Borza et al. 2012). In another study using integrin subunit α2-deficient mice, Rubel and colleagues showed that the loss of integrin α2β1 in Alport mice delayed renal fibrosis (Rubel et al. 2014). Furthermore, the role of integrin subunit α11 in renal fibrosis was investigated by us in which fibrotic mice had an increased expression levels of α11 in fibroblasts located in the tubulointerstitial space. In addition, the clinical transcriptomic data revealed a significant increase in mRNA expression of the ITGA11 gene in patients with renal interstitial fibrosis (Bansal et al. 2017).

2.4.4 Role of Integrin Subunit α3 in Renal Fibrosis

Zheng et al. investigated the role of the α3β1 integrin and E-cadherin in cell-cell adhesion with respect to renal fibrosis. Zheng and coworkers concluded that renal fibrosis after UUO and ischemia reperfusion (IR) was exacerbated in E-cadherin deficient mice due to upregulation of integrin α3β1 and an integrin α3-dependent enhancement of ILK-signaling in proximal tubular epithelial cells (Zheng et al. 2016).

2.4.5 Role of Integrin Subunit α8 in Renal Fibrosis

Integrin α8 appears to have an anti-fibrotic role in adult kidneys (Hartner et al. 2002; Marek et al. 2016). Hartner et al. showed that lacking integrin subunit α8 showed increased susceptibility to glomerular capillary destruction as compared to wild-type mice. However, Hartner and colleagues found that integrin α8 did not seem to play a key role in the development of renal fibrosis (Hartner et al. 2002). Marek and coworkers found that integrin subunit α8 deficiency resulted in more severe renal fibrosis and concluded that integrin subunit α8 seemed to attenuate UUO-induced tubulointerstitial renal fibrosis via regulation of TGF-β signaling, reducing fibroblast activation and/or immune cell infiltration (Marek et al. 2016).

In conclusion, the integrin αv family has a dominant role in facilitating EMT and has an overexpression in myofibroblasts in renal fibrosis. The role of integrin subunit β1 in renal fibrosis seems to depend on the location within the kidney and on the cell type. Overexpression of integrin subunits α2, and α3 correlated with severity of fibrosis, while integrin subunit α8 appears to reduce fibrotic effect. Yet, more studies are needed to verify these observations.

2.5 Integrins in Tumor Fibrosis

In contrast to normal tissue, cells in the tumor tissue divide uncontrollably due to genetic mutations. Tumors can roughly be divided in two categories, namely benign and malignant. Benign tumors usually grow slowly and, more importantly, do not metastasize, while malignant tumors grow rapidly and tend to metastasize (Patel 2020). The tumor microenvironment (TME) or tumor stroma, which consists of nonmalignant cellular and noncellular components, supports tumor cells to survive, progress, and become resistant to therapies (Balkwill et al. 2012; Jorge et al. 2020; Wang et al. 2017). The fibrotic part of the TME is commonly known as the tumor stroma, which consists of cancer-associated fibroblasts (CAFs) and ECM (Whiteside 2008). In the mid-1980s, tumors were described as “wounds that do heal” by Dvorak, and evidence was presented that the progression of the tumor and its stroma are induced by the activation of the host’s wound-healing response. However, this

wound-healing response in tumors is continuously activated and seems to have much in common with the onset and progression of fibrosis that is discussed earlier in this chapter (Dvorak 1986). In addition, chronic fibrosis, such as IPF, increases the risk of developing lung cancer (Qunn et al. 2002). Therefore, it seems that there is a direct link between fibrosis and malignant tumors.

Progression of certain malignant tumors is characterized by chronic inflammation and an increased activation of CAFs (Piersma et al. 2020). Like fibroblasts, the population of CAFs is heterogenous within and among tumors (Mhaidly and Mechta-Grigoriou 2020). Subtypes of CAFs include myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs) (Boyd et al. 2021). Among all stromal cells within the TME, CAFs can make up 70–90% of the total cells and play a key role in tumor progression and invasion. Their prominent role within the TME is the deposition of ECM molecules, a process which is also referred to as desmoplasia (Chandler et al. 2019). By increasing the amount of ECM molecules due to the fibrotic response in tumors, the density of the tumor stroma increases as well. Cancers of the breast, lung, pancreas, and colon, which all have high fatality rates, are known to have dense stromal networks (Conklin and Keely 2012; Hosein et al. 2020; van Pelt et al. 2018; Xi et al. 2017), and the relation between poor survival and high stromal densities has been reported in many studies (Calon et al. 2015; Liu et al. 2016; Piersma et al. 2020; Wu et al. 2016).

As it becomes clear in the previous sections, signaling between various cells and the ECM is mainly mediated by integrins. Integrins within the TME, expressed by many types of cells, can have different functions in terms of tumor progression and survival which depends on the type of cancer (Chandler et al. 2019). In tumors with (dense) stromal networks, remodeling of the ECM is facilitated by CAFs. Since this chapter mainly focuses on fibrosis, the role of integrins in CAFs will be mainly described here. We will start by discussing the most researched integrin subunit in tumor fibrosis, namely integrin subunit α11.

2.5.1 Role of Integrin Subunit α11 in Tumor Fibrosis

In the last decade, the research has been focusing on the role of integrin subunit α11 (Helms et al. 2020; Iwai et al. 2021; Navab et al. 2016; Primac et al. 2019; Schnittert et al. 2019; Smeland et al. 2020; Zeltz et al. 2019; Zhu et al. 2007). One of the first published articles on integrin subunit α11 is the work from Zhu et al. which investigated the role of integrin subunit α11 which was upregulated in human non-small cell lung carcinoma (NSCLC). In this study, embryonic fibroblasts from wild-type and integrin subunit α11-deficient mice were implanted alone or co-implanted with lung adenocarcinoma cells, squamous cell carcinoma cells, or large-cell lung carcinoma cells in immunodeficient mice. Tumor growth was reduced in which fibroblasts were derived from integrin subunit α11-deficient mice, indicating that integrin subunit α11 has an important role in the growth of lung cancers (Zhu et al. 2007). In addition, work by Navab and colleagues

demonstrated that integrin α11β1 has a role in the regulation of stromal stiffness and the promotion of tumorigenicity and metastasis in NSCLC (Navab et al. 2016). More recently, an integrin subunit α11-positive subtype of CAFs in NSCLC was shown by Iwai and colleagues (Iwai et al. 2021). In pancreatic ductal adenocarcinoma (PDAC), the most severe type of pancreatic cancer, we observed an overexpression of integrin subunit α11 in clinical samples which was colocalized with CAFs. Furthermore, we found that subunit α11 was also upregulated in pancreatic stellate cells (PSCs) upon activation with TGF-β1 and knocking down of subunit α11 using shRNA inhibited PSC-induced tumor cells migration (Schnittert et al. 2019). Furthermore, a recent study revealed that only a minor fraction of myCAFs in PDAC originate from PSCs (Helms et al. 2020), which play a significant role in modulating the tumor microenvironment. This suggests that the overexpression of α11 associated with α-SMA+ CAFs in PDAC samples might be associated with PSC-derived myCAFs. Recently, overexpression of α11 in various types of cancers, including breast cancer, lung cancer, and pancreatic cancer, was reported (Zeltz et al. 2019). Zeltz and colleagues found co-localization of α11 with αSMA, a common marker for CAFs, while the colocalization of α11 with fibroblast-specific protein 1 (FSP1) was poor in most types of cancer (Zeltz et al. 2019). Others have identified integrin subunit α11 as a positive subset of pro-tumoral CAFs in breast cancer and showed that its overexpression on CAFs correlated with aggressiveness of breast cancers in human (Primac et al. 2019; Smeland et al. 2020).

2.5.2 Role of Integrin Subunits α5 and α3 in Tumor Fibrosis

Another type of integrin that is often overexpressed in CAFs, is integrin subunit α5 which is investigated by few (Kuninty et al. 2019; Miyazaki et al. 2019). We demonstrated that integrin subunit α5 overexpression in the tumor stroma of PDAC patient samples was largely associated with αSMA+ CAFs. Importantly, the overexpression of subunit α5 was well-correlated with the poor survival of patients, suggesting it as a prognostic marker. Furthermore, we showed that knocking down of integrin subunit α5 in PSCs resulted in reduced collagen deposition and slower tumor growth. Our novel antagonist against α5, the so-called AV3, significantly reduced desmoplasia in different PDAC tumor models (Kuninty et al. 2019). Furthermore, a novel mechanism for CAFs-dependent cancer cell invasion was proposed by Miyazaki and coworkers. By using lung adenocarcinoma cells and pancreatic cancer cells in 3D-matrices, they found that cancer cells could adhere to CAF-produced fibronectin through integrin subunit α5, which appeared to help the cancer cells to migrate in the ECM (Miyazaki et al. 2019).

There are only few studies that showed the role of integrin subunit α3 in CAFs. The research conducted by Cavaco and colleagues showed a correlation between CAF differentiation and increased expression of integrin subunit α3. Furthermore, the study revealed that integrin subunit α3 has a crucial role in maintaining CAF phenotype (Cavaco et al. 2018).

2.5.3 Role of Other Integrins in Tumor Fibrosis

Integrins α1β1 and α2β1 are profoundly expressed on fibroblasts (Zeltz and Gullberg 2016). However, research on the expression of integrin α1β1 in CAFs seems to be limited to colon carcinoma (Boudjadi et al. 2013; Rodriguez et al. 2009). In a study conducted by Rodriguez et al., integrin α1β1 expression on CAFs appeared to be important for fibroblast differentiation as well as neovascularization and connective tissue organization (Rodriguez et al. 2009). Boudjadi and colleagues showed higher mRNA levels of integrin subunit α1 in CAFs as compared to their corresponding margin tissues (Boudjadi et al. 2013). Integrin α2β1 is widely expressed in various cells in tumors and has shown beneficial effects as well as negative effects on tumor progression in different types of cancer (Zeltz and Gullberg 2016). Yet, the role of integrin α2β1 in CAFs still remains to be explored. There are suggestions that integrin α2β1 on CAFs could alter the stiffness of the ECM which has previously been observed in tumor models (Levental et al. 2009). Attieh and colleagues showed, by using CAFs from various sources, that integrin αvβ3 plays a part in the α5β1-mediated fibronectin fibrillogenesis and has a pivotal role in tumor invasion. By inhibiting integrin subunit β3, Attieh and coworkers demonstrated abrogation of CAF-mediated cancer cell invasion (Attieh et al. 2017).

Altogether, the expression of integrin subunit α11 seems to be a hallmark of CAFs within the TME of different types of cancers. Studies on the expression of integrin subunits α3 and α5 in CAFs seem to be limited to lung and pancreatic cancer. The role of collagen-binding integrins α1β1 and α2β1, which are profoundly expressed on fibroblasts, is still a field to be explored. Expression of the αvβ3 integrin was shown to have an important function in CAF-mediated cell invasion. However, not much is known about the precise role of integrin αvβ3 in CAFs.

2.6 Conclusion

Integrins are an important class of receptors which are involved in the maintenance of normal physiology as well as in the control and onset of different pathologies. In fibrosis they play a major role in the regulation of the disease progression. Since the fibrotic tissue is rich in ECM, the role of integrins becomes substantial, especially on myofibroblasts and CAFs. While integrins are major receptors for binding to the ECM and thereby regulate cell adhesion, cell migration, and differentiation, they also act together with several growth factor receptors to control their activities. Furthermore, their bidirectional sensing of chemical and mechanical signals has a great importance in controlling the cellular response of cells which eventually becomes essential for controlling the pathophysiological processes in these fibrotic diseases.

Despite the identification of integrins more than two decades ago, there is still a gap in understanding this large family of receptors in terms of their roles in both the

maintenance of normal physiology and in different pathological events. Understanding the role of integrins in tissue fibrosis and tumor stroma is a highly interesting area of research, as it may lead to novel therapeutic targets for developing therapeutics against these diseases. The most studied integrin family in different tissue fibrosis is the αv family which includes αvβ1, αvβ3, and αvβ6 integrins. They have been shown to be upregulated in myofibroblasts and play a key role in the activation of latent TGF-β and are, therefore, considered pro-fibrotic. This knowledge led to the clinical development of different molecules against αv family, namely cilengitide (a cyclic peptide against αvβ3 and αvβ5 by Merck KGaA), etaracizumab (an antibody against αvβ3 by Abergrin) and GLPG0187 (a broad spectrum αv inhibitor, Galapagos). All of them failed at different stages of clinical trials due to either lack of efficacy or off-target adverse effects (Slack et al. 2021). Yet, many inhibitors against the αv family including monoclonal antibodies, small molecules and peptides are in clinical development. Since it is not well understood which specific αv-containing integrin is pivotal in a particular disease, it becomes challenging to design selective inhibitory molecules against one or more αv integrins. Apart from the αv family, other integrin subunits such as α5, α2, and α11 are of high interest in the field of fibrosis. Although some inhibitory molecules against α5 have been developed, none of them reached clinical approval.

To apply integrins as therapeutic targets, many more challenges remain to be addressed. One of them is the proper target validation of integrins in the pathological tissues. Since integrins exist as heterodimers, it is crucial to specifically identify the integrin subunit as a target because an integrin subunit can be a partner for many other integrin subunits, exemplified by αv and β1 subunits. In addition, in vitro cell culture may display artifacts for the overexpression of certain integrins due to unnatural culturing conditions such as stiff surfaces, dependency on coatings and growth factors. As described in this chapter, mechanosensing and growth factors are major factors responsible for the induction of integrin expression levels. Next issue is that many studies use gene silencing techniques to demonstrate the role of a specific integrin and one needs to be careful that knocking down of a subunit may affect the expression levels of other integrins, which may result in biased outcomes. Also, complete knockout animal models to demonstrate the biological significance of integrins in a disease is not optimal as the total knockdown might have adverse effects on other cells and tissues.

Conclusively, integrin receptors are proven to be vital therapeutic targets in many fibrotic and malignant diseases but their therapeutic potential yet remained to be proven in the clinic. Nevertheless, ongoing efforts to understand their significance in various pathologies and to design new specific inhibitors against these receptors warrant successful integrin-targeted therapeutics in near future.

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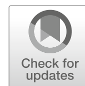
Integrins in Cardiac Form, Function, and Disease



Ana Maria Manso, Andreas Romaine, Geir Christensen, and Robert S. Ross

Abstract Integrins are cell surface receptors expressed ubiquitously including on cardiac cells, which are critical for maintaining homeostasis in the cardiovascular system in health and function as key modulators of cardiac disease. They govern most facets of cardiac myocyte and fibroblast cellular function, act as important bidirectional mechanoreceptors, and interact with and respond to the extracellular matrix and its varied components. In this chapter, we focus on the growing body of knowledge identifying which of the integrin family members are expressed in the heart in health and disease, delineating the expression patterns of integrins in cardiac myocytes and fibroblasts, while highlighting important lessons learnt from cellular and animal models of cardiac disease. We will also highlight aspects of integrin biology obtained from humans, as available.

Integrins lack intrinsic enzymatic activity to transduce mechanical to biochemical signaling, therefore, we will also discuss some of the identified binding and adapter proteins involved in integrin-mediated cell signaling relevant to the heart. We will present the currently understood therapeutic potential of select integrins in specific

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cardiac diseases, such as that following myocardial infarction or development of various cardiomyopathies. Most of this work has been largely unexplored in human cardiac disease. Finally, we highlight some of the pitfalls that have hampered our understanding of integrin and integrin-related protein targeting in heart disease and discuss the reemergence of integrins as attractive therapeutic targets.

1 Introduction

Integrins are multifunctional receptors that are ubiquitously expressed and are best known for their role in adhesion, providing means for cells to interact with their extracellular environment and extracellular matrix (ECM). In this chapter, we intend to provide a current and comprehensive discussion of integrins in the cardiovascular system, focusing mainly on their role in the myocardium as opposed to the vasculature. Specific attention will be to their functions in cardiac myocytes (CMs) and fibroblasts.

In their adhesive role integrins are key organizers that not only cement cells in their specific location—durably or transiently, but also assist in directing tissue architecture, and thus are key for normal organismal development. Yet, in addition to their critical role in formation of ECM-cytoskeletal junctions, integrins are instrumental in many other events in cells and organs including those in the cardiovascular system (Fig. 1). In CMs they can modulate key biochemical signaling events, modify hypertrophic growth responses, protect the CMs from ischemic stress and regulate ion channel function. In addition, integrins convert mechanical perturbations to biochemical ones (mechanotransduction) and alter the remodeling process that occurs with numerous myocardial pathologies, particularly as they function in cardiac fibroblasts.

In this chapter we will provide details on integrin expression patterns in the heart and information on integrin function in CMs and fibroblasts, from work in vitro and in vivo. Much of the in vivo work will detail studies in animal models but will be expanded to data in humans as possible. Some details will be provided on integrin-associated adapters and signaling proteins that are necessary for integrin function and that have received significant study in the myocardium. In this, we will also explore the role of integrins in cardiac disease, and finally, elaborate on future directions ripe for discovery.

Integrin Expression in Cardiomyocytes Mammals express 18α and 8β integrin subunits, which heterodimerize to form 24 receptors. The integrin subunits range in size from 80–180 KDa molecular weight. In the CMs, the integrin heterodimers most highly expressed are α1β1, α5β1, and α7β1, which are predominantly collagen (Col), fibronectin (FN), and laminin (LN) binding receptors, respectively (Babbitt et al. 2002; Brancaccio et al. 1998; Simpson et al. 1994) (Fig. 2). In addition, high transcript levels of α9 and αv have also been detected on human cardiac myocytes (Litvinukova et al. 2020), α3 was detected in rat adult CMs (Terracio et al. 1991), α6

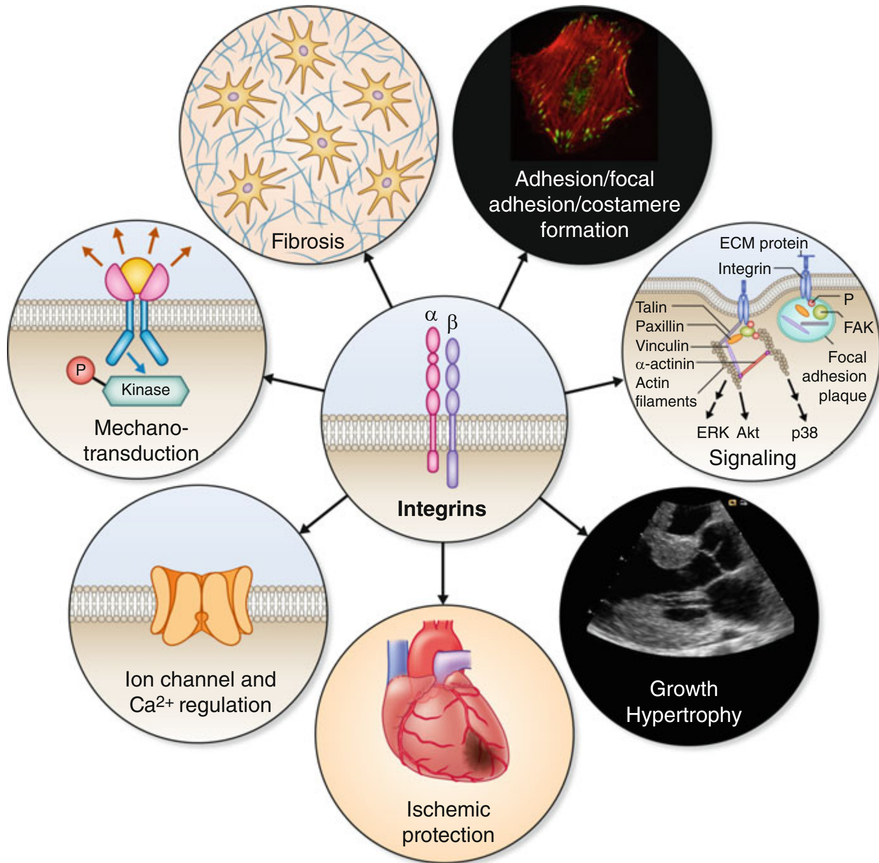

Fig. 1 Integrins have multiple functions in the heart. Heterodimeric integrin receptors (center) are best known for their role in adhesion but as shown here, have many other roles in the myocardium. This ranges from their function in transmitting mechanical signals (mechanotransduction) and other types of signaling events; normal and pathological growth responses including hypertrophy; ion channel regulation and also ischemic protection and an extensive role in the fibrotic process

was identified in neonatal mouse heart (Brancaccio et al. 1998) and also found to have differential expression on mouse atrial vs ventricular adult CMs (Wiencierz et al. 2015). While β1 is the dominant β integrin subunit expressed in CMs, β3 and β5 have also been identified in some studies, but further studies are needed for validation of the expression of these two subunits (Suryakumar et al. 2010; Johnston et al. 2009; Le Gat et al. 2001; Manso et al. 2017).

Though this portion of the chapter centers on CMs, we need to consider that integrin subunit expression can vary temporally, by cell type, and with disease, as occurs in all organs. Thus, there are unique integrin profiles in CMs vs. fibroblasts or

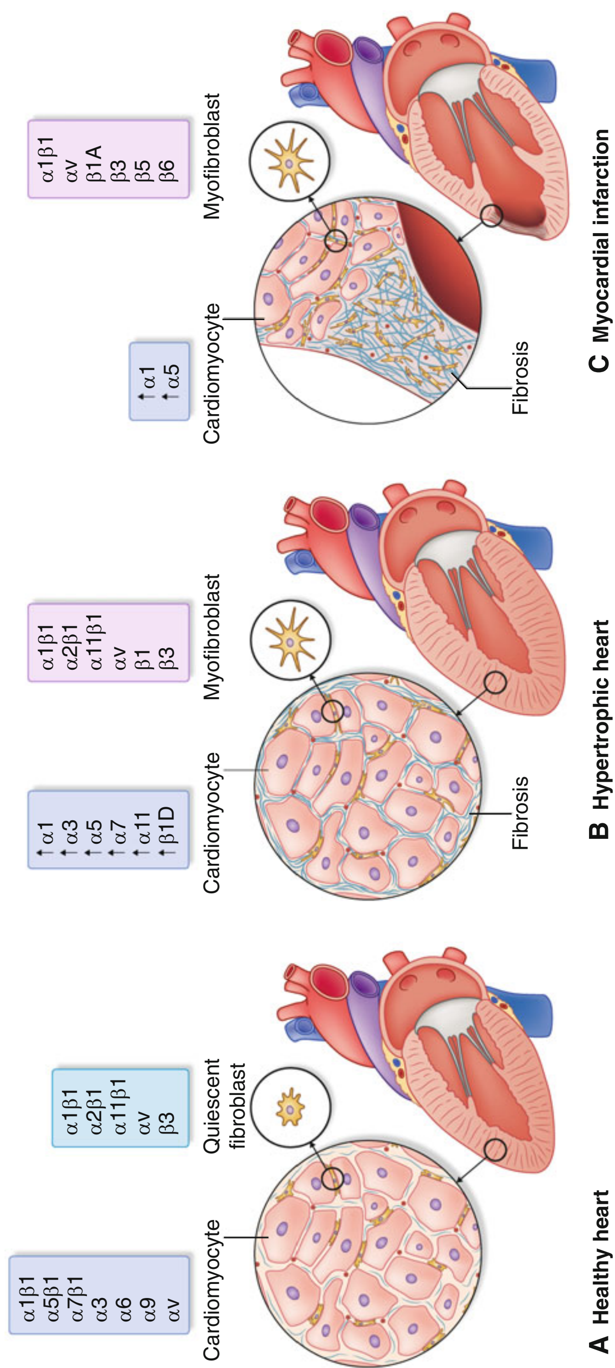

Fig. 2 Expression of integrin subunits in cardiac myocytes and fibroblasts. (**a**) Expression of integrins in the healthy heart. (**b**) Expression of integrins in the hypertrophic heart. (**c**) Expression of integrins in the heart following myocardial infarction

endothelial cells, in fetal vs. neonatal or adult CMs, and also in normal vs. diseased heart. For example, while the α5 subunit is highly expressed in fetal and neonatal CMs, α7 replaces α5 at birth and becomes the main integrin subunit in adult CMs (Brancaccio et al. 1998). Subunit expression can also switch and return to the fetal form when the heart is stressed for diseased. For instance, α5 and α7 subunits significantly increase following ischemic insult or post-myocardial infarction (MI) (Nawata et al. 1999). Hemodynamic overload that generates cardiac hypertrophy can also increase the transcript levels and protein expression of α1, α3, α5, α7, α11, and β1D integrins (Romaine et al. 2018; Babbitt et al. 2002; Terracio et al. 1991).

Integrin Splice Variants Alternative splicing of various subunit transcripts adds even further complexity to the integrin repertoire. For example, β1 integrin has 4 isoforms. Two of these are cytoplasmic domain splice variants β1A and β1D, which are both expressed in myocytes. The A-form is expressed predominantly in the embryo, and the D-form is expressed highly in the adult myocyte (Belkin et al. 1996; van der Flier et al. 1997). Early studies in knock-out (KO) and knock-in β1D mouse models demonstrated that both isoforms are not interchangeable (Baudoin et al. 1998). Subsequently, it was shown that these isoforms have varied affinity for focal adhesion (FA) proteins and the actin cytoskeleton (Pfaff et al. 1998). β1D stabilizes costameres and ECM binding, a property important for the continuously contracting adult CMs (Belkin et al. 1997). Studies demonstrated that β1D may have these properties compared to the A-isoform, due to its relative enhanced affinity for talin (Tln) (Anthis et al. 2010), and its ability to limit paxillin recruitment, leading to stabilized integrin dynamics (Soto-Ribeiro et al. 2019). Further, β1D, but not β1A, binds and stabilizes the ryanodine receptor (RyR2) in CMs transverse (T)-tubules (Okada et al. 2013; Wang et al. 2020).

Like the different subunits, integrin isoform expression can also change with disease. For instance, β1D is downregulated post-MI and POL. With this, CMs may not be able to interact appropriately with the ECM, leading to decreased function of the post-MI heart (Sun et al. 2003; Schips et al. 2019). Further work is clearly necessary to understand how the detailed structural differences between the A and D isoforms lead to their varied function. α7 integrin also has multiple alternatively-spliced variants, with α7B being the dominant one expressed in normal adult CMs (Brancaccio et al. 1998).

Integrin as Signaling Receptors Though integrins do not possess their own enzymatic activity, they are potent bidirectional signaling receptors, converting events outside the cell to intracellular signals and vice versa (Fig. 3). When ECM binds to extracellular integrin domains, intracellular adaptor proteins including integrin-linked kinase (ILK), FA kinase (FAK), paxillin, vinculin (Vcl), talin (Tln), kindlin, and Src are assembled. Signaling proteins are then activated, effecting a process termed "outside-in" signaling. In this manner, integrins can propagate signals to pathways such as Akt, JNK, ERK, p38, or NFκB.

In addition to outside-in signaling, a variety of cytoplasmic events can assemble proteins that bind to integrin tails, leading to integrin activation and increased ECM

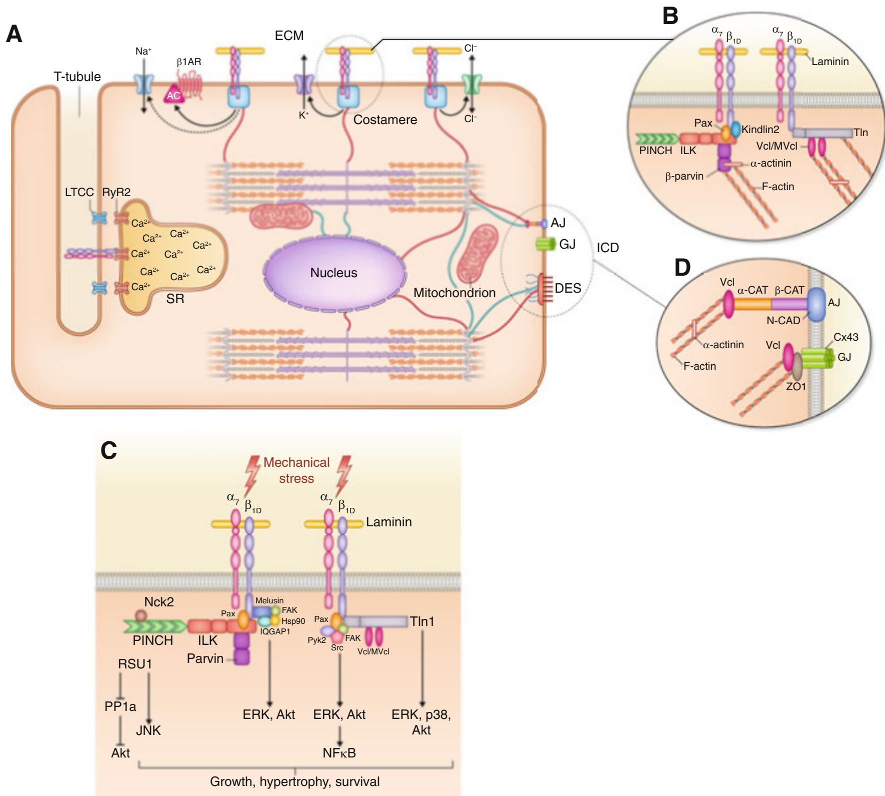

Fig. 3 Integrin location, complex formation, signaling, and mechanotransduction in the myocyte. (**a**) Overview of integrin and related proteins in the cardiac myocyte. Integrins contact the ECM as they traverse the sarcolemmal membrane at the costamere, where they can interdigitate to the sarcomere as they connect to Z-line structures. In this location they can transmit information even to the nucleus. Integrins are also localized at the T-tubules where they interact with the ryanodine receptor, RyR2 at the sarcoplasmic reticulum. They also regulate the trafficking and activity of several ion channels such as Na^{+}, Cl^{-}, and L-Type Calcium Channels, as well as β1 adrenergic receptors. (**b**) Enlarged view of integrins at the costamere. Here, integrin α7β1D, a predominant laminin receptor in the mature CM bridges the ECM to the sarcomere, across the costamere where the integrin forms a complex of structural and signaling proteins such as integrin-linked kinase (ILK), focal adhesion kinase (FAK), paxillin (Pax), vinculin (Vcl), talin (Tln), kindlin, PINCH, parvin, actinin, and even actin. This allows connection of the ECM to the cellular cytoskeleton and perhaps even the sarcomere. It also allows propagation of signals bidirectionally across the cell membrane. (**c**) Illustration of some integrin signaling pathways in the cardiac myocyte. When integrins bind ECM ligands (e.g., laminin as illustrated here), they assemble proteins on their intracellular cytoplasmic domains and ultimately orchestrate signals down a variety of pathways such as those produced through Akt, JNK, ERK, p38, or NFkB. In turn, cellular events such as myocyte growth, hypertrophy, cell survival, or death can occur. Of note is that mechanical events occurring outside the cardiac myocyte can stimulate the ECM–integrin interaction and also lead to intracellular biochemical changes, a process termed "mechanotransduction." See text for details. (**d**) Enlargement of integrin interacting proteins found at the intercalaged disc (ICD). Vinculin interacts with α-CAT and ZO-1 in adherens junctions (AJ) and gap junctions (GJ), respectively, promoting the stabilization of these structures in cardiomyocytes. See text for details. (ILK = integrin-linked kinase; Mito = mitochondria; Pax = paxillin; Tln = talin; Vcl/MtVcl = vinculin and metavinculin, respectively; FAK = focal adhesion kinase; LTCC = L-Type calcium channel; RyR2: Ryanodine

binding, a process termed "inside-out" signaling. Inside-out signaling has not been explored extensively in CMs. Further complexity occurs since integrins may cooperatively signal with other receptors in CMs such as cytokines, growth factors, and even adrenergic receptors. These cooperative interactions are critical for functions within the CMs, but extended discussion about this topic is beyond the focus of this chapter. The reader is referred to other pertinent papers on this subject (Ross 2004; Belkin et al. 1997; Pentassuglia and Sawyer 2013; Maldonado and Hagood 2021).

Lessons Learned About Myocardial Integrins and Associated Proteins from Non-mammalian Models: Fly and Fish As with many proteins, important knowledge about myocyte integrin function comes from work in fly and fish: *D. melanogaster and D. rerio. Drosophila* expresses five α and two β subunits, while zebra fish contain the vertebrate setup of 24 integrins (Mundell 2013). In addition to the 24 integrins expressed by mammals, zebra fish also express a large set of integrin paralogs derived from a genome duplication event (Mould et al. 2006; Bergen et al. 2022). Several of these duplicated genes have mutated into pseudogenes, although some exhibit important functions including integrin α3b which is involved in CMs proliferation and contractility (Yu and Hwang 2022). Genetic studies in these simpler organisms have proven useful since fewer compensatory responses of alternative integrin subunits occur following deletion of any one integrin subunit as compared to mammalian models. We also touch upon work in the heart studying key integrin adapter proteins here since they are crucial for integrin function.

Fly heart is of course quite varied from that in man, being a tube in an open circulatory system. Yet, work in *Drosophila* has revealed the importance of integrins and most importantly, integrin-associated proteins in early cardiogenesis and aging. For instance, reduced expression of the sole *Drosophila* talin at any time during the fly lifespan led to heart tube dysfunction that could not be restored by its re-expression (Bogatan et al. 2015). Loss of Tln disrupted heart cell migratory dynamics, morphogenesis, and polarity (Vanderploeg and Jacobs 2015). Likewise, if expression of both *Drosophila* kindlin 2 orthologs were reduced in fly CMs, a severe cardiomyopathy resulted, due to failure of the CMs to develop as a functional syncytium (Catterson et al. 2013).

Flies have been particularly useful for aging studies given their short lifespan. These studies also focused mostly again on integrin-linked proteins. For instance, moderate global reduction of ILK in the fly increased lifespan, while CM-specific reduction of ILK as well as several other integrin pathway proteins (e.g., parvin (pat-6), Tln, paxillin, and pinch) prevented aging-related decline in cardiac performance (Nishimura et al. 2014). In contrast, CM-restricted increases in Vcl

Fig. 3 (continued) Receptor 2; SR = sarcoplasmic reticulum; Mito = Mitochondria; AJ = Adherens junction; DES=Desmosome; GJ = Gap Junction; α-CAT = alpha-catenin; β-CAT = beta-catenin; N-CAD = α-cadherin; Cx43 = connexin-43; ZO-1: Zonula occludens-1; AC = adenylyl cyclase

expression enhanced organization of CMs myofilaments, augmented contractility and also produced longer lifespan (Kaushik et al. 2015).

Studies on integrins and related proteins in *D. Rerio* are comparatively new compared to those in fly. Zebra fish have been useful for studying heart development. α5 integrin was found essential to preserve left-right symmetry, in agreement with work in mice. Morpholino knockdown of kindlin-2 that is important in integrin activation resulted in hypoplastic, dysmorphic hearts that had contractile abnormalities (Dowling et al. 2008). Cardiac valve formation was promoted by α5β1 signaling and Tln1 (Gunawan et al. 2019), while combined loss of α4 and α5 caused formation of two heart tubes, cardia bifida (Schumacher et al. 2020), linking cooperative function of these integrins to differentiation and proper migration of both endocardial and myocardial cells in cardiac development.

Zebra fish was also used as a model to explore the role of integrins in postnatal function. The ILK-Pinch-Parvin (IPP) complex was shown essential for mechanosensing in the heart and that ILK mutations or inactivation caused cardiomyopathy (Bendig et al. 2006; Brodehl et al. 2019; Hannigan et al. 2007; Meder et al. 2011). Gene reduction of paxillin and FAK also caused abnormal cardiac function and did so by destabilizing vinculin expression in the cardiac myocyte (Hirth et al. 2016).

Lessons from Rodent Models Genetic manipulation of integrins in the mouse has proven to be quite informative in deciphering their role in cardiovascular diseases. First, we will highlight models where integrins were directly interrogated, and then as already discussed some in the fly and fish studies above, provide information on mouse integrin adapter proteins which also were essential in moving the field forward. Some of this data will be summarized in Tables 1, 2, and 3.

Integrin Models in Development Several mouse models have been created to assess the role of integrins in cardiac development. β1 integrin has been extensively evaluated with multiple models (Table 1). Since it is ubiquitously expressed throughout the organism, with its global deletion, early embryonic lethality was seen (Stephens et al. 1995). To study it specifically in CMs required use of a floxed allele. When the β1 subunit was excised from CMs early in cardiogenesis using Nkx2.5-Cre, ventricular compaction was perturbed, and CMs proliferation was reduced (Ieda et al. 2009). While the mice showed generally normal heart structure, the β1 mutant hearts showed smaller ventricles and enlarged atria, suggesting cardiac dysfunction. Nkx2.5-Cre was chosen to delete the floxed β1 integrin gene since it expresses Cre intensely in the developing ventricle and outflow tract by early time points when cells are first directed toward the cardiac lineage (McFadden et al. 2005). Heart development continued for several days in these mice after excision of the floxed β1 integrin gene likely because the β1 integrin protein has a long half-life. Indeed, another study using $\beta1^{flox/-}$ mice, crossed with CM-specific troponin-T (TnT)-Cre shows that β1cKO embryos began to die by E13.5. Discontinuity of the compact layer of the myocardium was evident in these mice, with resultant hemorrhage through the myocardial wall into the pericardium, leading to lethality by E14.5 (Zhang et al. 2019). This study further demonstrates the essential role of β1 during

Table 1 Integrin myocardial mouse models

Integrin	Type	Cardiac phenotype	Ref.
β1	β1 cardiac myocyte-specific (β1$^{flox/flox}$ Nkx2-Cre) knock-out	Perinatal lethality Decreased cardiomyocyte proliferation Ventricular compaction defects	Ieda, M., et al., Dev Cell, 2009. 16(2): p. 233-44
	β1 cardiac myocyte-specific (β1$^{flox/-}$ cTnT-Cre) knock-out	Embryonic lethality at E13.5 Discontinuity of the compact layer Reduced expression of fibronectin, laminin, and perlecan	Zhang, Z., et al., Circulation, 2019. 139(12): p. 1554-1556
	β1 cardiac myocyte-specific (β1$^{flox/flox}$ MLC2V-Cre) knock-out	Progressive myocardial fibrosis and dilated cardiomyopathy	Babbitt, C.J., et al., Histochem Cell Biol, 2002. 118(6): p. 431-9
	β1 cardiac myocyte-specific (β1$^{flox/flox}$ αMHC-MerCreMer) knock-out	Increased damage under ischemia-reperfusion (I/R). Intolerant to POL.	Okada, H., et al., J Clin Invest, 2013. 123(10): p. 4294-308 Li, R., et al., Am J Pathol. 2012 Mar;180(3):952-962
β1D	β1D cardiac myocyte-specific (β1D$^{flox/flox}$ αMHC-MerCreMer) knock-out	Ventricular arrhythmias before cardiac dysfunction Cardiac dysfunction and fibrosis	Wang, Y., et al., Circulation, 2020. 141(18): p. 1477-1493
	β1D global knock-in (No β1A expression)	Embryonic lethality by E16.5	Baudoin., et al., Genes Dev 1998 Apr 15;12 (8):1202-16
	β1D global knock-out (β1D$^{-/-}$)	Mild cardiac functional abnormalities	Baudoin., et al., Genes Dev 1998 Apr 15;12 (8):1202-16
α7	α7 global knockout (a7$^{-/-}$)	No cardiac changes reported	Mayer. U., et al., Nat Genet. 1997 Nov;17 (3):318-23
	α7 β1 cardiac transgenic OE (αMHC -Cre)	Cardiac protection under I/R	Okada, H., et al., J Clin Invest, 2013. 123(10): p. 4294-308
α5	α5 global knockout (a5$^{-/-}$)	Embryonic lethality. Abnormalities in the cardiac left-right symmetry. Embryonic lethality by E10.5. Abnormalities in outflow tract formation	Pulina, M.V., et al., Dev Biol, 2011. 354(2): p. 208-20 Mittal, A., et al., Dev Biol, 2013. 381(1): p. 73-82
	α5 cardiac transgenic OE (αMHC-Cre)	No detectable adverse cardiac effects	Valencik and McDonald. Am J Physiol Heart Circ Physiol. 2001 Jan;280(1): H361-7.
	α5 gain-of-function truncation cardiac	Perinatal lethality Electrocardiographic abnormalities. Cardiac fibrosis	Valencik and McDonald. Am J Physiol Heart Circ

(continued)

Table 1 (continued)

Integrin	Type	Cardiac phenotype	Ref.
	transgenic OE (αMHC-Cre)		Physiol. 2001 Jan;280(1): H361-7
	α5 β1 cardiac transgenic OE (αMHC-Cre)	No cardiac differences vs WT under I/R	Okada, H., et al., J Clin Invest, 2013. 123(10): p. 4294-308
β3	β3 global knockout ($\beta3^{-/-}$)	Blunted hypertrophy with increased dilation and decreased cardiac function and survival under POL Decreased fibrosis under POL	Johnston R.K., et al., FASEB J. 2009 Aug;23 (8):2759-71 Ren. J., et al., J Mol Cell Cardiol. 2007 Feb;42 (2):367-77 Balasubramanian. S., et al., PLoS One. 2012;7(9):e45076

cardiogenesis. Global KO of α5 integrin showed that this integrin subunit was not needed for cardiac chamber specification, but when deleted, caused abnormalities in outflow tract formation, likely due to defects in fibronectin-mediated adhesion and fibroblast growth factor (FGF) 8 signaling (Mittal et al. 2013).

Postnatal Integrin Models In addition to the studies of integrin function during development, other work has shown how important integrins are in the postnatal heart. When the β1 integrin gene was excised specifically in ventricular myocytes late in development using a myosin light chain (MLC)-2 ventricular (v) Cre model, β1 was reduced soon after birth, leading to progressive myocardial fibrosis and development of dilated cardiomyopathy (DCM) (Shai et al. 2002). These mice were also intolerant of hemodynamic loading and had increased damage following challenge with ischemia-reperfusion (Okada et al. 2013; Shai et al. 2002). Likewise, induced CM-specific excision of the β1 integrin gene in the adult CMs led to a blunted hypertrophic response and defective hypertrophic-stress signaling in the intact heart (Cheng et al. 2012). Moreover, adrenergic-mediated signaling was abnormal in the isolated β1 integrin-deficient myocyte. These studies proved the critical role of β1 integrin in basal cardiac function during adulthood and in the response to stress conditions, such as hemodynamic loading and ischemia-reperfusion (I/R).

While the above studies on β1 integrin ablated expression of all β1 forms in the CMs, recall that there are two isoforms of β1-A and D, with D being dominantly expressed in striated muscle. A β1D integrin tamoxifen (Tamo)-inducible KO mouse model has recently been generated and exhibited cardiac dysfunction with fibrosis 3 months after Tamo injection (Wang et al. 2020). Deletion of β1D for only 2 weeks, a time point preceding overt structural and functional phenotypes, resulted in ventricular arrhythmias, RyR2 Ser-2030 hyperphosphorylation, SR Ca^{2+} leak, delayed afterdepolarizations, and triggered activities under isoproterenol stress.

Table 2 Myocardial mouse models of integrin-associated proteins (Part a)

Integrin related protein	Type	Cardiac phenotype	Ref.
Tln1	Tln1 cardiac myocyte-specific (Tn1$^{flox/flox}$ αMHC-Cre) knock-out	Blunted hypertrophy and fibrosis under POL	Manso, A.M., et al., J Biol Chem, 2013. 288(6): p. 4252-64
Tln2	Tln2 global knockout (Tln2$^{-/-}$)	No functional or structural cardiac changes Downregulation of β1D expression (Tln1 upregulation)	Manso, A.M., et al., Proc Natl Acad Sci U S A, 2017. 114(30): p. E6250-E6259
Tln1/Tln2	Tln1/Tln2 (Tln2$^{-/-}$/Tln1$^{flox/flox}$ αMHC-Cre) knock-out	Downregulation of β1D expression Loss of membrane integrity Dilated cardiomyopathy and premature death	Manso, A.M., et al., Proc Natl Acad Sci U S A, 2017. 114(30): p. E6250-E6259
	Tln1/Tln2 (Tln2$^{-/-}$/Tln1$^{flox/flox}$ cTnT-Cre) knock-out	Embryonic lethality at E11.5 Discontinuity of the compact layer	Chen, C., et al., Pediatr Cardiol, 2019. 40(7): p. 1401-1409
Vcl	Vcl$^{+/-}$	ICD morphological abnormalities Widened QRS complex. Sudden death and heart failure under POL.	Zemljic-Harpf, A.E., et al., Am J Pathol, 2004. 165(3): p. 1033-44
	Vcl cardiac myocyte-specific (Vcl$^{flox/flox}$ αMHC-Cre) knock-out	Sudden death and DCM Downregulation of β1D and cadherin expression and Cx43 mislocalization	Zemljic-Harpf, A.E., et al., Mol Cell Biol, 2007. 27 (21): p. 7522-37
MtVcl	MtVcl global knockout (MtVcl$^{-/-}$)	No changes in tissue architecture. Normal hypertrophic response to POL	Kanoldt, V., et al., Nat Commun, 2020. 11(1): p. 6403
Kindlin2	Kindlin2 cardiac myocyte-specific (KN2$^{flox/flox}$ αMHC-Cre) knock-out	Embryonic lethality	Zhang, Z., et al., Circ Heart Fail, 2016. 9(8)
	Kindlin2 cardiac myocyte-specific (KN2$^{flox/flox}$ cTnT-Cre) knock-out	Embryonic lethality at 11.5 Discontinuity of the compact layer. Reduced expression of fibronectin, laminin, and perlecan	Zhang, Z., et al., Circulation, 2019. 139(12): p. 1554-1556
	Kindlin2 cardiac myocyte-specific (KN2$^{flox/flox}$ αMHC-Cre) knock-out	Embryonic lethality	Zhang, Z., et al., Circ Heart Fail, 2016. 9(8)

(continued)

Table 2 (continued)

Integrin related protein	Type	Cardiac phenotype	Ref.
	Kindlin2 cardiac myocyte-specific (KN2$^{flox/flox}$ bMHC-Cre) knock-out	Downregulation of β1D expression Dilated cardiomyopathy and premature death	Zhang, Z., et al., Circ Heart Fail, 2016. 9(8)
	Kindlin2 cardiac myocyte-specific (KN2$^{flox/flox}$ αMHC Mer-Cre-Mer) knock-out	Downregulation of β1D expression Dilated cardiomyopathy and premature death	Zhang, Z., et al., Circ Heart Fail, 2016. 9(8)
ILK	ILK muscle specific (ILK$^{flox/flox}$ mcK-Cre) knock-out	Sudden death and DCM Lethal arrhythmogenic cardiomyopathy	White, DE., et al., Genes Dev. 2006 Sep 1;20 (17):2355-60 Quang KL., et al., 2015. Circ Arrhythm Electrophysiol. 2015 Aug;8(4):921-32
	ILK cardiac transgenic OE (αMHC-Cre)	Cardiac hypertrophy	Lu, H., et al. Circulation, 2006. 114(21): p. 2271-9
	ILK ^{S343D} cardiac transgenic OE (αMHC-Cre)	Cardiac hypertrophy	Lu, H., et al. Circulation, 2006. 114(21): p. 2271-9
	ILK ^{R211A} cardiac transgenic OE (αMHC-Cre)	Modulation of cardiac contractility. Cardiac protection following DOX	Traister, T., et al., Nat Commun. 2014 Sep 11;5:4533

These results add new information about the role of integrin β1D deficiency in the pathogenesis of cardiac arrhythmias.

Perturbation of α5 integrin function was produced by inducible expression of a truncated α5 "gain-of-function" transgene in the adult heart (Valencik et al. 2006). Expression of this transgene caused rapid deterioration of ventricular function, rhythm abnormalities with loss of Cx43 containing gap junctions, and increased expression of calreticulin. To test the effects of α7β1, this laminin binding integrin was overexpressed in dystrophic mice and was found to maintain muscle integrity, increase regenerative capacity and hypertrophy, and decreased cardiomyopathy (Liu et al. 2012; Burkin et al. 2005). Interestingly, the protective effect in the myocardium was indirect as the transgene was expressed in skeletal muscle, so the mechanism of this effect requires further exploration. Some of our own recent work found that CM-specific transgenic expression of α7β1D integrin protected the myocardium from ischemia/reperfusion injury, while α5β1D did not (Okada et al. 2013).

Recent studies highlighted the importance of integrin regulation at the CM membrane in a variety of cardiac disease states and examined its role in membrane stability. One study demonstrated that thrombospondin-3, a matricellular glycoprotein, promoted sarcolemmal destabilization during stress induced by pressure overload or MI, by reducing trafficking of integrins to the cell membrane (Schips

Table 3 Myocardial mouse models of integrin-associated proteins (Part b)

Integrin related protein	Type	Cardiac phenotype	Ref.
PINCH1	PINCH1 cardiac myocyte-specific (PINCH1$^{flox/flox}$ MLC2V-Cre) knock-out	No functional or structural cardiac changes	Liang, X., et al., Mol Cell Bio. 2005 Apr;25 (8):3056-62
	PINCH1 cardiac myocyte-specific (PINCH1$^{flox/flox}$ cTnT-Cre) knock-out	Susceptibility to injury after MI	Liang, X., et al., Circulation. 2009 Aug 18;120 (7):568-76
PINCH2	PINCH2 global knockout (PINCH2$^{-/-}$)	Susceptibility to injury after MI	Liang, X., et al., Circulation. 2009 Aug 18;120 (7):568-76
PINCH1/PINCH2	PINCH1/PINCH2 cardiac myocyte-specific (PINCH1$^{flox/flox}$ PINCH2$^{-/-}$ cTnT-Cre) knockout	Progressive signs of heart failure and death at 4 weeks	Liang, X., et al., Circulation. 2009 Aug 18;120 (7):568-76
b-Parvin	b-Parvin global knockout (b-Parvin$^{-/-}$)	No functional or structural cardiac changes (a-Parvin upregulation)	Kruger, M., et al. Cell. 2008 Jul 25;134(2):353-64
Melusin	Melusin global knockout	Prevention of cardiac dilation under POL	Brancaccio, M., et al., Nat Med, 2003. 9(1): p. 68-75
	Melusin cardiac transgenic OE (αMHC)	Mild cardiac hypertrophy Protection against the transition toward cardiac dilation under POL Protection against I/R Protection against MI	De Acetis, M., et al., Circ Res, 2005. 96(10): p. 1087-94 Penna, C., et al., Basic Res Cardiol. 2014 Jul;109(4):418 Unsold, B., et al., Cardiovasc Res, 2014. 101(1): p. 97-107
FAK	FAK cardiac myocyte-specific (FAK$^{flox/flox}$ MLC2a-Cre) knock-out	Embryonic lethality at E14.5 Thinned ventricular walls Reduced myocyte proliferation	Peng, X., et al., Proc Natl Acad Sci U S A, 2008. 105(18): p. 6638-43.
	FAK cardiac myocyte-specific (FAK$^{flox/flox}$ MLC2V-Cre) knock-out	Blunted hypertrophy to POL	Peng, X., et al., J Clin Invest, 2006. 116(1): p. 217-27
	S-FAK cardiac transgenic OE (bMHC)	Protection against I/R	Cheng, Z., et al., Vasc Biol. 2012 Apr;32 (4):924-33
FRNK	FRNK cardiac transgenic OE (FRNK $^{flox/flox}$ Nkx2.5-Cre)	Embryonic lethality at E15.5 Ventricular non-compaction Reduced myocyte proliferation	DiMichele, L.A., et al., Circ Res, 2009. 104(10): p. 1201-8

(continued)

Table 3 (continued)

Integrin related protein	Type	Cardiac phenotype	Ref.
	FRNK cardiac transgenic OE (FRNK $^{flox/flox}$ MLC2V-Cre)	Blunted hypertrophy to POL and Ang II	DiMichele, L.A., et al., Circ Res, 2006. 99(6): p. 636-45
Pyk2	Global knockout (Pyk2$^{-/-}$)	Ventricular tachyarrhythmia under parasympathetic stimulation	Lang, D., et al., Am J Physiol Heart Circ Physiol 301: H975–H983, 2011

et al. 2019). Another paper linked integrins to rhythm disturbances in arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) (Wang et al. 2020). Extending this initial finding to studies using mouse and cells showed that loss of β1D may occur by ERK1/2 activation and then ubiquitination and degradation of integrin β1D. These studies provide novel insights into the role of integrin deficiency in cardiac diseases and may have important implications for the future development of new therapies to treat these conditions.

Integrin-related Adaptor Proteins in the Rodent Heart Since integrins do not possess their own enzymatic or actin-binding activity, adaptor proteins which bind to the cytoplasmic tails of integrin subunits enable bidirectional signaling and mediate integrin activation. As mentioned above, since these adaptors play a crucial role in integrin function, it is important to also discuss the role of some of these proteins in the myocardium (See Tables 2 and 3).

ILK-PINCH-Parvin: The IPP Complex In cardiac muscle ILK, PINCH and parvin co-localize at the costamere forming the IPP complex (Liang et al. 2009). Studies in adrenergically-stimulated neonatal rat ventricular myocytes (NRVMs) demonstrated that the IPP complex functioned in the hypertrophic response in an integrin-dependent manner and that this complex protected the myocytes from apoptosis (Chen et al. 2005). By extension, transgenic overexpression of ILK in mouse CMs was shown to produce cardiac hypertrophy (Lu et al. 2006), while increased delivery of ILK via adenoviral gene therapy in rats provided beneficial remodeling and improved cardiac function after MI or doxorubicin-induced DCM in rats (Ding et al. 2009).

Subsequent studies have shown that mice that lack ILK, parvin, or PINCH function in CMs exhibit fibrosis, show disaggregation of CMs, and ultimately develop a lethal DCM (White et al. 2006; Liang et al. 2009). In all these models, destabilization of the IPP complex was associated with reduced Ser473 phosphorylation of Akt. In recent studies, CM ILK deletion in mice has been also associated with a lethal arrhythmogenic cardiomyopathy due to changes in ion channels and structural remodeling (Quang et al. 2015). ILK was also shown to modulate cardiac contractility through an interaction with the sarco-endoplasmic reticulum Ca^{2+}-

ATPase (SERCA)2A/phospholamban (PLN) module in the sarcoplasmic reticulum (Traister et al. 2014).

The Talin-Vinculin Complex Extensive work has focused on this complex that is crucial for integrin function. Our lab showed that during embryogenesis, ***Tln1 and Tln2***, the two talin isoforms, are both highly expressed in mouse CMs (Manso et al. 2013). In adult mouse CMs, Tln1 protein expression becomes reduced and Tln2 becomes the main Tln form. With cardiac pressure overload (POL), Tln1 protein expression increased in CMs where it specifically becomes localized to costameres, suggesting that it is involved in the adaptive mechanisms triggered in the stressed heart. Indeed, following POL, Tln1 CM-specific KO mice (Tln1cKO) had blunted hypertrophy, decreased fibrosis, preserved cardiac function, and attenuated kinase signaling, compared to littermate controls.

Since Tln2 was found to be the most abundant talin isoform in adult CMs, it was suggested that this isoform may have a key structural role connecting integrins and the sarcomeres at the cardiac costameres. Yet, global deletion of Tln2 in mice caused no abnormalities in heart as Tln1 became upregulated in CMs (Manso et al. 2017). Tln2 loss increased integrin activation, although levels of the muscle-specific β1D-integrin isoform were reduced by 50%. Yet as one might predict, when both CM Tln isoforms were deleted, β1D integrin and other costameric proteins were subsequently depleted from the cell, membrane integrity was compromised; heart failure and premature death then occurred. Therefore, it appears that CM Tln1 can substitute for Tln2 and preserve heart function, but loss of all Tln forms causes heart muscle instability and leads to DCM. CM-specific KO of all Tln forms in early embryogenesis resulted in thinner myocardial walls together with intrapericardial hemorrhage and embryonic lethality by 11.5 (Chen et al. 2019).

Vinculin is highly expressed at both costameres and intercalated disks (ICDs) in CMs, so it is difficult to dissect its unique functions at these two cellular regions. A 68-amino acid insert splice-variant isoform of Vcl, termed metavinculin (MVcl), is also expressed in muscle (Belkin et al. 1988). To assess Vcl function in heart, much work has been performed in mouse models. Homozygous global Vcl KO mice ($Vcl^{-/-}$), null for both Vcl and MVcl, die by E10.5 (Xu et al. 1998). The heterozygous Vcl KO ($Vcl^{+/-}$) mice were found to have ICD abnormalities, widened QRS complexes, and intolerance when challenged with POL. Some $Vcl^{+/-}$ displayed sudden death and others developed heart failure (Zemljic-Harpf et al. 2004). To study CM Vcl more specifically, CM-specific KO Vcl mice (cVclKO) were constructed and found also to show sudden death in a subset of these mice that still had preserved contractile function, with the remainder of the surviving cVclKO going on to develop DCM and die by 6 months of age, with abnormal adherens junctions and reduced expression of β1D integrin (Zemljic-Harpf et al. 2007). An MVcl isoform-specific KO mouse was recently generated, that left the expression of the ubiquitously expressed Vcl form intact (Kanoldt et al. 2020). The MVcl KO had no basal abnormalities and a response to POL similar to control mice. Therefore, loss of MVcl alone does not impair heart muscle development or myocardial function.

Kindlins Kindlin-2 (KN-2) is the only kindlin expressed in CMs and is localized at costameres (Zhang et al. 2016). When KN-2 was ablated during early development in a CM-specific fashion (KN2-cKO), mice began to die at E11.5 with hemorrhage within the myocardial wall likely due to the reduction of multiple ECM components and loss of the basement membrane. β1 integrin activation was reduced in this model, indicating that KN-2 is required for β1 integrin activation in CMs (Zhang et al. 2019). KN2-cKO died earlier than β1 integrin CM-specific KO mice when gene excision was produced using the same TnT-Cre, indicating a possible integrin-independent role of KN-2 during cardiogenesis. When KN-2 was deleted late in gestation, heart failure and premature death occurred with a DCM phenotype (Zhang et al. 2016). Integrin β1D protein expression was significantly downregulated in these adult hearts, indicating the essential role of KN-2 in maintaining β1D at the costameres and cell membrane stability. These data indicate the important role of KN-2 in the heart.

FAK, Pyk2, and Paxillin FAK is a ubiquitously expressed non-receptor tyrosine kinase that plays a major role in integrin-mediated signal transduction (Fukamachi and McCarthy 2005). Its activity is regulated by a truncated, C-terminal domain of FAK, termed FAK-related non-kinase (FRNK) (Nolan et al. 1999). FAK is localized to CM costameres and ICDs. Early studies demonstrated that FAK was rapidly activated by mechanical stretch, hemodynamic loading, and agonists which stimulate hypertrophic responses (Domingos et al. 2002; Babbitt et al. 2002; Torsoni et al. 2003). With in vivo activation FAK relocates from the perinuclear region to the costameres, and in cultured CMs it accumulates in the nucleus where it binds and activates MEF2, then Jun kinase and a hypertrophic response (Cardoso et al. 2016). In doing so, FAK increased the expression of Jun kinase, a potential means to orchestrate the hypertrophic response of CMs. FAK has also been identified to play a role in glucose transport in CMs (Viglino and Montessuit 2017). With loss of FAK, CM glucose transport is reduced, an important consideration given the growing interest in use of FAK inhibitors as anticancer therapeutics (Lv et al. 2021). Work has also implicated FAK in mitochondrial function in CMs (Chang et al. 2021). A recent study with human induced pluripotent stem cell (iPSC)-derived cardiomyocytes demonstrated that FAK modulates viscoelastic properties of cardiac myofibrils, highlighting its role as a regulator of the cardiac mechanical properties (Taneja et al. 2020).

With early developmental CM-specific FAK gene KO or FRNK-mediated reduction of FAK activity, lethality occurred by E14.5–15.5 with thinned ventricular walls and reduced myocyte proliferation (Peng et al. 2008; DiMichele et al. 2009). Yet with perinatal CM FAK deletion or FRNK-mediated inhibition in CMs, mice survived normally but had a blunted hypertrophic response (Peng et al. 2006; DiMichele et al. 2006). FAK was also implicated in I/R injury in that postnatal loss of CM FAK increased infarct size and CM apoptosis after I/R, while enhancing CM FAK activity led to the converse result (Cheng et al. 2012).

Proline-rich tyrosine kinase-2 (Pyk2) is a tyrosine kinase related to FAK that is detected in both sarcoplasm and costameres in CMs, where it co-localizes with

paxillin (Heidkamp et al. 2005). Studies using rat CMs suggest Pyk2 remodels the cytoskeleton and mediates cardioprotective and proapoptotic pathways (Guo et al. 2006; Hirotani et al. 2004; Melendez et al. 2004; Menashi and Loftus 2009). Pyk2 has also been suggested to be involved in both cytosolic and mitochondrial Ca^{2+} handling (Heidkamp et al. 2005; O-Uchi et al. 2014). Finally, inhibition of Pyk2 signaling after MI improved survival and LV function, and also modified LV remodeling responses (Hart et al. 2008).

Melusin Melusin is a muscle-specific protein that binds to the β1 integrin cytoplasmic domain. With POL its expression increased during the compensatory phase and returned to basal levels during the transition to heart failure (De Acetis et al. 2005). To test the function of this integrin-binding protein more specifically, global melusin KO mice were constructed. When the melusin KO were subjected to POL, DCM resulted (Brancaccio et al. 2003), akin to the β1 integrin CM-specific KO mice discussed above. In contrast, when melusin was overexpressed directly in CMs, increased activation of pro-survival signaling and beneficial LV remodeling occurred, with evidence of preserved contractile function following prolonged POL (De Acetis et al. 2005). Melusin overexpression also protected against cardiac ischemia-reperfusion injury (Penna et al. 2014) like α7β1D integrin, as well as MI and post-MI myocardial rupture (Unsold et al. 2014).

2 Myocyte Integrin and Integrin-Complex Protein Function in Large Animal Models and Humans

Work in larger animal models, and a small amount of data in humans, has been performed to further elucidate the function of integrins. A recent single-nucleus transcriptome study in human cardiac cells found that like mice and rats, α7 and β1 are the main integrin subunits in adult human CM populations (Litvinukova et al. 2020). That was also high transcript levels of α9 and αv in CMs, though these findings required further validation.

Canine models of MI, mitral valve regurgitation and ventricular pressure overload, have been used to evaluate the role of myocardial integrin-related signaling. For example, when mitral regurgitation was produced by chordal rupture in a dog model, collagen accumulation and FAK-related signaling were reduced despite increased left ventricular (LV) stress kinase signaling (Sabri et al. 2008). It was hypothesized that the loss of ECM synthesis modulated integrin expression in this volume-overload model. In another study, β1D integrin protein expression was increased in a model with ventricular hypertrophy and accompanying diastolic dysfunction produced by prolonged complete atrioventricular (AV) block (Donker et al. 2007).

Investigations have also evaluated integrins in heart tissue from cardiomyopathy patients treated with left ventricular assist devices (LVAD) (Birks et al. 2005; Hall et al. 2007; Dullens et al. 2012), comparing expression in samples obtained at the

time of implantation vs. ones following explantation of the LVAD, or native heart removal at transplantation. Some data showed α5 and β5 integrin expression increased slightly, while α7, β1, and β6 decreased, others noted α1, α6, and α10 transcripts were significantly different between pre- and post-LVAD samples in DCM, and that α5, α6, and β6 varied in ischemic cardiomyopathy (ICM) samples. No differences in these integrins were detectable by immunohistochemistry, likely since this technique is not particularly sensitive and unfortunately, Western blotting was not performed. Another group compared failing with non-failing heart tissue and showed that β1D integrin protein was decreased by 36% in ICM vs. controls, though β3 integrin levels were not changed (Pfister et al. 2007). In contrast, a different group of investigators found β1D protein was significantly increased upregulated in both ICM and hypertrophic cardiomyopathy (HCM) LV vs. healthy controls (Wang et al. 2020). Clearly, this observational work in man requires additional study.

Like for the other organisms, studies on integrin adapter proteins have also been instructive. Human heart samples showed an increase in Tln1 expression and localization to the costameres in DCM compared to non-failing samples, in agreement with the mouse studies (Arimura et al. 2009). Multiple mutations of MVcl and more rarely Vcl have been associated with both human DCM and HCM (Vasile et al. 2006b; Olson et al. 2002; Vasile et al. 2006a). While detection of Vcl variants is relatively rare in cardiomyopathies compared to some other gene mutations, its detection rate is much higher in the pediatric population than when evaluated in adult cardiomyopathy patients (Pugh et al. 2014). Further, in line with work in mice discussed above, Vcl variants have also been linked to sudden nocturnal death syndromes in humans, perhaps due to alterations in sodium currents (Cheng et al. 2017b; Cheng et al. 2017a). Variants of Vcl along with another key CM protein tropomyosin (TPM1) have also been shown to combinatorially link to genetic cardiomyopathy in a large family (Deacon et al. 2019).

Though rare, other integrin-complexed proteins have also been associated with cardiomyopathies. For instance, a melusin mutation was found in a DCM patient, but not in control samples (Ruppert et al. 2013), while mutations in ILK were identified in 1/736 patients with DCM and variants also were detected in ARVC/D patients (Knoll et al. 2007; Brodehl et al. 2019). ILK was found to increase in human cardiac hypertrophy (Lu et al. 2006) and when forcibly overexpressed, improved cardiac function post-MI in swine (Lu et al. 2017).

3 Integrins, Integrin-related Proteins, and Ion Channels

Studies have also connected integrins and their related proteins to ion channel function in the CM. Some initial work by Lester's group identified that the arginine-glycine-aspartate (RGD) ECM adhesion site on inward rectifying K^+ (GIRK) channels allowed binding of these channels to integrins (McPhee et al. 1998). Though these initial studies used Chinese Hamster Ovary (CHO) cells as a model, they were

some of the first to identify that integrin-GIRK binding was necessary for proper localization and function of ion channels. Perhaps more relevant are studies using rat CMs. Mechanical perturbation of ECM engaged integrins, mobilized Kv1.5 potassium channels from intracellular stores, and increased their trafficking and expression on the CM sarcolemma (Boycott et al. 2013). A series of studies also identified that integrins regulate L-type Ca^{+2} channel (LTCC) function in development and disease (Wang et al. 2000; Wu et al. 2001; Hescheler and Fleischmann 2002; Cheng et al. 2004). Laminin binding to β integrins modulated LTCC and β_1-adrenergic receptor function and was also linked to adenylate-cyclase and autonomic regulation of cardiac function, leading to hypotheses that this connection was important in cardiac hypertrophy and failure (Wang et al. 2000; Wang et al. 2009b). Further, FAK modulated Cl^- channels and currents in rat and rabbit CMs, further suggesting that cardiac rhythm might be modulated by integrins and their partner proteins (Browe and Baumgarten 2003; Walsh and Zhang 2005), as was shown in some of the mouse models discussed above. Thus, there is a growing body of work implicating integrins and integrin-linked proteins in ion channel function and cardiac rhythm.

4 Integrins and Mechanotransduction in Cardiac Myocytes

Mechanotransduction is defined as the conversion of mechanical forces into chemical signals. Mechanosensitive proteins in CMs localize at the sarcomeres, at cell–cell contacts, cell–matrix adhesions, and the costameres, many of the sites where integrins are found. Active forces such as pressure or volume loading, or passive forces which influence cell stiffness or cause cell deformation, may modify mechanosensory function (Ward and Iskratsch 2020). Passive stiffness is greatly influenced by the ECM composition that surrounds CMs, thus implicating integrins also in this process. Integrins are the principal transmembrane receptors associated with mechanotransduction, but as we emphasized above, they lack the intrinsic enzymatic activity to transform mechanical inputs into chemical signals. To do so requires the integrins to complex with various intracellular kinases (e.g., FAK) and adapter proteins (e.g., Tln) as we have touched upon already above. We will specifically elaborate on integrins and mechanotransduction here.

Mechanotransductive properties of integrins and related proteins were first explored using NRVMs, as well as in vivo rat POL models. A series of studies showed that mechanical stretch of NRVM acted via a β1 integrin-dependent pathway to rapidly activate FAK and then ERK, Akt, and JNK, followed by the increase of hypertrophic genes (Torsoni et al. 2005; Lal et al. 2007). Likewise, subjecting the rat heart to just a few minutes of POL induced a rapid activation of FAK, followed by stimulation of Src, Grb2, ERK, and Akt (Franchini et al. 2000; Babbitt et al. 2002). Further amplification of these concepts came with mouse models. When β1cKO mice were subjected short-term (minutes) POL, blunted activation of downstream stress kinases occurred (Li et al. 2012), which was associated with a reduced hypertrophic response when the animals were subjected to longer-term POL. Similar

results were observed in Tln1cKO mice (Manso et al. 2013). All these data demonstrate the essential role of integrins, in orchestrating a proper mechanotransductive response in the myocardium.

Mechanotransduction through integrins have been also linked to electrical coupling in CMs. Integrins provide mechanoelectrical coupling between cardiac cells by producing mechanical stress on gap junctions. For example, integrin-dependent mechanical strain was found to increase CX-43 expression in NRVMs (Shanker et al. 2005). Since integrin expression is altered post-MI, some investigators have proposed that integrin-mediated mechanoelectrical feedback might predispose the heart to arrhythmias and even sudden cardiac death (Dabiri et al. 2012). While an intriguing hypothesis, this connection requires further study.

Direct mechanotransductive properties of integrins can be appreciated since mechanical forces applied to integrins can be transmitted deep into the cell via the cytoskeletal network, even reaching the nucleus (Wang et al. 2009a). This connection has been proposed to be one that might quickly alter nuclear shape and impact chromatin organization, DNA replication, gene transcription, and RNA processing (Wang et al. 2009a). These direct effects indeed were hypothesized to occur much more rapidly than typical membrane receptor-mediated chemical changes. CM integrin-LN binding has indeed been shown to modulate the β-adrenergic response, affect cellular excitability, action potential morphology, Ca^{2+} metabolism, force development and resting tension (Wang et al. 2000). This represents a unique physical response in which integrins can alter the sensitivity to a soluble mitogen that affects both electric and mechanical responses.

Having discussed integrins and integrin-related protein function in CMs, we will now consider their function in cardiac fibroblasts.

5 Integrins and Related Proteins in Cardiac Fibroblasts and Fibrosis

An Introduction to Fibrosis, Extracellular Matrix, and Cardiac Fibroblasts Cardiac diseases often present with fibrosis, defined as the excess accumulation of ECM. This can result in stiffening of the heart and potentially cause a decrease in cardiac function, leading to frank heart failure (Segura et al. 2014; Manso et al. 2009; Frangogiannis 2021, 2019). The formation of fibrotic scars may initially be beneficial following conditions such as MI where they might protect from rupture of the myocardial wall (Frangogiannis 2017), yet since the heart has limited self-healing capacity, these scars can ultimately impair cardiac function (Talman and Ruskoaho 2016).

Alterations to the structure and function of the heart occur during the development of cardiac disease. While many of the changes in the size, mass, and geometry of the heart can be readily attributed to alterations in the CMs, important alterations can be also seen to occur in the ECM when the heart is examined microscopically. We shall discuss how alterations in the abundance, structure, and composition of the

ECM occurs in various cardiac diseases, implicating fibroblasts, the main ECM-producing cell type in the heart (Hortells et al. 2019), as central mediators.

Integrins bind ECM fibrils and fibroblasts use these interactions to sense and respond to alterations in their external environment. Beyond their role as cell anchoring links, since integrins are involved in the bidirectional transfer of biochemical signals across the fibroblast cell membrane, as they do in CMs, they mediate fibroblast cell behavior, including the production and turnover of the ECM. We shall therefore highlight the role of integrins in mediating cardiac fibroblast (CF) function and by extension, the ECM, with a focus on understanding the contribution of integrins to cardiac disease pathogenesis.

Cardiac Fibroblasts While it is generally described that CFs deposit and maintain the ECM in physiological conditions, details on how to define the specific characteristics of a CF remain a contentious issue that has yet to be resolved (Furtado et al. 2014; Pinto et al. 2016). Despite attempts to categorize fibroblasts in organs aside from the heart based on the expression of cell surface protein expression, no consensus yet exists on what constitutes a CF. To add further complication to this concept, is recent work in organs other than the heart such as lung, showing that in development and disease, non-fibroblast cells types, including macrophages, smooth muscle cells, and pericytes, also have the capacity to produce collagen (Ben Shoham et al. 2016; Kusuma et al. 2012; Tsukui et al. 2020; Reyfman et al. 2019). Previously it was assumed that this property was an exclusive attribute of fibroblasts.

Defining CFs based on their production of ECM has therefore led to their misclassification in several prior studies, in which endothelial and epithelial cells were shown to have a propensity in vitro for producing ECM (Myers and Tanner 1998; Kusuma et al. 2012). The more stringent classification of the CF has led in recent years to revisit the relative cell composition of the heart. Historically, CFs were thought to represent up to 60% of the cellular composition of the mammalian heart, however now that more sophisticated techniques in cell sorting are available, this figure is thought closer to 13% of the total cells, at least in the mouse heart, although large variations in cellular composition have been observed between species (Pinto et al. 2016; Souders et al. 2009). An important factor in explaining the variations between studies is the method of cell isolation utilized by each study, with some protocols favoring the recovery of non-CM cell fractions at the expense of destroying the fragile CMs and thus introducing the biased recovery of cell types (Zhou and Pu 2016).

When studying CFs, their activation state is also an important consideration. Tissue resident adult CFs can be broadly divided into three activation states: the mature fibroblast, the activated fibroblast, and the myofibroblast. Mature fibroblasts are responsible for producing basal levels of collagens, to maintain a homeostatic turnover in health and are commonly defined by a low expression of α-smooth muscle actin (αSMA). Once activated, mature fibroblasts largely express the marker periostin and undergo rapid proliferation. These activated fibroblasts may then undergo further differentiation into myofibroblasts, which in addition to periostin now express αSMA. Myofibroblasts produce and secrete increased levels of ECM

proteins, thus contributing to wound repair and fibrosis. An additional fibroblast activation state has also recently been described, termed the matrifibrocyte (Fu et al. 2018). These cells, which appear to occur as the scar further matures, are observed to lose their expression of αSMA and their proliferative capacity over time. Lineage tracing studies showed that matrifibrocytes gained a specialized gene profile that supports the maturing scar, include genes more commonly detected in chondrocytes and osteoblasts. Still, the matrifibrocyte clearly displayed a unique gene signature from these other cell types.

Previously, studies of CFs used only a handful of markers, such as periostin, discoidin domain receptor 2 (DDR2), and αSMA. The use of individual cardiac fibroblast markers, including DDR2 which was long thought to be specific for fibroblasts but has since been challenged, has limited effectiveness in specifically identifying all cardiac fibroblast subpopulations and has been reviewed in detail elsewhere (Doppler et al. 2017). However, recent work utilizing gene sequencing and proteomics has unveiled new insight into CF heterogeneity. For instance, single-cell RNA-seq data has identified at least seven distinct clusters of fibroblast subsets from human heart (Litvinukova et al. 2020) with fibroblasts exhibiting the greatest heterogeneity of the cardiac cell populations in one recent mouse study (McLellan et al. 2020).

The Cardiac ECM As in other organs and tissues, the cardiac ECM primarily provides an interface and rigid support structure for tethering the myocytes. However, local systemic insults to the heart can trigger alterations to the ECM that promote disease. The role of fibroblasts in cardiac fibrosis has attracted increasing attention (Kong et al. 2014; Travers et al. 2016).

Broadly, the ECM can be divided into fibrillary and non-fibrillary components which interact within the extracellular space. In mammals, type I collagen is the predominant isoform expressed in the cardiac interstitium, representing up to 90% of the fibrillar ECM (Silva et al. 2020). Type III collagen accounts for up to 10% of the remaining myocardial collagen. The pericellular matrix and basement membrane, which form the direct interactions with cell surface receptors such as integrins, are mainly composed of type IV collagen, fibronectin, laminins, glycosaminoglycans, and proteoglycans. These varied components act as a local reservoir for growth factors and enzymes and can also mediate ECM/cell interactions via integrin outside-in signaling (Hynes 2009; Bourgot et al. 2020).

Cardiac Fibroblast Activation The activation of CFs is regulated by both mechanical and chemical factors that often act in unison, which can be triggered through the cell membrane or intracellularly. The mechanical forces that fibroblasts are exposed to in the heart are dynamic, altered in health, during the cardiac cycle, and also during cardiac disease. Mechanical stimulation can promote CF activation either directly or through paracrine signaling of neighboring cardiac cells such as endothelial cells and myocytes. Identifying the receptors responsive to mechanical stimulation is a possible avenue to cardiac disease therapy.

As discussed earlier in the chapter, the conversion of a mechanical stimulus to a biochemical response is referred to as mechanotransduction. Mechanotransduction

can act directly at the local cell level, but also indirectly through regulation of the ECM. Both CMs and fibroblasts express stretch-sensitive molecular elements that may trigger adverse remodeling of the heart (Tavi et al. 2001; Herum et al. 2017), therefore mechanosensing by cardiac cells is an important pathway that may be modulated for therapeutic gain in the future. As mentioned, the ECM is a rich reservoir of growth factors and cytokines. Traction forces generated between the engagement of cells and the ECM can trigger the release of ECM-sequestered factors. One important driver of myofibroblast differentiation is transforming growth factor β (TGF-β), which is secreted by cells into the ECM and sequestered in a latent complex through association with latency-associated propeptide (LAP) and the subsequent binding to TGF-β binding protein 1 (LTBP-1) in the ECM. Integrins have been identified as part of the mechanosensing machinery of cardiac cells and modulators of TGF-β expression and activation, however whether fibroblastic cells within the heart express specific integrins shall now be explored.

CFs in the healthy heart have been shown to express integrin α1β1, α2β1, α11β1, and β3 integrins (Gullberg et al. 1990; Dullens et al. 2012; Talior-Volodarsky et al. 2012) (Fig. 2). Perhaps the most comprehensive assessment of integrin expression by CFs to date has been from a recent large transcriptomic approach to assessing differentially expressed genes between distinct human CF cell clusters (Litvinukova et al. 2020). Of the seven sub-clusters of fibroblasts identified, only one displayed high collagen I and III gene expression, a profile of TGF-β responsive genes and exhibited high expression of the integrin αv. Several integrins, including the αv integrins, recognize and bind the RGD (Arg-Gly-Asp) motif within LAP. Interaction of LAP with these integrins triggers two distinct mechanisms of TGF-β activation. It has been suggested that integrin αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8 transmit cell traction forces thereby mechanically releasing the latent TGF-β1 complex (Campbell et al. 2020; Annes et al. 2004; Sarrazy et al. 2014). In contrast, integrin αvβ8 can also activate TGF-β1 in a proteolysis-dependent manner by binding simultaneously to matrix metalloproteinases (MMPs) and the latent TGF-β1 complex, promoting the enzymatic release of TGF-β1 (Mu et al. 2002).

Mechanical strain and ECM stiffness can also have direct effects on CFs mediated through outside-in integrin signaling, promoting fibroblast activation and differentiation (van Putten et al. 2016). Mechanical cues can directly promote myofibroblast activation, a phenotype that can be reversed by reducing substrate stiffness (Schroer and Merryman 2015; Huang et al. 2012). Integrin mechanosensing may also be modulated by other ECM factors, including hyaluronan. Hyaluronan is a non-sulfate glycosaminoglycan produced endogenously in the heart and can interact directly with cell membrane receptors or indirectly alter integrin mechanosensing through interaction with integrin ligands (Chopra et al. 2014). Activation of CFs by mechanical cues is mediated by the FAK signaling pathway and activation of the mTOR complex. FAK depletion has been shown to inhibit the stretch-induced activation of CFs (Dalla Costa et al. 2010). The MAPK signaling cascade is also involved in fibroblast activation, with ERK2 and JNK1 demonstrated to be activated by mechanical stretch in rat CFs (MacKenna et al. 1998). Interestingly, ERK2 activation could only be blocked by targeting integrin α4, α5, and the RGD-binding integrins

Table 4 Integrin mouse models with ECM phenotypes

Integrin	Type	Cardiac phenotype	Ref.
β1	β1 heterozygous ($\beta1^{+/-}$) knock-out	Reduced fibrotic and hypertrophic response following β-adrenergic stimulation	Krishnamurthy et al., Hypertension, 2007. 49(4): p.865-72
β3	β3 global knockout ($\beta3^{-/-}$)	Reduced ECM accumulation in the heart in response to pressure overload. In isolated fibroblasts, β3 confers responsiveness to PDGF stimulation and regulates fibroblast proliferation and migration	Balasubramanian et al., PLoS One, 2012. **7**(9): e45076
α11	α11 transgenic (β-actin promoter)	Spontaneous development of cardiac fibrosis in aged mice	Romaine et al., Acta Physiol, 2018. 222(2): e12932
	α11 global knockout ($a11^{-/-}$)	Following STZ treatment to induce diabetes, α11 knock-out mice show reduced cardiac fibrosis development	Civitarese et al., Am J Physiol Heart Circ Physiol, 2016. 311(1):H96-H106

simultaneously, suggesting both RGD-binding and RGD-independent integrins are involved in the ERK2 response to mechanical stimulation. In contrast, JNK1 activation could not be blocked with the same inhibitors, indicating that other integrins or mechanosensitive receptors are involved in its activation following stretch. It should be noted, however, that signaling involved both upstream and downstream of fibroblast activation has not been elucidated between the now identified subsets of fibroblast that have been demonstrated in mice and men, as mentioned above. It therefore remains an open field of research to determine whether distinct arms of the MAPK signaling cascade, for example, are relevant in the activation of specific fibroblast subpopulations, which would have important ramifications for therapeutic targeting.

Despite efforts to screen the repertoire of integrins expressed by CFs from healthy tissue, the integrin repertoire in disease, however, is poorly categorized. The re-expression of the fetal gene program is a hallmark of several pathological heart conditions (Taegtmeyer et al. 2010), therefore the de novo expression and altered expression of integrins shall now be discussed in the context of heart disease. We also highlight studies using integrin mouse models that report cardiac ECM phenotypes (Table 4).

Valve Disease The normal functioning of valves in the healthy heart is critical to maintaining adequate cardiac output. Valvular heart disease (VHD) can arise from several clinical manifestations including congenital valve disease, calcific aortic valve disease—as commonly occurs with normal aging, rheumatic heart disease, and infective endocarditis. Stiffening of the aortic valve is prevalent in several VHD and shares several pathological features of atherosclerotic lesions formation in the vessels. Namely, damage to the endothelium of the valves promotes the activation and migration of fibroblasts which in turn drives fibrosis. The chronic thickening of the valve that results from this activation is known as aortic valve sclerosis and in concert with immune cell infiltration drives stenosis of the valve.

Human cardiac valvular interstitial cells (VICs), the predominant cells in heart valve leaflets, are distinct from CFs yet bear several functional and physiological properties of fibroblasts, including taking on a myofibroblast state in disease settings (Liu et al. 2007a). Human VICs express the highest levels of α1, α2, α3, α4, α5, and β1 integrins (Latif et al. 2005). Yet, integrin expression by VICs appears to be species dependent, with bovine VICs expressing high levels of integrins α9β1 and αvβ3 (Wiester and Giachelli 2003).

Studies utilizing VICs have revealed that integrins are crucial for mediating their interaction with the ECM and subsequently modulate valve structure and specifically calcification. Heart valves require both flexibility to alter their shape rapidly and yet also need sufficient rigidity to endure the high pressure gradient present across the valve. VICs are highly plastic cells and must transition on demand between quiescent, stem cell-like and myofibroblastic phenotypes, to maintain valve integrity. This plasticity is lost in calcified valves (Bogdanova et al. 2019), suggesting the maintenance of VIC plasticity as a possible therapeutic intervention in valvular disease.

As one example, blocking integrin α5β1, but not integrin α2β1, leads to increased calcification (Gu and Masters 2010). In vitro, TGF-β1 stimulation promotes VICs to differentiate into myofibroblast-like cells (Walker et al. 2004), as defined by increased α-SMA, while VIC plasticity is rescued by TGF-βR inhibition (Wang et al. 2021). Proteomics has revealed significant alterations in the ECM composition of activated VICs and stenotic valve tissue (Bertacco et al. 2010; Li et al. 2015; Lim et al. 2020), however integrin expression was unchanged, suggesting altered integrin activation state rather than increased integrin expression may promote VHD development.

Periostin, a matricellular protein and integrin ligand, is highly expressed in the developing heart and can directly bind collagen type I, suggesting it may regulate the structural integrity of the heart valves and myocardium (Norris et al. 2007). Mutant mice with targeted deletion of the periostin gene develop calcific aortic valve disease through modulation of Notch1 signaling (Tkatchenko et al. 2009). Through interaction with β1 or β3-integrin, periostin has been demonstrated to be involved in the differentiation of endocardial cushion cells, so crucial in valve formation and also in the induction of collagen type I (Markwald et al. 2019). As periostin signaling can promote fibroblastic differentiation in the developing valves, it is also possible it plays similar roles in the adult heart and the development of fibrotic and calcified valves.

Pressure Overload, Cardiac Remodeling, and Fibrosis As discussed extensively above, POL promotes remodeling of the heart, which manifests as hypertrophy, driven largely by altered myocyte activation as described earlier in our discussion of CMs. POL can also produce fibrosis in the myocardium. The activation of CFs to myofibroblasts leads to excessive ECM production and cardiac fibrosis, which in turn increases the stiffness of the heart and contributes to diastolic dysfunction. Diastolic dysfunction is consistently associated with adverse outcomes in patients suffering from heart failure with preserved ejection fraction (HFpEF), a condition with increasing prevalence and poor prognosis (Pfeffer et al. 2019).

Physiological, as compared to pathological cardiac remodeling has revealed differential integrin expression in health and disease. Burgess and colleagues cultured fibroblasts derived from rats conditioned with exercise training or hypertension and identified that integrin β1 expression was elevated while conversely, integrin α2 was reduced, in both conditions. However, interestingly, integrin α5 expression was elevated in exercise trained and reduced in the hypertensive group (Burgess et al. 2002). In contrast, integrin αvβ5 expression was shown to be elevated in cardiac tissue from spontaneous hypertensive rats (SHR) compared to hearts from normotensive controls, with in vitro inhibition of this integrin reducing SHR-derived fibroblast activation (Perrucci et al. 2018).

One study, utilizing a pig model of heart failure produced by chronic rapid atrial pacing, isolated and sub-cultured LV DDR2-positive fibroblasts from failing hearts and showed a decrease in the relative abundance of the β1 integrin monomer compared to control healthy cells (Flack et al. 2006). Whether these results reflect species dependent differences in integrin regulation in cardiac disease states, or experimental differences in regard to the cell isolation, fibroblast selection criteria, and culturing conditions, remains to be elucidated.

Balasubramanian and colleagues investigated the role of β3 integrin in the cardiac remodeling response to POL. They discovered β3 integrin null mice exhibited reduced cardiac ECM accumulation and by isolating fibroblasts determined that β3 confers responsiveness to platelet-derived growth factor (PDGF) stimulation and downstream fibroblast activity including proliferation and migration (Balasubramanian et al. 2012).

We have also demonstrated that the integrin α11 subunit is upregulated in non-myocyte cardiac cells following aortic banding used to produce POL and that its overexpression correlated with development of cardiac fibrosis in aged mice (Romaine et al. 2018).

Myocardial Infarction and Fibrosis MI leads to ischemic cell death and promotes the replacement of damaged tissue with a fibrotic scar. While initially reparative, replacement fibrosis often extends over time beyond the region of cardiac damage, detrimentally affecting healthy myocardial tissue and thereby reducing cardiac function. Animal models of MI, particularly ones utilizing rodents, have resulted in a wealth of knowledge concerning the pathophysiology and mechanisms of ischemic heart disease, however there are important differences between the models that should be considered. We present the current evidence of integrin involvement in the continued activation of fibroblasts and myofibroblasts within fibrotic scars.

As reviewed by Lindsey et al., an important variable between MI models is whether the heart is reperfused or not (Lindsey et al. 2021). Clinically, the importance of re-establishing blood flow has led to rapid reperfusion strategies for patients suffering ST-elevated MI (STEMI), however it is acknowledged that reperfusion can lead to the paradoxical worsening of cellular dysfunction and increase infarct size, due to the so-called ischemia-reperfusion (I/R) injury. Reperfused MI models may therefore more closely recapitulate the clinical situation, while non-reperfused MI models, that often transition rapidly to heart failure, may therefore represent the

sub-population of MI patients who are not reperfused. This type of pathology also can occur as a result of coronary artery bypass surgery, when the heart is placed on "bypass" and then later has restoration of normal coronary flow.

Shah and colleagues isolated CFs from murine hearts subjected to sham and non-reperfused MI, and employed mass spectrometry (MS) to analyze regional differences in protein expression (Shah et al. 2021). They discovered integrins α1, αv, β1, β3, β5, and β6 were upregulated and that these integrin changes coincided with increased collagen I and collagen III expression from fibroblasts isolated from MI tissue, compared to sham. In contrast, an earlier study by Nawata et al. observed integrin α1 and α3 expression by "fibroblast-like" cells in infarcted regions of post-MI rat hearts, however these cells may have been non-fibroblasts (Nawata et al. 1999). Indeed, histological staining by the authors revealed a similar pattern of integrin expression in coronary vessels located in infarcted areas, likely derived from vascular endothelial cells. A time-course study by Sun et al. used in situ hybridization to identify integrin β1 mRNA was increased in the peri-infarct area of non-reperfused MI on day 3 post-MI, peaking at day 7, before declining between days 14–28 (Sun et al. 2003). Staining for the integrin β1A isoform revealed enrichment in inflammatory and fibroblast-like cells, while integrin β1D positive cells were primarily identified as myocytes. Interestingly, β1A staining was also observed by CMs from post-MI hearts and absent in sham-operated hearts, consistent with the activation of a fetal gene program in CMs following MI.

Angiotensin II (Ang II) is a major mediator of the both the cardiac hypertrophic and fibrotic remodeling that occurs in several cardiac diseases (Gavras and Gavras 2002; Schnee and Hsueh 2000). Ang II induces the upregulation of αv, β1, β3, and β5 integrins in rat CFs, with blocking of integrin αvβ3 reducing Ang II-enhanced fibroblast attachment and motility (Graf et al. 2000; Kawano et al. 2000). Ang II-treated rats upregulate the expression of integrin α8 around fibrotic cardiac vessels, showing prominent staining on vascular smooth muscle cells and in αSMA positive cells that may represent activated fibroblasts (Bouzeghrane et al. 2004). This coincides with a recent publication demonstrating Ang II-mediated activation of a pro-fibrotic CF phenotype required integrin β1 expression (Titus et al. 2021). The authors demonstrated that Ang II promotes FN production by CFs, which in turn activates integrin β1, promoting further collagen production. Interestingly, FN is the endogenous ligand for integrin α8β1, although the role of this heterodimer was not directly explored.

Osteopontin is a glycoprotein that can act as an integrin ligand and is found upregulated in several cardiac diseases, including post-MI (Singh et al. 2010). Ang II-induced cardiac fibrosis is impaired in osteopontin-deficient mice, and osteopontin appears to regulate CF growth and attachment, likely through integrin involvement (Collins et al. 2004). Collagen gel contraction by rat CFs induced by osteopontin is blocked by inhibition of β3 integrin, suggesting osteopontin regulates integrin-mediated cell contractility (Nunohiro et al. 1999).

Integrin-binding proteins have also been implicated in the pathogenesis of MI-induced cardiac fibrosis. Calcium- and integrin-binding protein 1 (CIB1) was initially categorized as a novel integrin αIIb binding partner, but has since been

shown to interact with integrin α2, α3, α4, α5, α11, αM, αL, and αV (Leisner et al. 2016). CIB1 knockdown in mice attenuates MI-induced cardiac hypertrophy and reduces collagen I and III upregulation (Hu et al. 2021). However, whether an interaction with integrins in required for this response was not explored. Isoproterenol is a synthetic beta-adrenoreceptor agonist that induces myocardial damage. L-isoproterenol infusion in β1 integrin heterozygous knock-out mice results in reduced cardiac hypertrophy and fibrosis compared to WT-isoproterenol treated mice suggesting β1 integrins are important in β-adrenergic receptor-stimulated myocardial remodeling (Krishnamurthy et al. 2007).

TGF-β signaling is induced in the infarct region following MI, with increased activation of Smad2 and Smad3 observed in interstitial cells (Dobaczewski et al. 2010). Fibroblast specific loss of Smad3 promotes increased myofibroblast proliferation and larger scars, with Smad3-deficient fibroblasts having baseline decreased expression of integrins α2, α5, and β3 (Kong et al. 2018). Interestingly, Smad3 activation appeared to promote myocyte death, although whether this may also be attributed to a differential expression of integrins was not studied.

Secreted protein, acidic, and rich in cysteine (SPARC) is a non-structural component of the ECM, which appears to have its expression and activity regulated by integrins (De et al. 2003; Sturm et al. 2002). SPARC is transiently upregulated in the infarcted region of the heart, two days to one month following MI induction in mice, which is ablated with in vivo inhibition of integrin αv (Wu et al. 2006). The areas of the myocardium that stained locally for SPARC were actin negative and contained high densities of nuclei, suggesting that the SPARC producing cells were non-myocyte, infiltrative, and highly proliferative. Mouse embryonic fibroblasts migrated more strongly toward FN-coated chambers in the presence of SPARC compared to just FN or SPARC alone, suggesting SPARC may mediate CF migration. Further, targeting a pro-fibrotic PDGFRβ+ cell population in cardiac tissue with integrin αv depletion protects against Ang II-induced cardiac fibrosis (Murray et al. 2017). While integrin αv regulates SPARC expression, it also directly modulates latent TGF-β1 activation through binding of the RGD motif present in LAP as described earlier. Pharmacological blockage of integrin αv reduces MI-induced cardiac fibrotic remodeling in mice (Bouvet et al. 2020). Integrin αv was found to be expressed by the majority of a stromal cell population expressed in the heart that is pan-stem cell marker Pw1/Peg3 positive ($PW1^+$). In vitro blockade of integrin αv in the presence of latent TGF-β reduced the ability of $PW1^+$ cells to activate TGF-β, indicating targeting integrin αv may reduce fibroblast activation.

Cardiomyopathy As introduced above, cardiomyopathies consist of a broad set of diseases whereby the cardiac muscle exhibits mechanical and/or electrical conduction disorders. In addition to DCM, HCM, and ARVC/D introduced above, restrictive (RCM) is another type of cardiomyopathy. Due to the high prevalence of cardiomyopathies present in patients with diabetes, the term diabetic cardiomyopathy was coined. However, diabetic cardiomyopathy has elements that overlap with the other defined cardiomyopathies, for example diabetic cardiomyopathy may be a non-ischemic form of DCM and is associated with a higher cardiovascular risk

profile and mortality in HCM patients, compared to non-diabetic patients (Wasserstrum et al. 2019; Zaveri et al. 2020). The precise definition of diabetic cardiomyopathy remains unsettled.

Alterations in the composition of the cardiac ECM in DCM were reviewed by Louzao-Martinez and colleagues (Louzao-Martinez et al. 2016). They identified that several genes associated with integrin regulation and function were upregulated in DCM, including connective tissue growth factor (CTGF) and neuron-derived neurotrophic factor (NDNF). Although the findings suggest increased integrin signaling in DCM, the precise role of integrins in DCM onset and progression remains to be explored.

Streptozotocin (STZ) injection promotes type 2 diabetes in rodent models. Li and colleagues demonstrated that the integrin β1/ERK1/2 pathway is activated, while Akt signaling is reduced, following STZ treatment of mice (Li et al. 2021). Further, the authors observed these signaling alterations could be restored, together with decreased collagen expression, through transfection of Klotho-cDNA. Klotho is single-pass transmembrane protein that regulates members of the fibroblast growth factor (FGF) family and has been implicated in the pathogenesis of diabetes. Soluble Klotho, which is reduced in diabetes, binds to integrin β1 and mediates the cardiac fibrotic response through regulation of ERK1/2. STZ-induced diabetes in rats promotes integrin α11 expression in CFs, with siRNA knockdown of integrin α11 in human fibroblasts reducing TGF-β2 and αSMA expression (Talior-Volodarsky et al. 2012). The same group treated integrin α11 KO mice with STZ to induce diabetes and found no overall improvement in diastolic function however observing a reduction in cardiac fibrosis (Civitarese et al. 2016).

Although arrhythmogenic cardiomyopathies can be readily attributed to defects in CM function, perhaps in part mediated by integrin involvement as elaborated on earlier, there is a significant association between fibrosis and the incidence of more generalized presentation of a wide range of cardiac arrhythmias (Kazbanov et al. 2016). For example, the presence of interstitial fibrosis, as observed following MI and POL, can impair local transverse conduction (Li et al. 1999). Therefore, in addition to the direct effects of integrin activation on myocyte arrhythmogenicity, integrin activation in CFs may promote fibrosis and thereby indirectly propagate cardiac arrhythmias.

It is challenging however to delineate cause and effect, as fibrosis may be consequential to the arrhythmia itself. Targeting fibrotic remodeling and observing the incidence of arrhythmia may seem an initially ideal approach to answer such a question, however clinical trials in humans have had conflicting results. Statins and ACE inhibitors can inhibit cardiac fibrosis, however candesartan (an angiotensin receptor blocker) or pravastatin (a statin) did not significantly reduce atrial fibrillation (AF) relapse following cardioversion to restore normal rhythm (Kazbanov et al. 2016; Tveit et al. 2004). Other studies, however, indicate that the early targeting of fibrosis may be beneficial in reducing the incidence of new-onset arrhythmias (Tveit et al. 2004). This complies with human and animal studies of reverse cardiac remodeling indicating that fibrosis may be irreversible beyond an established point

and thereby the timing of early drug therapy initiation is crucial for positive outcomes (Nagaraju et al. 2019; Ruppert et al. 2018).

Signaling Downstream of Integrin Engagement in Cardiac Fibrosis The signaling events downstream of integrin engagement in response to increased mechanical stress present in the heart following POL or MI has also been explored. In particular, FAK signaling (discussed above) has attracted considerable attention due to its multifaceted involvement in fibrosis present in several organs. Inhibiting FAK in vivo following MI in mice resulted in decreased fibrosis and improved LV function (Zhang et al. 2017). Similarly, siRNA knockdown of FAK in cultured adult rat fibroblasts impaired myofibroblast differentiation, and further, the mTOR complex was shown to be a critical mechanism downstream of FAK activation necessary for the mechanical stretch-induced activation of fibroblasts (Dalla Costa et al. 2010). FAK is also required for TGF-β-mediated induction of JNK phosphorylation in mouse embryonic fibroblasts (Liu et al. 2007b). Stimulation of CFs by TGF-β1 induces the activation of the three major subgroups of MAPKs—JNK, ERK1/2, and p38-MAPK. Inhibiting each of these MAPKs reduces TGF-β1-stimulated lysyl oxidase (LOX), an extracellular enzyme important for crosslinking collagen and is associated with cardiac fibrosis (Voloshenyuk et al. 2011).

FAK has also been identified as an important signaling bridge between integrin-mediated cell attachment and actin polymerization, which plays an important function in fibroblast motility and mechanosensing (Romero et al. 2020). Cultured CFs exhibit FAK activation that coincides with stress fiber organization of αSMA, however αSMA does not appear to be sufficient to induce cell contraction (Shinde et al. 2017). Instead, αSMA may modulate the proliferative activity of CFs, therefore it may regulate fibroblast cell behavior independent of cell contraction.

6 Integrins as Therapeutics in Cardiac Disease

Despite the multiple important roles of integrins predicated in the heart, the translation of this knowledge to clinical therapies continues to be evasive. As outlined in this chapter, integrins play important roles in multiple cell types in the heart that mediate cardiac disease. This, together with the relatively easy accessibility of integrins at the cell surface, makes integrins a potentially attractive therapeutic target. We assess previous attempts to target integrins in the heart and more recent on-going clinical trials, highlighting the hurdles and future promise of integrin-targeted therapies for cardiac disease.

Considerable research efforts have been directed to modulating the immune response observed in cardiac disease, particularly following MI, in part through integrin targeting. Although we direct the reader to other chapters in this book covering immunomodulation by integrins, we highlight some of the main study outcomes of this body of work with cardiac relevance. The HALT-MI study sought to investigate whether targeting the leukocyte integrin αLβ2 (CD11/CD18) using an

antibody (Hu23F2G) would reduce the infarct size of patients having suffered an acute MI who were treated with primary angioplasty (Faxon et al. 2002). Despite the drug being well tolerated, the primary end point of infarct size did not show a significant reduction following antibody treatment. Similarly, the LIMIT AMI study which tested the efficacy of a recombinant humanized monoclonal antibody directed against CD18, a leukocyte integrin subunit (β2), in patients following acute MI, failed to reduce infarct size or improve coronary blood flow (Baran et al. 2001).

To date, the most successful clinical targeting of integrins in cardiovascular disease has been inhibitors of the RGD-binding integrin αIIbβ3 in the treatment of thrombotic cardiovascular events and ACS. While this work may be extensively discussed elsewhere in this volume, we will touch upon this topic briefly here. Today, the intravenously administered integrin αIIbβ3 antagonists abciximab, eptifibatide, and tirofiban are given according to NICE recommendations as "bailout glycoprotein inhibitors" when cardiologists performing primary percutaneous coronary intervention (PCI) observe a significant intracoronary thrombus burden. The use of these inhibitors is associated with increased risk of bleeding and thrombocytopenia, however clinical evidence suggests their benefits outweigh the risk (Germing et al. 2010). Integrin αIIbβ3 antagonists inhibit fibrinogen binding to the plasma membrane of platelets, therefore preventing their aggregation and act as antithrombotic agents (Lippi et al. 2011). Next generation inhibitors have sought to reduce the incidence of thrombocytopenia. RUC-4 is a novel small molecule inhibitor of integrin αIIbβ3, showing promising positive results in improving coronary flow prior to PCI and limited infarct size in STEMI patients (Kereiakes et al. 2020). Allosteric binding inhibitors, generally either blocking the orthosteric site or altering the activation state of the integrin by locking it in an inactive state, are also in development. The disintegrin TMV-7 has been demonstrated in one promising pre-clinical study to prevent occlusive thrombosis in vivo, without altering the activation state of the integrin and thereby acting via a distinct mechanism compared to TMV-2 (Kuo et al. 2017).

Most recently, a higher thrombotic burden in numerous vessels has been shown in patients with COVID-19 infection and has thus increased the need to use integrin αIIbβ3 antagonists, from 9% in patients with non-COVID STEMI to as high as 59% in comparable COVID-19 patients (Koutsoukis et al. 2021; Kelham et al. 2021). Therefore, the use of integrin αIIbβ3 antagonists is likely to remain important in the context of pro-inflammatory infections with high thrombotic burden.

However, the targeting of integrins in cardiac tissue resident cells such as myocytes and fibroblasts has been largely unexplored. As identified in this chapter, TGF-β isoforms are important mediators of both myocyte and fibroblast function and activity, regulated both upstream and downstream of integrin engagement. It should be noted that while targeting TGF-β signaling has potent anti-fibrotic effects both in vivo and in vitro, the concentrations of pharmaceutical agents required to elicit beneficial outcomes are highly toxic and therefore have had limited their application in human disease (Sweeney et al. 2020). As we have reviewed, integrins have specific expression patterns in the healthy and diseased heart, in addition to varied expression in specific cellular subsets in the heart. Therefore targeting integrins in

heart disease may modulate local TGF-β signaling in a more efficacious manner than systemic TGF-β targeting.

Evaluation of integrin and integrin-associated protein expression may also have prognostic value in cardiac disease states. Antihistamine-induced cardiotoxicity studied in rat CMs identified alterations in the integrin signaling pathway that may predict cardiac QT prolongation and cardiac arrhythmia (Yun and Kim 2015). The integrin αvβ3-selective radiotracer, ^{18}F-fluciclatide, has been utilized in positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI) of the heart in patients following recent acute MI, as a clinical marker of healing activity (Jenkins et al. 2017). ^{18}F-fluciclatide uptake associated with regions of tissue healing, where predominantly endothelial cells expressing integrin αvβ3 accumulate during re-endothelialization and angiogenesis. There is limited evidence, however, that shows whether increased integrin αvβ3 expression after MI is favorable, with a recent study citing low sample size as the reason for not demonstrating this important connection. Therefore, whether ^{18}F-fluciclatide has cardiac clinical prognostic value (Makowski et al. 2021) remains unclear.

The recently renewed interest in integrin therapies has been largely driven by improved mechanistic understanding of the role of integrins in fibrotic disease. In several pre-clinical studies reviewed in this chapter, the targeting of select integrins has improved myocyte function and reduced cardiac fibrosis (Okada et al. 2013; Balasubramanian et al. 2012). However, hurdles that are both generic to pharmaceutical drug development and others that perhaps are more integrin-specific exist in the setting of cardiac disease treatment. Firstly, the use of animal models, particularly small rodents, so often used to model cardiac disease, is often not directly translatable to human conditions and this has led to questions both on the efficacy and therapeutic window available for integrin targeting. Even results from larger animal models have proved difficult to directly extrapolate to human disease. Several cardiac conditions, such as hypertension, develop and manifest over decades in humans, while vastly accelerated disease models such as aortic banding in a rodent model, that recapitulates some aspects of these human conditions, are produced in a matter of days to weeks. Secondly, targeting specific integrins specifically in the heart is problematic. Several studies do not delineate the integrin heterodimers that are relevant in cardiac disease, by investigating and targeting integrin beta-subunits that form multiple integrins. Given the importance of integrins in multiple organs, targeting beta-subunits that form multiple heterodimers is likely to elicit high toxicity and off-target effects, as observed with TGF-β inhibition. The ability to specifically target integrins on, for instance, a CM or cardiac fibroblast remains currently evasive. Similarly, targeting single integrins in complex cardiac diseases may not be a promising approach, as multiple integrin heterodimers may be responsible for the same biological end process. Thirdly, the integrin antagonist agents utilized in pre-clinical studies, which predominantly use RDG mimetics in the case of the RGD-binding integrins, are often not suitable for oral route administration as they are charged at physiological pH (Slack et al. 2021). Thus, much work is necessary before we can consider how best to potentially manipulate integrin biology in the heart to provide useful therapeutics.

7 Conclusions and Future Perspectives

Integrins are critical component of the cell surface repertoire of both CMs and CFs, where they govern the homeostatic function of the heart in health and also regulate the development of several cardiac diseases. Although strides have been made to understand the role of specific integrins in cardiac disease through the use of animal models and recent genomic approaches, gaps remain in our understanding that has hampered the translation into therapeutic interventions.

As we have discussed, integrins appear to be an important factor in the development of hypertrophy—both physiological hypertrophy that can be beneficial and pathological hypertrophy that can be detrimental and be a component in the evolution of various types of cardiomyopathies, and even arrhythmias. Yet despite this knowledge, no therapeutic approaches targeting integrins or integrin-related proteins in CMs are currently being extensively explored for primary cardiac disease, other than that being used as anti-platelet agents, as noted above. Clearly, this would require the ability to target both specific cell types, such as CMs or fibroblasts in the heart, and a specific repertoire of integrins. Lack of specificity and potential off-target effects would be of great concern given, for instance, the ubiquitous expression of many integrin receptors. New therapeutic strategies such as the use of gene therapy with adeno-associated viruses that have muscle tropism, combined with CM-specific promoters, might assist with some of these issues, but clearly new work is required to explore this approach.

Still, like the development of many therapeutics that have long lag times, we must put these somewhat pessimistic views aside. New molecular tools in light microscopy and nanoengineering have recently allowed a deeper understanding of the organization and dynamics of integrins and intracellular regulators in living cells. To date, these approaches have not been extensively investigated using CMs or fibroblasts. Use of these and other new types of studies are necessary to better understand how integrins are involved in mechanotransduction in the heart, how integrins propagate numerous signaling events both rapidly and in the longer term, and how they do so in a cell-type restricted manner. Whether modulating mechanotransduction in the heart will be truly beneficial in cardiac homeostasis and disease is currently unknown.

In addition, the understanding now that fibroblasts are a heterogeneous population even in the heart itself, with multiple subsets and various differentiation states, has furthered the cardiac fibrosis field, but too, made it appreciated that it is complex. Conversely, the expression of specific integrins in these cell populations is poorly categorized and if we are to target integrins in disease states that cause cardiac fibrosis, we must first begin with identifying the integrins relevant in the pro-fibrotic fibroblast subpopulations. The targeting of resident cardiac fibroblasts in human heart disease also remains largely unexplored.

With the extensive past investigations, and current evolving interest in integrins in the heart, we would suggest that future work will allow development of therapeutics that might modify integrins in cells within the heart to be useful in our

armamentarium against the wide range of globally important cardiovascular diseases.

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The authors note no pertinent employment, financial, or non-financial interests that directly or indirectly would influence this work. All work performed directly by any of the authors and cited herein was approved by the authors home institutions' appropriate animal and/or human use review committees and has been performed in accordance with the ethical standards as per the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Portions of the text have been modified from prior work of the authors (Israeli-Rosenberg et al. 2014; Chen et al. 2019).

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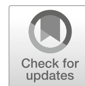
Integrin α8 and Its Ligand Nephronectin in Health and Disease



Carole L. Wilson, Chi F. Hung, and Lynn M. Schnapp

Abstract The α8 integrin is a classic arginine-glycine-aspartic acid (RGD)-binding subunit that partners exclusively with the β1 subunit. α8 is expressed by mesenchymal cells, particularly vascular and visceral smooth muscle cells (SMCs) and highly contractile fibroblasts, that are associated with basal laminae. While α8 recognizes typical RGD-containing ligands such as fibronectin, it shows an exceptional affinity for nephronectin (NPNT), a basement membrane protein that has both an RGD and a synergistic binding site for α8. NPNT was identified as the key signaling ligand for α8 in kidney morphogenesis. Although both α8 and NPNT contribute to the development of other organs, such as hair follicles, lack of phenotypic differences in the global KOs as compared to wild-type are often explainable by compensatory mechanisms. Studies using either cells or mice deficient in α8 indicate that, in general, this integrin subunit is required for the maintenance of SMC differentiation and is protective against the sequelae of injury, especially in the kidney. Although less is known about NPNT in tissue injury, its expression also appears to correlate with repair. Finally, dysregulated expression of NPNT and α8 has been recently described in several cancers, suggesting that these proteins may be novel targets for future therapeutic intervention.

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1 Introduction

Integrin α8 was first identified as a novel α subunit in chicken (Bossy et al. 1991), later followed by cloning of the human and mouse versions (Schnapp et al. 1995a, b; Muller et al. 1997; Denda et al. 1998a, b). It belongs to the arginine-glycine-aspartic acid (RGD) subfamily of integrins and heterodimerizes with only the β1 subunit. As is typical of RGD integrins, α8β1 binds substrates such as fibronectin, tenascin C, and vitronectin, as well as the latency-associated peptide of TGFβ. A new ligand with a particularly robust affinity for integrin α8 emerged in studies of kidney development in the α8-deficient mouse, and this ligand was termed nephronectin (NPNT) (Muller et al. 1997; Linton et al. 2007). In this chapter, we will review the biology of both integrin α8 and NPNT and the latest developments in understanding the myriad roles of these proteins in health and disease.

2 Integrin α8 Structure and Expression

Integrin α8 was first cloned from chick brain and embryo cDNA libraries (Bossy et al. 1991) and subsequently cloned from human and mouse libraries (Schnapp et al. 1995a, b; Muller et al. 1997; Denda et al. 1998a, b). α8 dimerizes exclusively with the β1 subunit (Bossy et al. 1991; Schnapp et al. 1995a, b). Like other α subunits, α8 has a putative signal peptide sequence, seven repeats in the extracellular domain, a hydrophobic region consistent with a transmembrane domain, and a short hydrophilic cytoplasmic domain in the C-terminus. Modeling of the extracellular region shows that it contains the β-propeller domain, a thigh domain, and two calf domains that are typical of α subunits (Fig. 1). Human α8 is most closely related to α5, αv,

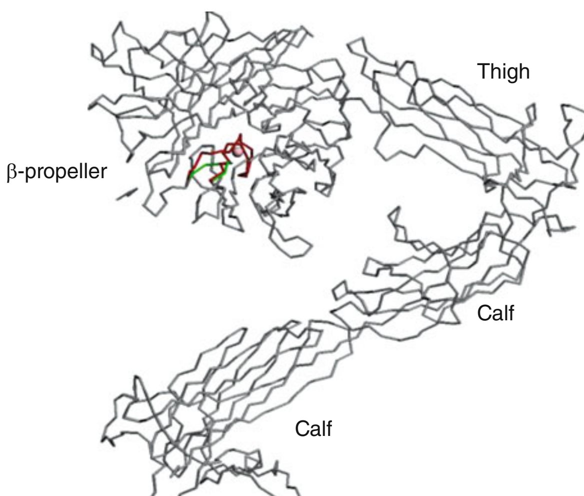

Fig. 1 Predicted structure of the α8 extracellular domain. Modeling of this domain reveals a typical α subunit structure: β-propeller domain, thigh domain, and two calf domains. Divalent cations are shown as spheres in the β-propeller domain

and αIIb (36–43% homology), all of which contain RGD sequences and have similar genomic organization (Ekwa-Ekoka et al. 2004). This subset of α subunits also lacks the I or A domain responsible for ligand binding in other integrins (reviewed in (Hynes 2002)). α8 differs from α5, αv, and αIIb in its β-propeller domain, in which there are conserved clusters of basic amino acid residues in the loops between blades 1 and 2 and within blade 3 (Sato et al. 2009). Of note, α8 has an RGD sequence in the last repeat in the extracellular domain. This motif has been found in all species for which the α8 sequence is known—including human, mouse, rat, chick, cat, cow, bat, horse, and rabbit—with the exception of the duckbill platypus, which has an RGG. Three-dimensional modeling using α5 as a template indicates that the RGD is surface exposed in the β-propeller region (our unpublished data). However, it is not known if this motif has a biological function in α8.

Early studies showed α8 expression is associated with the basal laminae similar to other integrins that interact with ligands in this type of matrix. This finding was the first indication that α8 has ligands in the basement membrane (BM), a theme that will emerge again in later sections. Notably, the majority of cell types that express α8 have a contractile nature: mesangial cells in the kidney, smooth muscle cells of the intestine and vasculature, arrector pili cells of the hair follicle, and (myo)-fibroblasts in the alveolar wall.

3 Major Ligands of Integrin α8

α8 shares several common ligands with other α subunits in the RGD-binding family of integrins. Fibronectin (FN) is an extracellular matrix ligand for α8 through its RGD peptide sequence (Muller et al. 1995; Schnapp et al. 1995a, b). α8 binding to FN increases cell survival through the phosphoinositide 3-kinase (PI3K) pathway (Farias et al. 2005). Vitronectin (VN), tenascin-C (TN-C), and osteopontin (OPN) were also identified as ligands for α8, although demonstration of binding of α8 to these proteins has not been consistent across all studies (Schnapp et al. 1995a, b; Varnum-Finney et al. 1995; Denda et al. 1998a, b).

The latency-associated peptide (LAP) of TGFβ1 and TGFβ3 contains an RGD sequence and is a ligand for α8 (Lu et al. 2002). LAP noncovalently associates with mature TGFβ and prevents TGFβ binding to its receptor. In addition, this complex, known as the small latent complex, interacts with latent TGFβ-binding proteins to form a large latent complex that becomes incorporated into the extracellular matrix (ECM). Conformational alterations to LAP, mediated by αv-containing integrins binding to the RGD-sequence on LAP, result in the activation of TGFβ1 and TGFβ3 (Munger et al. 1998, 1999; Annes et al. 2002). α8 also binds to LAP-TGFβ, similar to other RGD-binding integrins. However, when expressed in non-contractile cells (SW480 and CHO), α8 binding did not activate TGFβ1 or TGFβ3 (Lu et al. 2002; Nishimichi et al. 2021). Instead, α8 binding promoted cell adhesion, spreading, and proliferation on LAP-TGFβ1 through phosphorylation of focal adhesion kinase and extracellular signal-regulated kinase (Lu et al. 2002). In contrast, when α8 was

expressed in highly contractile cells with significant levels of α-SMA stress fibers, such as activated stellate cells or fibroblasts, binding to latent TGFβ in vitro resulted in its activation (Nishimichi et al. 2021). These experiments demonstrate that the latent complex of TGFβ affects cell behavior and that it is a biologically relevant ligand for α8. Increased expression and deposition of TGFβ complexes, as well as other RGD-containing ECM molecules such as FN, are characteristic of fibrotic responses. Taken together, these in vitro studies suggested that the interaction of this integrin with these substrates in vivo may impact the persistence of mesenchymal cells and that α8 could contribute to the development of organ fibrosis.

A novel, high-affinity ligand for α8, called nephronectin (NPNT), was later discovered through the characterization of the α8 knockout mouse. Details on NPNT and its interactions with α8 are discussed in later sections. Other ligands identified through studies using α8-deficient mice are also described.

4 Generation and Initial Characterization of Integrin α8-Deficient Mice

Conventional gene-targeting techniques were used to disrupt the gene for mouse integrin α8 (*Itga8*) by replacement of the exon encoding amino acids 362–424 with a neomycin-resistance cassette (Muller et al. 1997). Mice with one targeted allele (*Itga8*$^{+/-}$) appeared normal. Breeding these heterozygotes produced mice with the genotype *Itga8*$^{-/-}$ (hereafter referred to as knockout (KO)) at about the expected Mendelian frequency. Absence of protein in KO mice was confirmed by Western blotting of extracts from neonatal lungs or immunohistochemical analysis of this tissue (Muller et al. 1997). Although some KOs survived into adulthood, most of the pups died early postnatally.

KO neonates had a spectrum of abnormalities in kidney morphogenesis: about half of the mutants had no ureters or kidneys, while the remaining KOs exhibited only kidney rudiments or had one or two kidneys that were generally smaller than WT (Muller et al. 1997) (Fig. 2). Integrins had long been proposed to play a role in the formation of the mammalian mature kidney from the metanephros (reviewed in (Kanwar et al. 2004)). Development of the kidney involves reciprocal epithelial-mesenchymal interactions: the ureteric bud invades undifferentiated mesenchyme, which undergoes epithelial transformation, followed by mesenchyme-induced branching of the bud and generation of nascent nephrons. In the α8-deficient kidney, growth and branching of the ureteric bud into the mesenchyme is impaired and epithelialization of the mesenchyme is defective (Muller et al. 1997). In developing WT kidneys, α8 localizes to mesenchymal cells that border the bud epithelium, suggesting that the ligand for this integrin resides in or close to the BM (Fig. 3). Of the known ligands of α8 at the time, only OPN showed the correct spatiotemporal pattern of expression in the kidney (Rogers et al. 1997); however, kidney development is normal in mice lacking OPN (Liaw et al. 1998), as well as VN (Zheng et al.

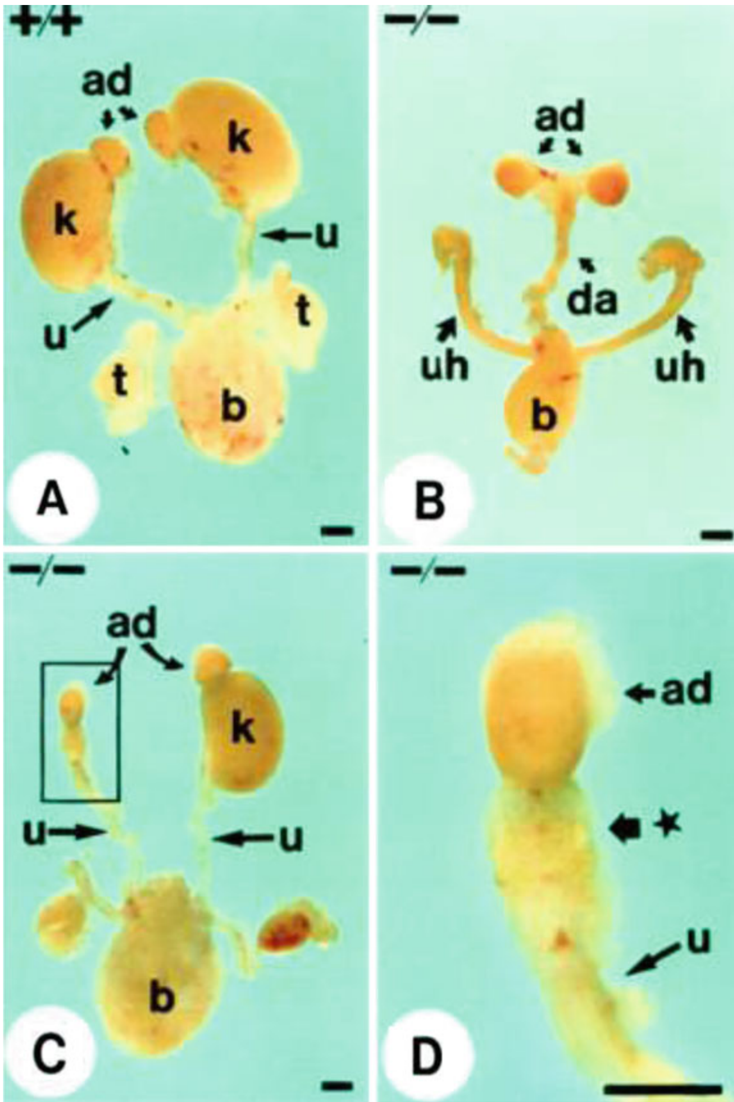

Fig. 2 Kidney phenotype in α8-deficient newborn mice. The urogenital tract was dissected from wild-type (**a**) or α8-deficient (**b–d**) mice. (**a**) In wild-type mice, the adrenal glands (ad), kidneys (k), ureter (u), bladder (b), and testes (t) were well developed. (**b**) The most seriously affected mutants had no ureter and kidney, but the bladder and adrenal glands were unaffected. Note that the dorsal aorta (da) and uterine horns (uh) were not removed in this dissection (**c**) One small kidney and one rudiment (boxed) from a mutant animal. (**d**) High magnification view of the rudiment (boxed in C). The star indicates the undifferentiated mesenchyme between the adrenal gland and the ureter. Scale bars, 100 μm. Reproduced with permission from Muller et al. (1997) Integrin alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell 88:603–613

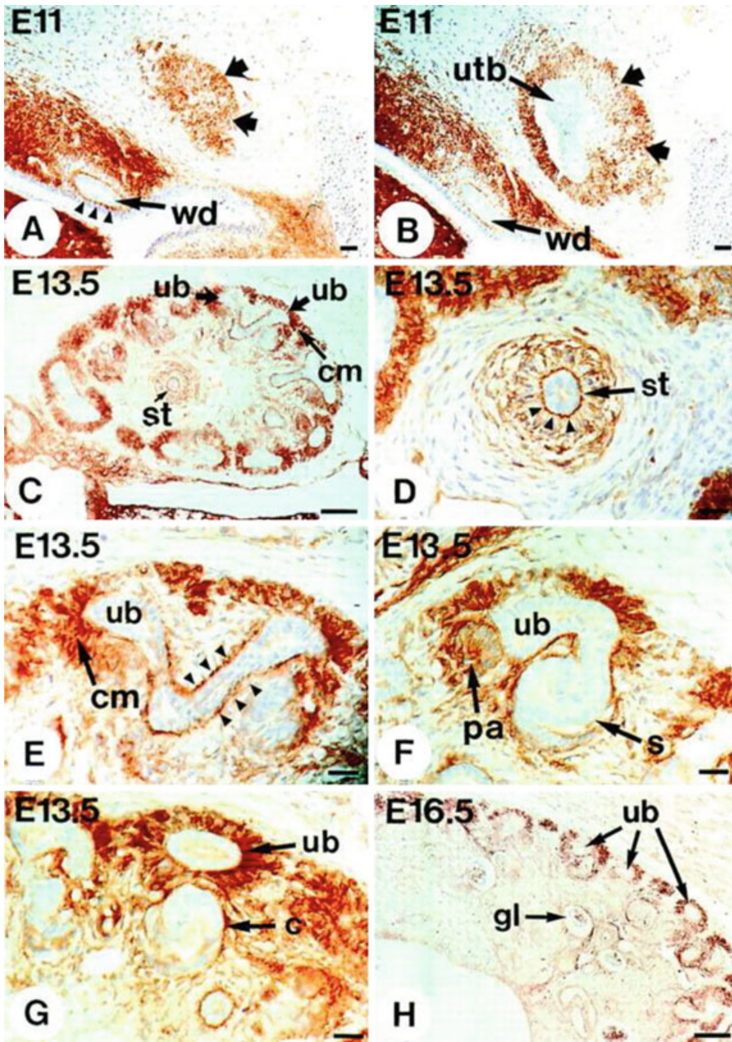

Fig. 3 Immunohistochemical localization of integrin α8 in the developing mouse kidney. (**a, b**) At E11, α8 is expressed in mesenchymal cells surrounding the Wolffian duct (wd), at the interface between the duct epithelium and the surrounding mesenchymal cells (arrowheads), in mesenchymal cells above the tip of the growing ureteric bud (utb) (**a**, wide arrows) and surrounding the ureteric bud (**b**, wide arrows). (**c–g**) At E13.5, α8 is expressed in the condensing mesenchyme (cm) surrounding the tips of the branching ureter (ub) (**c, e**) and in pretubular aggregates (pa) (**f**), but not in comma-shaped (c) bodies (**g**) or S-shaped (s) bodies (**f**). α8 is expressed at high levels in mesenchymal cells bordering the ureteric epithelium (**d** and **e**, arrowheads). (**h**) At E16.5, expression of α8 is seen in the outer layer of the kidney on condensing mesenchymal cells but not more internally on differentiated structures such as the glomerulus (gl). Scale bars, 100 μm (**c**, **h**) and 10 μm (**a, b, d–g**). Reproduced with permission from Muller et al. (1997) Integrin alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell 88: 603–613

1995) and TN-C (Saga et al. 1992) KOs. Thus, a novel ligand was postulated for α8 in the kidney, and this ligand was later identified as nephronectin (Brandenberger et al. 2001), as detailed below.

5 Nephronectin as a Ligand of Integrin α8 in the Kidney

To identify new ligands of α8 in the kidney, a soluble heterodimer composed of the extracellular domains of α8 and β1 fused to alkaline phosphatase (AP) was used; this fusion protein had previously been shown to recognize multiple ligands of α8β1 with binding characteristics equivalent to the full-length transmembrane protein (Muller et al. 1997; Denda et al. 1998a, b). The α8β1-AP fusion protein detected a unique protein in the 70–90 kDa range on Western blots of mouse kidney extracts, which was then cloned from a lambda phage library (Brandenberger et al. 2001). The protein was called “nephronectin” because of its putative involvement in kidney development. Coincident with the discovery of mouse NPNT, a novel α8β1 ligand was cloned from MC3T3-E1 osteoblast-like cells and called POEM (preosteoblast epidermal growth factor-like repeat protein with MAM domain) (Morimura et al. 2001). POEM was recognized to be identical to NPNT. The human gene was identified by database searches for EGF-like domain sequences (Huang et al. 2005).

From the deduced amino acid sequence, NPNT is a glycosylated protein with a putative signal peptide at the N-terminus and no transmembrane domain, both of which are features of secreted proteins. NPNT has 5 EGF-like repeats, a proline-rich mucin-like linker region containing an RGD sequence, and a C-terminal MAM (meprin-A5 protein-receptor tyrosine phosphatase μ) domain (Fig. 4). The RGD sequence is required for binding to α8β1 in vitro (Brandenberger et al. 2001). Rabbit antiserum against NPNT confirmed that the 70–90 kDa protein in kidney extracts corresponds to NPNT. Furthermore, NPNT and α8 could be co-immunoprecipitated, indicating they likely interact in vivo. The NPNT antibody also detected protein in

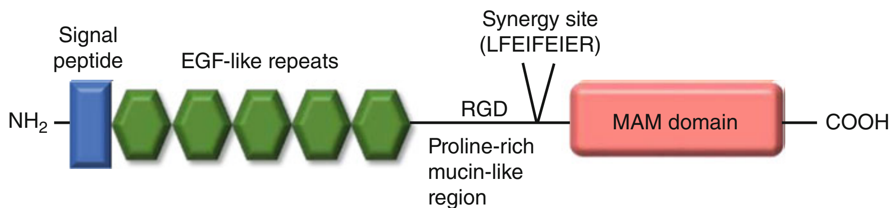

Fig. 4 Schematic of nephronectin (NPNT) structure. The NPNT protein has an N-terminal signal peptide sequence, EGF-like repeats, a proline-rich mucin-like linker region containing an RGD sequence and synergy site for high-affinity binding to integrin α8, and a C-terminal MAM domain found in other proteins such as meprin, A5 protein, receptor tyrosine phosphatase μ, and neuropilins. Adapted from Brandenberger et al. (2001) Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin alpha8beta1 in the embryonic kidney. J Cell Biol 154:447–458

the mouse kidney throughout development, where it localizes to the BM of ureteric buds and the mesonephric duct (from which the ureteric buds emerge). In situ hybridization experiments demonstrated that it is the branching epithelial cells in the developing kidney that express *Npnt* mRNA (Brandenberger et al. 2001). Thus, NPNT is ideally positioned for interactions with integrin α8 on adjacent mesenchymal cells.

NPNT KO mice recapitulate renal developmental abnormalities observed in α8 KO mice (Linton et al. 2007), demonstrating that NPNT-α8 interactions are essential for normal kidney formation. In a pattern strikingly similar to the α8 KO, about half of embryos homozygous for the *Npnt* null mutation lacked kidneys. The remainder of the KO cohort had one or two kidneys, which were generally smaller than normal (Linton et al. 2007). Like the α8 KO, there was a delay in the invasion of the metanephric mesenchyme by the ureteric bud at embryonic stage E11.5. In addition, once the bud had invaded at E12.5, there were fewer rounds of branching in NPNT-deficient kidneys.

Because NPNT localizes to the BM of the ureteric bud, the expression patterns of laminins and collagen IV, forming the core protein networks of this specialized matrix, were assessed. Immunostaining for these proteins, as well as FN, in NPNT KO embryonic kidney was indistinguishable from the WT (Linton et al. 2007). These findings led to the hypothesis that NPNT, acting through α8, more likely plays a role in signaling events in kidney morphogenesis rather than a structural function. After examining the expression of genes known to be involved in kidney development at E11.5, only glial cell line-derived neurotrophic factor (GDNF), a member of the TGFβ superfamily that is normally present in the metanephric mesenchyme, was not expressed in either the NPNT or the α8 KO at this time point. Haploinsufficiency of *Gdnf* on the α8-null background increased the kidney agenesis to 95% (Linton et al. 2007), further supporting a critical role of GDNF signaling following α8-nephronectin interactions during kidney development.

6 Additional Characteristics of Nephronectin

Although the RGD in the linker region of NPNT is clearly required for its binding to α8, the in vitro affinity of α8 for NPNT surpasses (by ~100-fold) that of all other ligands tested so far, including FN (Sato et al. 2009; Kiyozumi et al. 2012). To determine the basis for this high-affinity recognition of NPNT, recombinant deletion mutants of the linker segment were tested in binding assays (Sato 2009). The sequence LFEIFEIER, ten amino acids downstream from the RGD, was identified as a synergistic contributor to the binding of NPNT to α8. From binding analyses of recombinant forms with alanine replacements of residues within this sequence, EIE emerged as the key motif that operates in concert with RGD (Sato et al. 2009). Using model self-assembled substrates, another group confirmed the synergy between LFEIFEIER and RGD, but showed that each domain could independently promote adhesion via different sites on α8β1; in addition, they identified the FEI triad, rather

than EIE, as the critical motif (Sanchez-Cortes et al. 2011). Technical differences in the experiments may have accounted for the disparate findings. Interestingly, a homologue of NPNT, known as EGFL6 (or MAEG), has a similar domain structure (Buchner et al. 2000) and is a ligand for α8 (Osada et al. 2005), but lacks the synergy site.

NPNT binds other integrins in addition to α8, including αvβ3, αvβ5, αvβ6, and α4β7 (Brandenberger et al. 2001; Morimura et al. 2001). All but α4β7 are RGD-binding integrins (Hynes 2002), so presumably, they interact with the RGD in NPNT, but this has not been confirmed, nor has the basis for binding of NPNT to α4β7 been determined.

Consistent with its role as an ECM protein, NPNT is present in the basal laminae of many organs and tissues throughout mouse development (Brandenberger et al. 2001; Morimura et al. 2001). By immunoelectron microscopy, NPNT localizes to the BM sublamina densa region, which faces the mesenchyme (Kiyozumi et al. 2012). Thus, NPNT is ideally placed for interactions with α8β1 in this compartment, as depicted in Fig. 5. The Fraser syndrome-associated proteins FRAS1, FREM1 (also known as QBRICK), and FREM2 (Pavlakis et al. 2011) are required for orchestration of NPNT assembly into embryonic BMs (Kiyozumi et al. 2012). In addition, interactions of the MAM domain in NPNT with proteoglycans appear to play an important role in its localization to the BM (Sato et al. 2013). NPNT can also be detected in cell lysates and on the cell surface (Morimura et al. 2001; Kuek et al. 2016). Mutation of the RGD to RGE did not affect the localization patterns of recombinant NPNT (Morimura et al. 2001). However, deletion of the MAM domain reduced cell-surface binding of recombinant NPNT and led to its accumulation in the conditioned medium, suggesting that this region mediates protein-protein interactions apart from α8. The putative binding partner(s) for NPNT have not been identified, but appear to be cell-type specific.

7 Integrin α8 and Nephronectin in Tissue Development, Homeostasis, and Disease

In the previous section, we described the initial characterization of α8 and NPNT and the studies that established their critical role in mouse kidney morphogenesis. In this section, we summarize the current body of work that has been done to assess the regulation and function of α8 and NPNT in other organs and cells, as well as in kidney homeostasis and disease (summarized in Tables 1 and 2). To circumvent the kidney defects in global α8 and NPNT KOs, mice with a floxed allele for conditional deletion of these proteins were generated (Linton et al. 2007; Chan et al. 2010). In the α8 targeting construct, *lox*P sites were inserted in sequence flanking exons 29 and 30, which encode the transmembrane and cytoplasmic domains. In the *Npnt* floxed allele, exon 1 was targeted for excision by Cre recombinase (Linton et al. 2007). This conditional approach efficiently ablates mRNA and protein for each gene (Linton

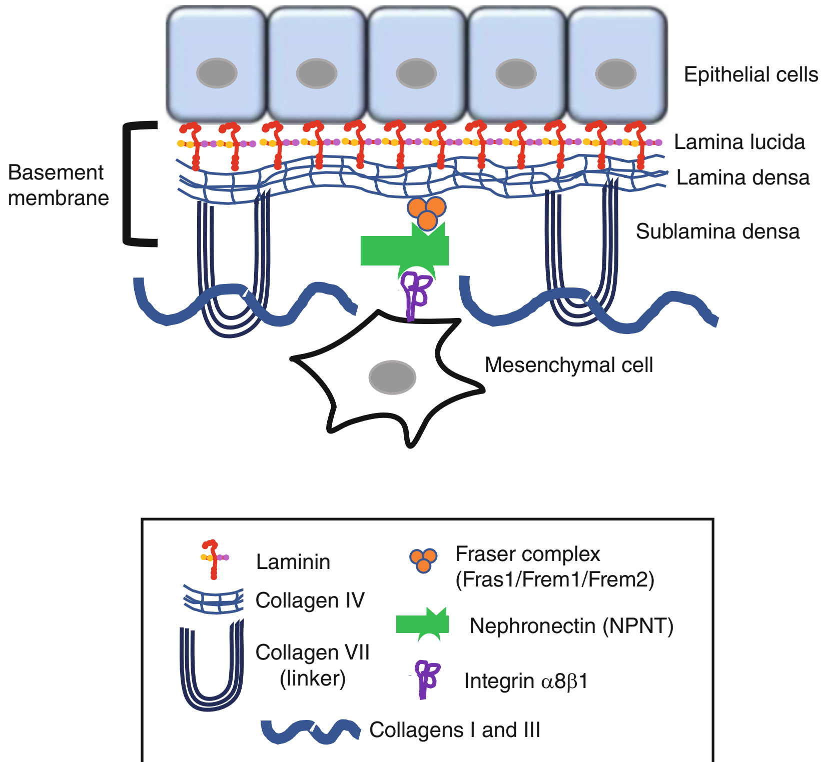

Fig. 5 Model for NPNT deposition and organization into embryonic basement membranes. In basement membranes, laminin and collagen IV are the primary structural proteins. NPNT is secreted by epithelial or mesenchymal cells and binds to Fraser complex proteins in the sublamina densa region, which anchors to the underlying mesenchymal collagens (I and III) via collagen VII. NPNT is well positioned to signal through integrin α8 on adjacent mesenchymal cells. Not depicted are accessory proteins in the different regions of the basement membrane or the interactions of NPNT with basement membrane heparan sulfate proteoglycans. Adapted from Kiyozumi et al. (2012) Basement membrane assembly of the integrin alpha8beta1 ligand nephronectin requires Fraser syndrome-associated proteins. J Cell Biol 197:677–689

et al. 2007; Chan et al. 2010; Khalifeh-Soltani et al. 2016, 2018; Hung et al. 2018; Zimmerman et al. 2018; Müller-Deile et al. 2021; Nishimichi et al. 2021). Mice with conditional deletion of α8 and NPNT are useful tools for unraveling their roles in different disease models.

Table 1 Outcome of integrin α8 deficiency in vascular SMCs compared to wild-type

Parameters	Mouse: KO	Rat: KD	References
Gene expression	↔ α-SMA ↔ desmin ↔ α5 and αv ↓ ECM	↓ SMC markers ↑ α-SMA ↑ α5 and αv	Zargham et al. (2005), Zargham et al. (2006), Marek et al. (2010), Volkert et al. (2014)
Proliferation	↔	↓	Zargham et al. (2007a, b, c), Marek et al. (2010)
Migration	NR	↑	Zargham et al. (2005)

KO knockout, *KD* knockdown (by siRNA), ↔, no change, *NR* not reported

8 Smooth Muscle Cells

8.1 Vascular Smooth Muscle Cells

The early studies on integrin α8 found the protein expressed on contractile cells, including smooth muscle cells (SMCs) in the aorta and other blood vessels, as well as visceral smooth muscle in the intestine (Schnapp et al. 1995a, b), with the highest expression of mRNA in the aortic smooth muscle tissue in mice, rats, and humans (Chen et al. 2001; Kitchen et al. 2013). This observation led investigators to focus on elements that may confer vascular smooth muscle (VSM) specificity (or selectivity) to α8 expression. The *Itga8* promoter contains CArG-like sequences (CC(A/T)$_6$GG), a motif that is found in SMC-restricted genes and binds serum response factor (SRF), a transcription factor that regulates some SMC differentiation genes (reviewed in (Owens et al. 2004)). However, analysis of *Itga8* promoter constructs in cultured cells showed no preference of the promoter for an SMC line over other cell types (Kitchen et al. 2013). This finding suggested that there are elements upstream of the promoter that potentially regulate α8 expression in SMCs (Kitchen et al. 2013). In support of this idea, ectopic expression of myocardin, a co-activator of SRF, in multiple SMC lines significantly enhanced levels of *Itga8* mRNA from the endogenous promoter (Kitchen et al. 2013).

Like the β1 subunit, expression of *Itga8* transcripts in cultured rat aortic smooth muscle cells was responsive to all-*trans* retinoic acid stimulation, and α8 is considered a delayed retinoid response gene, i.e., requiring de novo protein synthesis (Medhora 2000; Chen et al. 2001). Retinoids promote the retention of an SMC differentiated phenotype and suppress their migration and proliferation. De-differentiation of vascular SMCs from a contractile to a non-contractile phenotype must occur to allow their migration after injury. Knockdown of α8 by short interference RNAs (siRNAs) in rat vascular SMCs increases their migration, decreases expression of markers of differentiated SMCs, such as α-tropomyosin, calponin, and myosin heavy chain, and leads to the disassembly of actin stress fibers and focal adhesions (Zargham et al. 2005, 2006). Mouse *Itga8* KO vascular SMCs

Table 2 Summary of integrin α8 and NPNT expression in adult mice

Organ	Molecule	Expression pattern: normal	Expression pattern: injury	References
Kidney	α8	• Mesangial cells • Media of arterioles and arteries	• Increased glomerulonephritis, hypertension, and diabetic nephropathy • Induced in tubular epithelial cells and interstitial fibroblasts after a fibrotic stimulus	Hartner et al. (1999, 2002a, b, 2008, 2010, 2012), He et al. (2021)
	NPNT	• Mesangial matrix • Glomeruli BM • Subset of tubules	Increased in tubule epithelium by acute necrosis	Cheng et al. (2008), Zimmerman et al. (2018)
Lung	α8	• Interstitial cells of alveolar septa • SMCs of airways and vasculature • Fibroblasts in elastin-rich matrices	Increased during resolution phase of fibrosis	Levine et al. (2000), Schiller et al. (2015), Hung et al. (2018), Matsushima et al. (2020)
	NPNT	• Epithelial cells • Alveolar fibroblasts • Alveolar BM	Increased during resolution phase of fibrosis	Decaris et al. (2014), Schiller et al. (2015)
Liver	α8	SMCs of blood vessels and bile ducts	In activated stellate cells	Levine et al. (2000), Nishimichi et al. (2021)
	NPNT	Mesenchymal cells adjacent to portal and central veins	Increased in inflammatory regions	Inagaki et al. (2013)
Heart	α8	Fibroblasts in myocardium and valves	Increased in myofibroblasts	Thibault et al. (2001), Bouzeghrane et al. (2004), Hartner et al. (2009)
Hair follicle	α8	• Arrector pilus muscle (APM) • Dermal papilla cells	ND	Fujiwara et al. (2011)
	NPNT	• Bulge stem cells • Hair germ	ND	

BM basement membrane, *SMCs* smooth muscle cells, *ND* not done

show reduced transcripts for collagen III, fibrillin-1, and OPN, and an increase in expression of the matrix metalloproteinase inhibitor TIMP-1 and connective tissue growth factor (CTGF) (Volkert et al. 2014). However, there was no difference noted in the expression of α-smooth muscle actin (α-SMA) or its localization to stress fibers in KO vascular SMCs as compared to WT (Marek et al. 2010).

Proliferation of rat vascular SMCs was reduced by the knockdown of α8 through concomitant loss of RhoA, which normally associates with integrin α8 in the cell membrane (Zargham et al. 2007a, b, c). The RhoA pathway appears to be involved in α8-mediated reversion of dedifferentiated vascular SMCs to a contractile phenotype, as this reversion could be blocked by RhoA inhibitors (Zargham et al. 2007a, b, c). Other α integrins related to α8, including α5 and αv, are associated with stimulation of SMC proliferation and migration programs, and expression of these α subunits is increased with α8 silencing (Zargham et al. 2006). However, upregulation of α5 and αv was not detected in vascular SMCs isolated from α8 KO aorta (Marek et al. 2010). This finding, along with the lack of changes in other characteristics of α8 KO vascular SMCs, suggests that there are differences in how this cell type responds to acute (knockdown) vs chronic (knockout) absence of α8 (Marek et al. 2010). The results of these studies are summarized in Table 1.

In the large vessels of multiple species, α8 is normally expressed by vascular SMCs within the medial layer (Menendez-Castro et al. 2015). In the balloon injury model of the rat carotid artery, expression of α8 is reduced during the formation of the neointima (Zargham et al. 2005) but increases later during its constrictive remodeling (Zargham et al. 2007a, b, c). α8 is also diminished in atherosclerotic plaques in human carotids and advanced lesions of the aorta, as well as in mouse carotids after ligation-induced vascular damage (Menendez-Castro et al. 2015). Ligation of the left carotid artery in *Itga8*$^{+/-}$ and *Itga8*$^{-/-}$ mice produced lesions with a smaller lumen-to-media ratio and greater number of proliferating vascular SMCs than WT. However, there was no difference in neointima formation among the genotypes, although this occurred in less than 30% of the animals (Menendez-Castro et al. 2015). When the α8 null allele was crossed into *Apoe*$^{-/-}$ mice, which develop atherosclerosis spontaneously, there was an increase in the plaque-positive area in the aorta and aortic arch when compared to α8-replete *Apoe*$^{-/-}$ animals (Menendez-Castro et al. 2015).

8.2 Visceral Smooth Muscle Cells

Contraction of the visceral SM of the gastrointestinal tract regulates the rate of food transit from the stomach through the small intestine. In mice with conditional deletion of *Itga8*, lack of SM α8 increased gastric emptying and transit through the small intestine (Khalifeh-Soltani et al. 2016). These results were recapitulated by injection of WT mice with an α8 blocking antibody. In the conditional KO, there was decreased absorption of dietary fats and carbohydrates, and the mice gained less body weight and fat mass both on normal chow and a high-fat diet. These findings

phenocopied those observed in mice deficient in Milk fat Globule Epidermal Growth Factor like 8 (MFGE8), a known ligand of integrins αvβ3 and αvβ5 (Atabai et al. 2005) and modulator of SM contractions. MFGE8 was subsequently identified as a new ligand of α8β1 and these proteins co-localize in gastric SM. In contrast to vascular SM, α8 signaling through MFGE8 in visceral SM dampens RhoA activation through phosphatase and tensin homolog (PTEN)-dependent inhibition of PI3K (Khalifeh-Soltani et al. 2016). This differential effect of α8 on RhoA could be due to differences in GTPase activating factors and/or guanine nucleotide exchange factors in the cells; in addition, context of α8 activity may matter, as could the specific ligand(s) with which this integrin engages in tissues.

9 Kidney

In previous sections, we summarized the major role of the α8-NPNT pair in mouse kidney morphogenesis. Here, we will focus on a review of studies on the effect of integrin α8 and NPNT on kidney homeostasis in adult animals and the phenotype of α8- and NPNT-deficient kidney cells. In addition, we will address the contribution of α8 and NPNT to renal disorders in humans and animal (primarily rodent) models of disease.

9.1 Kidney Homeostasis

α8 is expressed in the glomeruli, which filters the blood, and in the media of arterioles and arteries in human and rodent kidneys ((Schnapp et al. 1995a, b; Sterk et al. 1998; Hartner et al. 1999); see Table 2). α8 co-localizes with the Thy1 antigen, which specifically marks the mesangial cells (Hartner et al. 1999). In both human and mouse kidneys, *Itga8* transcripts are highly and specifically expressed in mesangial cells (He et al. 2021). As noted previously, about half of inbred Itga8 KO mice fail to develop kidneys and die shortly after birth. Of the surviving mutants, the majority (76%) possessed two small kidneys, with the remainder (24%) having only one kidney (Hartner et al. 2002a, b). A significant number of the α8 KO kidneys displayed segmental malformations, manifested as cortical surface retraction, and some showed tubule collapse, dilatation, and atrophy, or infiltrates in the interstitium. Moreover, KOs exhibited a decreased ability to concentrate urine, possibly due to their having shorter nephrons than WT (Haas et al. 2003). Intra-arterial measure of blood pressure revealed an elevation of this parameter in KO mice.

Despite the localization of α8 to the glomeruli and large vessels, initial studies indicated that there were no obvious abnormalities in these structures in α8 KO kidneys (Hartner et al. 2002a, b). However, a later investigation revealed multiple minor alterations in the glomerulus, including a reduction in the number of nephrons,

an increase in glomerular volume and mesangial cellularity, an elevation in collagen IV and FN deposition, and abnormal expression of collagens I and III (Haas et al. 2003). Somewhat surprisingly, the function of the glomerulus was not significantly impaired, suggesting the development of mechanisms to compensate for the global loss of α8.

In the mouse kidney, NPNT is found in the mesangial matrix and BM of the glomeruli, as well as in a subset of tubules ((Zimmerman et al. 2018); see Table 2). The glomerular BM directly contacts the mesangial cells (Sakai et al. 1987). NPNT colocalizes with α8 at the base of capillary loops in special adhesion structures termed "mesangial pedestals" (Zimmerman et al. 2018). Conditional deletion of NPNT from either nephron progenitors or podocytes resulted in loss of mesangial pedestals (Zimmerman et al. 2018). In addition, there was an expansion of the mesangial cells in mutant kidneys and increased deposition of ECM, reproducing what was seen in α8 KO kidneys (Haas et al. 2003). Given these similarities, investigators have postulated that α8-NPNT interactions assist in providing mechanical stability to the glomerular tuft. This hypothesis was later supported in a model of hypertensive glomerular disease in the α8 KO (see below).

As was observed in the mouse, in the human kidney NPNT is a component of the glomerular ECM, where it localizes both to the BM and the mesangial matrix (Lennon et al. 2014). Stimulation of cultured podocytes by TGFβ results in decreased NPNT mRNA and protein, with concomitant upregulation of the microRNA miR-378a-3p. The 3′ untranslated region of the NPNT transcript in humans, mice, and zebrafish is a target of this microRNA (Kahai et al. 2009; Müller-Deile et al. 2017), as well as endothelial-derived miR-192-5p (Müller-Deile et al. 2021). Transfer of miR-192-5p to podocytes occurs via exosomes (Müller-Deile et al. 2021). Injection of an NPNT knockdown morpholino or miR-378a-3p/miR-192-5p mimics into zebrafish larvae at an early developmental stage suppressed NPNT levels and produced varying degrees of pericardial and yolk sac edema, indicating that loss of NPNT leads to glomerular leakage (Müller-Deile et al. 2017). Mice injected with the miR-378a-3p mimic exhibited reduced NPNT expression and increased albumin in the urine. Podocyte-specific deletion of NPNT in mice also resulted in significant proteinuria. In both mice and zebrafish, effacement of the podocytes was a consistent phenotype, as were structural alterations in the glomerular BM (Müller-Deile et al. 2017, 2021). Thus, NPNT is important to maintain the barrier function of the BM in the glomerulus.

9.2 Animal Models of Kidney Diseases

Because of the unique expression pattern of α8 in the kidney, potential roles of this integrin in renal diseases have been investigated (see Table 2 for a summary of expression changes with injury). In most of the experiments using α8 KO mice, WT controls were subjected to a uninephrectomy to normalize the total kidney mass to that of KOs. In rodent models of experimental glomerulonephritis, α8 expression

increases during the peak of nephritis, which involves transient proliferation and matrix deposition in the mesangium, then falls to control levels with resolution (Hartner et al. 1999, 2008). When α8 KOs were assessed, they had equivalent glomerular injury to WT. However, resolution was delayed in the KO kidneys, as evidenced by ongoing mesangial cell activation, proliferation, and apoptosis (Hartner et al. 2008).

To further investigate the role of α8 in glomerular disease, WT and α8 KO mice were treated with desoxycorticosterone-acetate (DOCA)-salt to induce a hypertension model that produces mechanical strain on the kidney (Hartner et al. 2002a, b). DOCA-salt loading in WT mice resulted in the upregulation of α8 protein in the glomeruli (Table 2). Although treated WT and KO mice developed a similar degree of hypertension and nephrosclerosis, KO kidneys showed increased glomerular cell activation (measured by α-SMA positivity), more frequent fibrin deposits, and a greater capillary destructive index than WT (Hartner et al. 2002a, b). Thus, absence of α8 appears to render the glomerulus susceptible to disruption from mechanical stress.

In addition to a role in maintaining structure in the glomerulus, α8 also preserves the integrity of the podocytes, which are specialized epithelial cells involved in filtration, as demonstrated in a model of diabetic nephropathy (Hartner et al. 2010). As seen in other models of glomerular disease, experimental diabetes resulted in increased expression of α8 (Hartner et al. 2010). The absence of α8 in the setting of diabetes led to more severe injury of the glomerulus, accompanied by higher urine levels of albumin, higher glomerulosclerosis scores, and decreased expression of podocyte markers (Hartner et al. 2010). Because there is increased deposition of FN and OPN in diabetic nephropathy (Fischer et al. 1998; Mason et al. 2003), reduced engagement of these ligands by α8 KO mesangial cells may have diminished their adhesion to the ECM, leading to weakened structural integrity of the glomerulus and defects in filtration (Hartner et al. 2010).

A recent study leveraged the high levels of integrin α8 expression in mesangial cells to develop a novel therapeutic approach for glomerulonephritis: hybrid liposome-nanoparticles modified with α8 antibodies were generated to deliver dexamethasone (anti-inflammatory) and captopril (anti-hypertensive) specifically to the mesangial cells. This approach was shown to alleviate both inflammation and fibrosis when the particles were injected into mice with mesangial proliferative glomerulonephritis (Zhou et al. 2022), demonstrating the therapeutic potential of α8-targeted drug delivery.

Given that mesangial cells stimulated with TGFβ, a profibrotic cytokine, increased expression of α8, a potential role for this integrin in kidney fibrosis has been explored (Hartner et al. 2012). Rodents were subjected to unilateral ureteral obstruction (UUO), which induces a tubulointerstitial injury and fibrosis characterized by increased expression of ECM genes and markers of cell activation (α-SMA, vimentin). In addition to the normal localization of α8 to mesangial and smooth muscle cells, α8 was found in tubular epithelial cells and interstitial fibroblasts after UUO (Hartner et al. 2012). When UUO was performed in α8 KOs that had two functional kidneys, KOs developed more tubulointerstitial damage, α-SMA-positive

fibroblasts, and collagen deposition compared to UUO-treated WT mice. A role for α8 in regulating cell turnover was ruled out by analysis of the proliferation marker PCNA, which showed no difference between the genotypes (Marek et al. 2016). Rather than promote fibrosis, α8 appears to be protective in the injured tubulointerstitium, possibly by dampening TGFβ signaling, fibroblast activation, and inflammation.

In studies with *Apoe*$^{-/-}$;*Itga8*$^{-/-}$ mice, plasma levels of creatinine and urea were elevated relative to *Apoe*$^{-/-}$;*Itga8*$^{+/+}$, indicative of kidney injury in this model (Menendez-Castro et al. 2015), although females show less pronounced disease than males (Marek et al. 2017). There was also a significant increase in glomerular size, as well as deposition of ECM in *Apoe*$^{-/-}$;*Itga8*$^{-/-}$ kidneys. However, it is not clear if this phenotype was primarily due to the absence of α8 or to the reduced renal mass in the KOs (Menendez-Castro et al. 2015).

In contrast to α8, there are currently no published studies assessing the role of NPNT in experimental models of glomerular disease. In a mouse model of acute renal tubular necrosis, the low level of NPNT expression in tubule epithelium normally seen at baseline was increased during the regeneration phase (Cheng et al. 2008).

9.3 Human Kidney Diseases and Developmental Disorders

Despite the large body of work demonstrating the involvement of α8 in experimental kidney diseases in the mouse, there is a paucity of evidence linking this integrin to disorders in the human organ. In autosomal-dominant polycystic kidney disease (ADPKD), α8 expression is elevated and occurs de novo in the epithelium (Zeltner et al. 2008). Furthermore, a polymorphism in the α8 promoter at position -414, in which a cytosine is replaced by a thymidine, is associated with earlier onset of end-stage renal failure than in ADPKD patients without this replacement (Zeltner et al. 2008). Recessive mutations in *ITGA8* were identified in two families with fetuses presenting with bilateral renal agenesis (Humbert et al. 2014). The mutation in one family changed the splice site preceding exon 29, which resulted in the loss of exon 28 and an in-frame deletion of 34 amino acids. This deletion ablates two β sheets in the second calf domain of α8 and may affect its conformation (Humbert et al. 2014). In the second family, each *ITGA8* allele contained a different mutation: on one allele, a 5-base pair deletion in exon 17 led to a frameshift and a truncated protein; on the second allele, a point mutation in exon 13 (G $>$ A) changed amino acid glycine at position 407 to arginine (Humbert et al. 2014). Transfection of an embryonic kidney cell line with *ITGA8* cDNA encoding the G407R mutation prevented cell-surface localization of α8 and reduced adhesion and spreading of the cells on NPNT (Humbert et al. 2014). In a separate cohort of 47 fetuses with bilateral renal agenesis, one fetus had a heterozygous missense mutation (C $>$ T) in *ITGA8* that changed threonine 255 to methionine in the β propeller domain; mutation

(s) in the other *ITGA8* allele of this subject were not assessed in the study (Humbert et al. 2014).

NPNT levels appear to be differentially regulated in kidney diseases in humans. Examination of the glomeruli proteome from patients with diabetic nephropathy in comparison to non-diabetic controls (Nakatani et al. 2012a, b) showed upregulation of NPNT, which localized to areas of mesangial expansion and glomerulosclerosis. NPNT immunoreactivity could distinguish diabetic nephropathy from other kidney disorders, as the protein was either weakly detected or absent in most glomerulonephritis diseases, hypertensive nephropathy, light chain deposition disease, focal segmental glomerulosclerosis, and amyloidosis (Nakatani et al. 2012a, b). In contrast to other reports (Lennon et al. 2014; Zimmerman et al. 2018), NPNT was not detected in the glomeruli of "normal" (non-diabetic) controls. Differences in anti-NPNT antibodies used may be one factor that accounts for the conflicting staining results.

As mentioned previously, NPNT expression is suppressed by microRNAs miR-378a-3p and miR-192-5p. In comparison to healthy control kidney tissue, there is increased detection of these microRNAs and a corresponding decrease in NPNT in the glomeruli of patients with idiopathic membranous glomerulonephritis and focal segmental glomerulosclerosis. By contrast, no change in NPNT or miR-378-3p was detected in minimal change disease or IgA glomerulonephritis (Müller-Deile et al. 2017).

In pediatric nephrotic syndrome, there is increased glomerular permeability and severe proteinuria, which generally improves with steroid therapy in some, but not all, patients. Serum levels of NPNT correlated with lower urinary albumin/creatine ratio and steroid-sensitive nephrotic syndrome (Watany et al. 2018), suggesting that NPNT could be a biomarker of glomerular regeneration and a predictor for responsiveness to treatment. The biological significance of soluble NPNT in peripheral blood is uncertain, although another group showed that serum levels of NPNT are slightly elevated in patients with lung silicosis compared to healthy volunteers (Lee et al. 2019). More work is clearly needed to validate the assays and to confirm NPNT as a reliable and useful serum biomarker.

10 Lung

10.1 Development and Homeostasis in the Lung

In the mouse, lung development begins at embryonic day 9.5 (E9.5), when the trachea separates from the foregut, and is characterized by a precise sequence of primary and secondary budding and branching to give rise to four lobes in the right lung (cranial, medial, caudal, and accessory) and one lobe in the left (reviewed in (Morrisey et al. 2010). In all the embryonic stages assessed (E12.5–E17.5), mesenchymal cells were positive for α8, which also co-localized with FN and α-SMA in perivascular and peribronchial areas (Wagner et al. 2003). In explant cultures of

lungs, the majority of α8 KO embryonic lungs exhibited dilated airways and reduced growth and branching (Benjamin et al. 2009); the branching defect is compensated in KO adults by an increase in the formation of new septa during the postnatal alveolarization stage (Cremona et al. 2020). In utero exposure of WT embryos to lipopolysaccharide (LPS) dampens α8 mRNA and protein in the lung and results in an arrest in airway branching, reminiscent of that seen in the α8 KO mouse embryos and in premature infants with bronchopulmonary dysplasia (Benjamin et al. 2009). Without α8β1, mesenchymal cells form less focal adhesions and stress fibers and migrate more rapidly on FN than WT, suggesting that these cells are not able to maintain the appropriate tension around developing airways (Benjamin et al. 2009). α8 expression is maintained in rodent adult lungs, where it localizes to interstitial cells within the alveolar septa, as well as vascular and airway SM ((Levine et al. 2000; Hung et al. 2018); see Table 2). A subpopulation of the α8 interstitial cells has contractile filaments, consistent with the theme of α8 as a marker of SM-like cells in tissues/organs. α8 expression in the adult mouse lung correlates with low Sca1 positivity and marks a fibroblast subtype present in elastin-rich connective tissue, such as the adventitia of the pulmonary vein (Matsushima et al. 2020). Similarly, in the human lung, $\alpha8^{high}$ fibroblasts are located in areas dominated by elastic fibers, including the pulmonary vein adventitia, alveolar septa, and bronchiole walls (Matsushima et al. 2020). NPNT is also expressed in branching epithelium, interstitial cells, and mesothelial cells in the mouse embryonic lung (Brandenberger et al. 2001; Kiyozumi et al. 2012). In the adult lung, *Npnt* is expressed by alveolar fibroblasts (Table 2), which are closely juxtaposed to epithelial/endothelial cell BMs and support the function of these cells (Tsukui et al. 2020).

In addition to the branching defects observed in α8 KO mouse lungs, the medial and caudal lobes of the right lung were fused in both embryos and adults (Benjamin et al. 2009; Cremona et al. 2020). The lung lobes form normally and are separate at embryonic day 13, but fuse later in development (≥ E16) (Fig. 6). Ex vivo incubation of the cranial and accessory lobes, which were not involved in fusion in vivo, results in fusion of these lobes when in proximity (Benjamin et al. 2009). The mesothelial lining of the lung surface was intact and indistinguishable from WT by conventional histology. The conclusion was that the absence of α8 may alter mesothelial cell function, with a possible contribution by abnormal mesenchymal cells, and promote aberrant tissue interactions during lung morphogenesis.

10.2 Injury and Fibrosis in the Lung

Injury of the lung with the chemotherapeutic agent bleomycin gives rise to a stereotypic fibrotic response that eventually resolves. Over the time course of fibrosis, the number of α8-positive cells increases within the parenchyma, and most of these cells also express α-SMA (Levine et al. 2000). To test if α8 plays a role in fibrosis, the floxed allele of *Itga8* was combined with a Pdgfrb-Cre transgene to delete the protein in PDGFRβ+ stromal cells (Hung et al. 2018). PDGFRβ

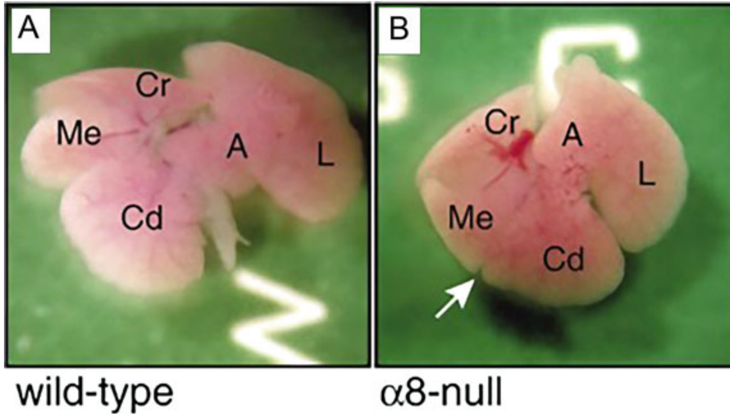

Fig. 6 Abnormal lung lobes in integrin α8-null embryos. (**a**) Wild-type lungs with distinctly separate left lung (L), cranial (Cr), medial (Me), caudal (Cd), and accessory (A) lobes comprising the right lung. (**b**) Lungs from an α8-null fetus showing fusion of the right medial (Me) and caudal (Cd) lobes. Arrow denotes fissure where lobes would normally be separated. Reproduced with permission from Benjamin et al. (2009) The role of integrin alpha8beta1 in fetal lung morphogenesis and injury. Dev Biol 335:407–417

expression increases in myofibroblasts and overlaps with α-SMA positivity in fibrotic areas of the lung and other organs (Henderson et al. 2013). After bleomycin administration, no differences in inflammation (at early time points) or fibrosis (at later time points) were found between Cre- controls and Cre+, other than increased collagen I expression in isolated Cre+, i.e., α8-deleted, cells (Hung et al. 2018). These findings suggest that α8 is dispensable for the development and progression of fibrosis in the lung, although this genetic approach may not have targeted all the relevant cell types.

By monitoring the extracellular proteome over 8 weeks post bleomycin, NPNT emerged as a matrisome-associated protein whose level decreases at the height of fibrosis but rebounds by day 56. A similar pattern of expression was noted for integrin α8, although it was found in a soluble fraction (Schiller et al. 2015). An analysis of the kinetics of the ECM after bleomycin showed there was an increase in newly synthesized NPNT in the guanidine-insoluble fraction, which reflects more stable proteins, but only at day 21 (Decaris et al. 2014). Taken together with the results from the fibrosis study using the α8 conditional KO, these data support the idea that both NPNT and α8 may have a role in fibrosis resolution and re-establishment of homeostasis.

10.3 Airway Obstructive Diseases

As we described earlier, conditional deletion of α8 in smooth muscle cells showed that signaling through this integrin and its novel ligand, MFGE8, regulates visceral SM contractions (Khalifeh-Soltani et al. 2016). In the lung, MFGE8 suppresses airway hyperresponsiveness by reducing SM contraction (Kudo et al. 2013). Tracheal rings isolated from mice with SM deletion of α8 showed an increase in contraction when incubated with IL-13 and then stimulated with methacholine, a cholinergic agonist (Khalifeh-Soltani et al. 2018). Normally, IL-13 activates the PI3K pathway and induces degradation of PTEN, and this degradation is prevented by the addition of recombinant MFGE8 to the experimental system. Exogenous MFGE8 did not have this protective effect on PTEN stability in the absence of α8 (Khalifeh-Soltani et al. 2018). There was no difference in contractility between WT and α8-deficient airway SM cells at baseline, unlike gastric SM (Khalifeh-Soltani et al. 2016). The results suggest that α8 is the key receptor for MFGE8 in airway SM cells.

Genome-wide association studies (GWAS) of chronic obstructive pulmonary disease (COPD) demonstrated that a locus containing *NPNT* was associated with decreased forced expiratory volume in one second (FEV_1) (Hancock et al. 2010) and FEV1/FVC (forced vital capacity), measurements of obstructive disease severity (Soler Artigas et al. 2015). Subsequent studies identified an intronic SNP, rs34712979, in NPNT as the most likely causal variant in this locus (Shrine et al. 2019) (Sakornsakolpat et al. 2019). This SNP gives rise to an alternative splice acceptor site that results in a 3-bp insertion encoding a serine residue near the N-terminus of NPNT (Saferali et al. 2020). Although it is unknown if and how this serine insertion affects the structure and/or function of NPNT, these findings point to new avenues of research into potential roles for NPNT and integrin α8 in airway dysfunction.

11 Other Organs

11.1 Liver

In normal liver, α8 protein is restricted to SM cells of blood vessels and bile ducts (Levine et al. 2000). During fibrosis, there is increased expression of α8 in areas of proliferation near ducts or fibrotic bands, in a distribution consistent with activated stellate cells, the equivalent of myofibroblasts in the liver (Nishimichi et al. 2021). Impaired a8 signaling, through deletion or antibody blockade, reduces markers of fibrosis in several models of the disease (Nishimichi et al. 2021). NPNT is weakly expressed in mesenchymal cells adjacent to portal and central veins (Inagaki et al. 2013). NPNT expression increases dramatically in fibrotic areas of cirrhotic livers (Inagaki et al. 2013). In a chronic hepatitis model, epithelial cells in bile duct

outgrowths were an additional source of NPNT. Ectopic overexpression of NPNT in the liver resulted in the formation of granuloma-like structures and exacerbation of acute liver injury (Inagaki et al. 2013). Deletion of either the RGD or synergy motif in ectopic NPNT reduced the number of granuloma-like clusters, suggesting that an RGD-binding integrin, likely α8, is involved. Thus, the expression pattern of NPNT and α8 in liver injury suggests a potential role of α8-NPNT signaling in liver fibrosis (Table 2).

11.2 Heart

Integrin α8β1 is expressed primarily in cardiac fibroblasts, and its expression is increased in different hypertension models (Thibault et al. 2001; Bouzeghrane et al. 2004; Hartner et al. 2009) (Table 2). α8 KO mice, in the setting of hypertension, showed loss of α-SMA-positive myofibroblasts, but left ventricular fibrosis, and other cardiac parameters of injury, were unchanged from WT. Although the NPNT KO mouse has not been used in similar hypertension models, in vitro experiments with rat neonatal cardiomyocytes demonstrated that their maturation state is better preserved on NPNT than FN (Patra et al. 2012). Taken together, these experiments support findings in other organs that α8 and NPNT influence cell differentiation.

In zebrafish, NPNT has a critical role in the development of the atrioventricular (AV) canal, which separates the atria and ventricles (Patra et al. 2011). Knockdown of NPNT in zebrafish embryos resulted in increased mortality, increased cardiac jelly, expansion of the AV canal, and impairment of AV valve formation. However, neither the α8 nor NPNT KO mouse shows an overt developmental phenotype in the heart, suggesting different roles for these proteins in mammals versus zebrafish.

11.3 Skin

In the skin, the base of the hair follicle contains stem cells in a structure termed the bulge. These bulge stem cells interact with a smooth muscle structure called the arrector pili muscle (APM), which mediates piloerection ("goosebumps"). NPNT is expressed by bulge stem cells and deposited in the BM, whereas α8 is expressed by cells in the APM (Fujiwara et al. 2011) (see Table 2). In the absence of NPNT, there is a small decrease in the number of hair follicles with an attached APM, and in the follicles that did develop, a significant number showed APM attachment above the bulge, where the NPNT homologue EGFL6 is localized (Osada et al. 2005). By contrast, a deficiency in α8 resulted in a loss of specificity for anchorage of the APM to NPNT, and attachment was observed at both NPNT- and EGFL6-expressing zones, likely via αv integrins (Fujiwara et al. 2011) (Fig. 7).

Below the bulge is the hair germ and a cluster of mesenchymal cells that comprise the dermal papilla, which, in concert with the surrounding environment, contributes

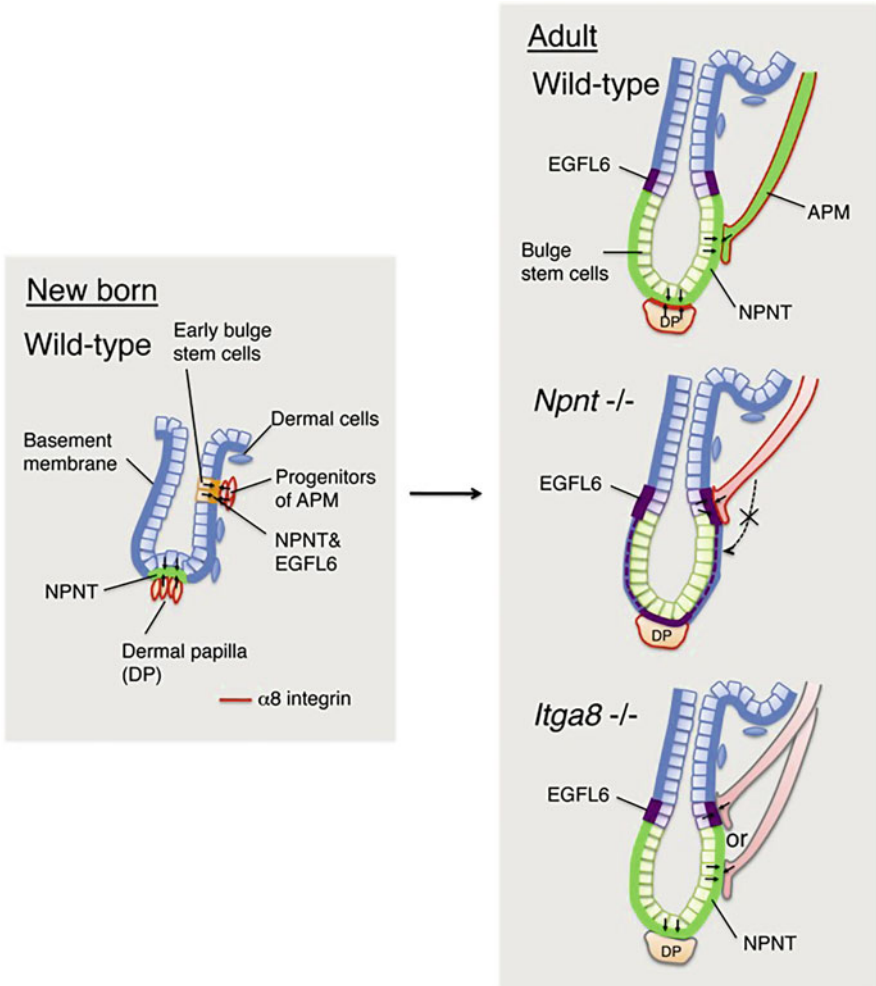

Fig. 7 Model depicting nephronectin (NPNT)-α8β1 interactions in the hair follicle bulge. During hair morphogenesis in neonatal skin, early bulge stem cells locally deposit NPNT in the bulge basement membrane. NPNT induces neighboring mesenchymal progenitors to differentiate into α8-positive arrector pili muscle (APM) cells, which adhere specifically to NPNT, establishing a stable anchorage to the bulge that is maintained throughout adult life. In the absence of NPNT, the APM attaches above the bulge, where there is compensatory upregulation of EGFL6. In the absence of α8, NPNT is still deposited in the bulge, but the selectivity of the APM interaction is lost, and muscles are anchored both to the NPNT-positive bulge and the EGFL6-positive upper bulge. Reproduced with permission from Fujiwara et al. (2011) The basement membrane of hair follicle stem cells is a muscle cell niche. Cell 144:577–589

signals that regulate follicle cycling. Wnt/β-catenin signaling upregulates NPNT expression in the hair germ, which in turn induces α8 expression in the dermal papilla (Fujiwara et al. 2011). So far, there have been no reports of an obvious effect

of α8 or NPNT deletion on the appearance of fur in adult mice. During development, NPNT is also detected in the basal laminae in the skin of mouse embryos (Brandenberger et al. 2001; Morimura et al. 2001; Kiyozumi et al. 2012); however, NPNT KO mice do not exhibit any defects in the epidermal-dermal junction (Linton et al. 2007). Given that EGFL6 is also expressed in the epidermal BM, it may compensate for the absence of NPNT here.

11.4 Brain and Nervous System

In chick embryos, α8 is strongly expressed in axon-rich regions of the central and peripheral nervous system, leading to the hypothesis that α8 might be involved in promoting axon outgrowth (Bossy et al. 1991). Later work showed that this immunolocalization pattern extends to other species (Einheber et al. 1996, 2001). In human brain, neuronal extensions in the hippocampus are immunoreactive for α8 (Schnapp et al. 1995a, b). In mice, conditional deletion of α8 in excitatory neurons of the forebrain resulted in impairment of long-term potentiation (strengthening of synapses involved in long-term memory) in the hippocampus, but basal synaptic transmission and other hippocampal-dependent processes involved in working memory were not affected (Chan et al. 2010). Although NPNT mRNA localizes to neuronal cell bodies and associated fibers in multiple regions of the mouse embryonic brain, its expression does not appear to overlap with that of α8 in the hippocampus (Morimura et al. 2001). Taken together, these findings suggest α8 may play multiple roles in neural development and memory formation, but more work needs to be done to identify its relevant ligands.

12 Cancer Biology

NPNT is elevated in highly metastatic mouse mammary tumors, where it localizes to the tumor epithelium (Eckhardt et al. 2005). The RGD and synergy site in NPNT mediate its role in promoting cell metastasis to the brain and enhancing cell viability (Steigedal et al. 2018; Toraskar et al. 2018; Magnussen et al. 2020). Involvement of the synergy site suggests that the relevant receptor is integrin α8β1, which is also upregulated in metastatic breast cancer (Magnussen et al. 2020). Mammary tumor cells engineered to overexpress NPNT have an increased ability to colonize the lungs when introduced into mice (Steigedal et al. 2018). Intriguingly, despite being a secreted protein, NPNT was also found intracellularly, as an anti-NPNT antibody detected the protein diffusely in the cytoplasm, in cytoplasmic granules thought to be exosomes, and in the nucleus of primary tumor cells and cells in metastatic lesions (Steigedal et al. 2018). Granular localization of NPNT in breast cancer tissue was associated with decreased survival rates in patients, but only when $<10\%$ of the tumor cells showed this staining pattern. This U-shaped relationship between NPNT

localization and survival has been observed with markers of other cancer types (see references in (Steigedal et al. 2018)).

Upregulation of NPNT is also observed in gastric cancer, where its expression is a predictor of poor prognosis (Mei et al. 2020), and in aldosterone-producing adenomas of the adrenal gland (Teo et al. 2017). NPNT expression is low in lymphoblastoid cell lines from patients with Laron syndrome, which is characterized by a deficiency in insulin-like growth factor 1 (IGF1) and decreased incidence of cancer (Sarfstein et al. 2020). Exogenous IGF1 stimulates NPNT expression in these cells, as well as breast and prostate cancer cell lines (Sarfstein et al. 2020). By contrast, NPNT expression is reduced in malignant melanoma cell lines and tissue, as compared to melanocytes (Kuphal et al. 2008). The synergy site in NPNT may be a unique motif to target in cancer and other diseases characterized by dysregulated expression of both NPNT and integrin α8 (Magnussen et al. 2021). Although the current evidence suggests that downmodulation of NPNT is cancer protective, further investigations into the potential role of NPNT in other types of cancer are clearly merited.

13 Conclusions and Future Perspectives

Integrin α8-NPNT interactions play a role in epithelial-mesenchymal cell crosstalk, particularly during tissue morphogenesis. Because α8 is not expressed by epithelial, endothelial, or immune cells, it has become a classic marker of stromal cells, particularly SMCs that are characterized by α-SMA expression and contractility. The association of α8-positive cells with BMs indicates that this integrin may have a preference for ligands in this specialized matrix, especially during development. The ligand with the highest measured affinity for α8 is NPNT, which is deposited into BMs and stabilized by Fraser syndrome-associated proteins and possibly interactions with proteoglycans during embryogenesis. Several seminal studies demonstrate that both α8 and NPNT are required for kidney development, where their interactions appear to have a signaling rather than structural role. Because α8 KO lungs present with developmental abnormalities, further studies to assess NPNT and its potential link to α8 in lung morphogenesis are warranted.

In the vasculature, α8β1 engagement, potentially by NPNT, induces and maintains a differentiated SM phenotype. α8 is upregulated in mesenchymal cells that populate fibrotic regions and appears to be a marker of myofibroblasts. Despite this association, global and cell-specific KOs of α8 have not demonstrated a significant impact of deletion of this receptor on the development of fibrosis in the lung, heart, or kidney. Most of the experimental models of injury, at least in the kidney, have demonstrated that lack of α8 leads to sustained tissue damage and impaired re-establishment of homeostasis. However, this propensity may be organ specific, as deletion of α8 in the liver, but not in the heart and lung, ameliorated fibrosis. NPNT has not been as extensively studied in models of organ injury. Regardless, like α8, expression of NPNT appears to be regulated by acute injury and is

associated with tissue remodeling. Overexpression of NPNT, as well as integrin α8, in cancer may be an uncontrolled manifestation of their normal roles in tissue repair. Given that mice with floxed alleles of *Itga8* and *Npnt* are available, these animals could be leveraged in future studies to assess further the function of these proteins in models of injury, repair, and carcinogenesis and their use as biomarkers of disease.

Compliance with Ethical Standards
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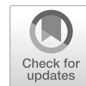
αv Integrin-Dependent TGFβ Activation in Cancer: A Brief Update



John F. Marshall, Pantelitsa Protopapa, and Natalie Allen

Abstract It is increasingly clear that TGFβ is probably the most important molecule promoting the immunosuppressive state of the tumour microenvironment (TME). As TGFβ is produced in an inactive latent form which is subsequently immobilized in the matrix or on cell surfaces, the molecules that can activate latent TGFβ represent potential targets for reducing the immunosuppression in the TME and enhancing the efficacy of both endogenous and therapeutic cancer-targeting immune effectors. Previously we have published the role of αv integrins in regulating TGFβ activity in cancer and this review provides an update to our earlier report. We describe particularly the progress made in the role of αvβ8 on modulating regulatory T cell (Treg) activity in a TGFβ-dependent manner and highlight that, although limited in number, clinical trials targeting integrins appear to be on the rise.

1 Introduction

The TGFβ family (for the purpose of this review TGFβ1, TGFβ2 and TGFβ3) are pleiotropic cytokines essential for tissue homeostasis including in development, wound repair and immune surveillance. However multiple studies over the last two decades show that TGFβ is also a primary factor in promotion of fibrosis (reviewed in (Henderson et al. 2020; Frangogiannis 2020; Caja et al. 2018; Kim et al. 2018)) in multiple organs and also promoting the latter stages of cancer (reviewed in (Caja et al. 2018; Derynck et al. 2021; Zhang et al. 2021; Batlle and Massagué 2019)). Since TGFβ is secreted as an inactive latent compound its ability to generate this pathology is controlled by mechanisms that release its biological potential. In 2019 (Brown and Marshall 2019) we reviewed the roles of αv integrins as they are major activators in vivo of latent TGFβ deposited in our tissues and on the membranes of certain cells. In this brief update, we shall review key biological developments that have revealed more detail about the biology in this field that is

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now driving multiple efforts to tackle these processes in the clinic. We shall not re-describe here much of the basic biology for integrin-TGFβ processes and refer readers to our original review (Brown and Marshall 2019).

2 Role of the GARP-LTGFB-αvβ8 Axis in Promoting Cancer

The inability of the host immune system or immune cell therapies to effectively eliminate cancers is linked strongly to the immunosuppressive environment of the tumour microenvironment (discussed in (Labani-Motlagh et al. 2020)). One of the major immunosuppressive cell types are regulatory T cells (Tregs) ($CD4^+$/$CD25^+$/$FOXP3^+$) as shown by the strong correlation between a high number of Tregs present and poor survival from many cancers (Labani-Motlagh et al. 2020; Shang et al. 2015; Ohue and Nishikawa 2019). While Tregs can mediate suppression of effector immune cells by a variety of direct (cell:cell) and secreted factor mechanisms (reviewed in (Sojka et al. 2008)) it is well established that in vivo Tregs can suppress CD8 cytotoxic effectors in a TGFβ-dependent manner; TGFβ-unresponsive CD8 T cells avoid Treg suppression and can promote tumour rejection (Chen et al. 2005). Moreover Choi and colleagues (Choi et al. 2020) showed that autocrine TGFβ1 signalling was essential to maintain the immunosuppressive Treg phenotype. While it is possible that Treg-produced TGFβ is not the sole source of TGFβ-driven CD8 T cell suppression, let us discuss recent developments concerning Treg activation of TGFβ.

The ability of Tregs to promote an immunosuppressive environment in cancer is driven by a complex of GARP (glycoproteins A repetitions predominant) covalently bound to latent-TGFβ1 (L-TGFβ) on the surface of Treg cells (Nakamura et al. 2001; Tran et al. 2009; Cuende et al. 2015) presented to integrin αvβ8 also expressed on Treg cells (Edwards et al. 2014; Worthington et al. 2015), either expressed on the same (cis-binding) or an adjacent (trans-binding) cell. The three-dimensional structure of TGFβ was solved by the Springer lab and suggested that the TGFβ homodimer was trapped non-covalently attached under two arms of the Latency Associated Peptide (LAP) 'strait-jacket' (Shi et al. 2011). Force-mediated stretching of this structure by αvβ6 (Shi et al. 2011; Munger et al. 1999) then 'released' the TGFβ. Whether the TGFβ diffused away was not clear. We discussed (Brown and Marshall 2019) the biological benefits of this pleiotropic cytokine TGFβ remaining localized in order to regulate spatio-temporal signalling and limit the potential pathological consequences of uncontrolled TGFβ signalling. The Nishimura lab has addressed this question (Campbell et al. 2020). To prove that the TGFβ cytokine need not be released from LAP in order to bind to its cell-surface receptors and generate TGFβ-dependent signals, the authors took Mink Lung Epithelial Cells expressing a TGFβ-reporter (PAI1-promoter-driven luciferase) and co-expressed GARP and a mutant TGFβ (TGFβ(R249A)) that was unable to separate from

LAP. These MLEC/GARP/ TGFβ(R249A) cells expressing the GARP:L-TGFβ (R249A) on their membranes were plated onto surfaces coated with immobilized extracellular domain of αvβ8. Significant and similar levels of TGFβ-dependent signals were detected in MLEC/GARP/ TGFβ(R249A) cells compared with MLEC cells expressing GARP and wild-type TGFβ. These data also confirmed that the TGFβ receptors on the same cells that expressed the GARP:L-TGFβ complex could bind to and be activated by the exposed TGFβ on the membrane of their own cell. Thus TGFβ cytokines exposed in activated latent-TGFβ, can remain localized to mediate signalling on adjacent cells (trans-activation) and host cells (cis-activation) (Campbell et al. 2020). Furthermore, since in this study the αvβ8 ligand was immobilized, it was clear that integrin-dependent force-mediated activation, as occurs with αvβ6 (Munger et al. 1999), was not required for αvβ8-dependent activation of L-TGFβ.

Studies have observed that inhibition of TGFβ globally or specifically in Treg cells results in more effective anti-cancer immunity. Mice bearing CD4 and CD8 T cells that were incapable of responding to TGFβ through expression of a dominant negative TGFβR construct (DN-TGFBR2), could not develop tumours after inoculation of two different syngeneic metastatic cancer cell types: EL4 thymoma or B16F10 melanoma (Gorelik and Flavell 2001). Depletion of either CD4 or CD8 populations resulted in growth of injected tumours showing the effects were T lymphocyte specific. Likely biological explanations are that CD4 cells expressing DN-TGFBR2 could not differentiate into Treg cells and cytotoxic CD8 cells expressing DN-TGFBR2 would maintain cytotoxicity even in the presence of TGFβ.

Tauriello et al. (2018) developed transgenic mice with combinations of colorectal driver mutations (Apc fl/fl, KrasLSL-G12D, Tgfbr2 fl/fl and Trp53 fl/fl) expressed inducibly only in intestinal stem cells by an Lgr5 promoter (called LAKTP mice). The presence of all mutations resulted in mice developing invasive carcinomas in over 90% of animals, metastases in over 40% of the mice and high TGFβ signalling (increased pSMAD3) in the tumour microenvironment (TME). Implantation of LAKTP organoids in the caecum resulted in similar cancers that again metastasized to liver. Pharmacological inhibition of TGFβ with the TGFBRII inhibitor galunisertib reduced primary tumour growth and prevented metastasis, an effect that was dependent on increased CD4 and CD8 cell efficacy. In mice with established liver metastases anti-PDL1 (which arose on metastases) was weakly therapeutic but combined with galunisertib, eliminated most metastases, enhanced cytotoxic effector cell activity and increased life expectancy (Tauriello et al. 2018). A related observation was made by Mariathasan and colleagues (Mariathasan et al. 2018) in Renal Carcinoma. Patients that exhibited resistance to anti-PD-L1 (atezolizumab) therapy exhibited a TGFβ-signalling signature in their TME and a reduced CD8 T cell infiltration in their tumours. Subsequent mouse studies confirmed that combined systemic antibody inhibition of TGFβ and PD-L1 promoted T cell penetration of tumours leading to enhanced anti-tumour immune responses and effective tumour therapy.

In each of these three studies targeting TGFβ directly would have suppressed the activity of Treg cells. Two recent studies have directly targeted the ability of Tregs to

make TGFβ as a means of studying their functions. GARP is encoded by the leucine-rich repeat containing protein 32 (LRRC32) gene (Ollendorff et al. 1994). Salem et al. (2019) generated mice that specifically lacked GARP on Treg cells by crossing Lrrc2 $^{fl/fl}$ mice with FOXp3 ^{YFP}-Cre + transgenic mice. The resultant Treg-$^{GARP-/-}$mice developed higher numbers of $CD4^+$/$FOXP3^+$ cells in spleen and thymus and exhibited higher fractions of activated ($CD44^+$/$CD62L^-$) $CD4^+$ and $CD8^+$ T cells. Mice developed pathologies in the colon consistent with chronic autoimmunity confirming that Treg cells required GARP to mediate immune homeostasis in the gut. In fact Treg-$^{GARP-/-}$ cells failed to migrate and remain in the colon, thought due to the significantly reduced expression of the homing receptor integrin αEβ7 (CD103) known to be regulated by TGFβ (Bain et al. 2017). Chronic inflammation-induced carcinoma was induced in Treg-$^{GARP-/-}$ mice using azoxymethane(AOM)/dextran sodium sulphate (DSS) administration. Compared with AOM/DSS treated wild-type mice, the Treg-$^{GARP-/-}$ developed smaller and fewer numbers of tumours associated with increased infiltration of $CD4^+$, but not $CD8^+$, T cells. This study genetically confirmed GARP on Treg cells is required to suppress gut immune homeostasis but also suppressed anti-tumour T cell responses. Recent studies using inhibition of GARP support these conclusions. Satoh et al. (2021) used anti-human GARP antibody DS-1055a in humanized mice bearing HT29 colon cancer tumours and reported reduction in CD4 + FOXP3+ Treg cells in the TME and resulted in strong anti-tumour responses.

In 2017 Stockis et al. (2017a) reported that αvβ8 is expressed at high levels on human Treg cells and using inhibitory antibodies to GARP and αvβ8, also reported that TGFβ activation on human Tregs was GARP and TGFβ-dependent. They extended these in vitro studies by examining the in vivo activity of GARP and αvβ8 on Tregs in a model of graft-versus-host-disease (GVHD). The highly immunodeficient NOD-SCID-gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice lack T, B and NK cells and allow engraftment of human cells. The authors injected human peripheral blood mononuclear cells (PBMC) into the mice inducing GVHD by day 23. The onset of GVHD was delayed to 86 days if autologous Treg cells were co-injected with the PBMCs. When NOD-SCID-gamma mice receiving both PBMCs and Tregs were also treated on day-1 and weekly thereafter with monoclonal antibody ADWA-11 that blocked αvβ8, the GVHD was significantly delayed to 35 days. Similar treatment with the anti-GARP antibody delayed GVHD onset to only day 26.

Recently (Dodagatta-Marri et al. 2021) the Sheppard lab again targeted the integrin αvβ8 on Treg cells with ADWA-11 to block their ability to activate TGFβ and thus suppress their tumour immunosuppressive function. Importantly, while monotherapy with either ADWA-11 or the checkpoint inhibitor anti-PD1 was moderately effective in suppressing growth of C57Bl/6 syngeneic carcinoma cell lines (CCK168-skin, EMT6-Breast, TRAMPC2-Prostate) combination therapy produced significantly prolonged survival (Dodagatta-Marri et al. 2021). Elimination of $CD8^+$ cells completely eliminated the anti-tumour effects of ADWA-11 treatment, implying that Treg-αvβ8 is responsible for CD8 T cell suppression of activity in cancer. The authors also showed that when mice bearing the radiosensitive

αvβ8-negative CT26 tumours were also treated with ADWA-11, this enhanced radiotherapy significantly, curing many mice who did not develop tumours when re-challenged with the same cell line.

An earlier study by Takasaka et al. (2018) reported that multiple tissues expressed pronounced levels of αvβ8 in associated carcinomas, including the lung (adeno- and squamous), ovary, skin, breast (DCIS and invasive ductal), prostate, colon, stomach, mouth and melanoma. To investigate its likely role in cancer, the authors established that MC38 (colon) and TRAMPC2 (prostate) carcinoma cell lines were both αvβ8 positive. Treatment with their αvβ8-specific blocking antibody C6D4 that blocks mouse and human αvβ8, abrogated growth of established tumours and significantly suppressed growth if combined with anti-PD1, 60% of tumours responding completely 70 days after the initial antibody treatment. C6D4 promoted increased tumour infiltration of CD8+ and CD4+ T cells and F4/80+ macrophages. Similar results were seen in the αvβ8-expressing LLC lung tumour-bearing mice exposed to C6D4 that also showed increased infiltration of activated NK cells (NK1.1 + IFNg+). Transcriptional studies showed suppression of αvβ8 restored the anti-tumour gene expression pattern of macrophages. Authors concluded that it was the tumour-expressed αvβ8 that suppressed the innate and immune cell tumour infiltrates. Taken together these studies show that simultaneous blockade of αvβ8 (or GARP) and PD-1, blocks Treg function, allows for tumour-specific CD8 cytotoxicity, increased tumour infiltration by innate and immune effectors and can complement the suitable standard therapy to generate cancer cures in mice (see Fig. 1). What is also clear from these studies is that αvβ8 represents a promising therapeutic target for improved treatment of cancer.

Anti-tumour immunity is also affected by GARP:L-TGFβ complex expressed on NK cells and platelets. Slattery et al. (2021) showed that peripheral blood NK cells from breast cancer patients were not fully functional with distorted mitochondria, dysfunctional metabolism and reduced IFNγ expression. This phenotype could be partially reversed by antibody inhibition of TGFβ. The authors noted that NK cells from many of the breast cancer patients showed increased GARP:L-TGFβ expression; when they used antibody inhibition of GARP this again significantly improved NK functionality recapitulating the effects of TGFβ blockade.

Platelets constitutively express GARP (Tran et al. 2009). When Rachidi and colleagues (Rachidi et al. 2017) generated mice where GARP was specifically deleted in platelets, they noted that levels of TGFβ in the peripheral blood were negligible. Moreover, when they knocked out the gene for TGFβ1 specifically in platelets, the TGFβ in peripheral blood remained similar to wild-type mice. The data suggest platelets are the main cell type regulating blood TGFβ but not the source of the TGFβ, and that they can activate latent TGFβ on other cells via their GARP expression. These studies did not examine which if any integrin was involved in this platelet-GARP upregulation of TGFβ in the blood. The authors proceeded to examine the role of platelet GARP on growth of syngeneic B16F1 melanoma and MC38 colon carcinoma tumours. Mice bearing platelet-deficient GARP, but not platelet-deficient TGFβ1, exhibited significantly suppressed growth of both B16F1 and MC38 tumours. In fact the anti-platelet drugs aspirin and clopidogrel treatment

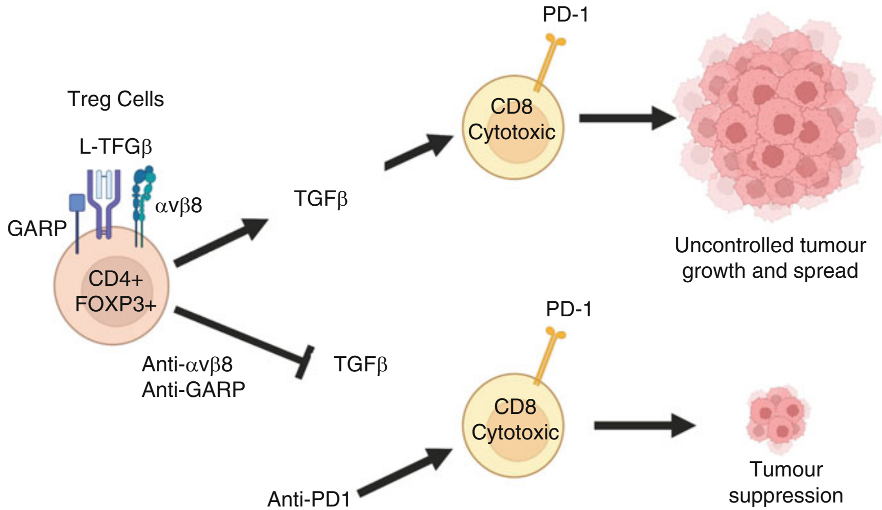

Fig. 1 Blockade of αvβ8 and PD-1 can restore tumour-specific CD8 cytotoxicity. Classical regulatory T cells (Tregs) that express CD4 and FOXP3, also express high levels of integrin αvβ8 and GARP bound to Latent-TGFβ. Treg cells activate TGFβ in an αvβ8-dependent manner and suppress the cytotoxic activity of CD8 T cells via a TGFβ-dependent mechanism, resulting in tumour growth and spread. In pre-clinical studies, antibody blockade of αvβ8 or GARP can inhibit the production of activated TGFβ by Treg cells, releasing CD8 T cells to kill cancer cells, a process enhanced by co-inhibition of PD-1. The result is inhibition or even elimination of established tumours. Created with Biorender

were sufficient to restore the anti-tumour cytotoxicity of adoptively transferred T cells. Metelli et al. (2020) again highlighted that platelet GARP:L-TGFβ is critical in tumour immunity. Excess numbers of platelets in the blood (thrombocytosis) occur in many cancers and are associated with poor survival. Metelli and colleagues discovered that the enzyme thrombin cleaved the GARP:L-TGFβ complex from platelets resulting in soluble GARP and GARP:L-TGFβ in cancer patient blood. This soluble GARP:L-TGFβ complex provided an immunosuppressive source of TGFβ since systemic treatment with thrombin-inhibitor altered the cellular tumour micro-environment towards effective tumour therapy, which was enhanced with anti-PD1 therapy (Metelli et al. 2020).

It is worth noting again that GARP is expressed not only by blood cells but by many other cell types, including hepatic stellate cells, mesenchymal stromal cells, endothelial cells, fibroblasts, and many types of cancer (reviewed in (Stockis et al. 2017b)). It is not clear that on non-Treg cells the GARP complexes with L-TGFβ and activates TGFβ in an integrin-dependent manner, but the wide expression of GARP does suggest the potential to locally regulate TGFβ activity. Additionally, the wide expression of both integrin αvβ6 (Saha et al. 2010) and αvβ8 (Slattery et al. 2021) on multiple types of cancer potentially offers many of these GARP-positive non-haemopoietic means of activating associated L-TGFβ. Additional data on

GARP-TGFβ axis is described in excellent recent reviews (Metelli et al. 2018; Bouchard et al. 2021).

3 Non-Treg Roles of αvβ8 Activation of TGFβ in Cancer

The functions and mechanisms of αvβ8 in biology are gradually being elucidated as beautifully described in (McCarty 2020). The cytoplasmic tail of the integrin β8 subunit is so divergent (Metelli et al. 2018), it does not retain the cytodomain motifs of other αv integrin β subunits that allow them to interact with multiple signalling and adaptor molecules (discussed in (Morse et al. 2014)). Thus the mechanisms of αvβ8-dependent functions in cancer are via its capacity to activate TGFβ or through novel intracellular signalling pathways unique to β8 (McCarty 2020).

Integrin αvβ8 is implicated strongly in the progression of glioblastoma (GBM) mostly through regulation of TGFβ. GBM progression is regulated significantly by TGFβ (reviewed in (Wick et al. 2006)). Roth and colleagues (Roth et al. 2013) sought to examine if expression of αv integrins might correlate with GBM disease. Immunohistochemistry revealed strong expression of αvβ5 and αvβ8, and low αvβ3. However when comparing Grade II versus the more aggressive Garde IV lesions, all three integrins showed significantly higher expression on Grade IV tumours, a result confirmed in separate studies (Schittenhelm et al. 2013). Knockdown of ITGAV, ITGB5 or ITGB8 with siRNA significantly and equally suppressed TGFβ signalling, leading authors to suggest αv integrin targeting as a therapeutic means to suppress GBM TGFβ. Vogetseder (Vogetseder et al. 2013) compared expression of αv integrins in primary tumours and brain metastases. Data showed that in brain metastases from breast, lung and renal cancer higher expression of αvβ8 (and αvβ3, αvβ5) was detected compared with primary tumours. Reyes et al. (2013) provided biological support for these largely observational studies. When LN229 GBM tumour cells were transduced with β8 shRNAs, the tumour inoculated into the brain parenchyma of mice grew larger but no longer exhibited the typical aggressive invasive activity. Biochemical analysis revealed that Rho GTPase (especially Rac1 and CDC42) activity was significantly enhanced in GBM cells transfected with β8 siRNAs. Subsequent pull-down studies revealed that integrin β8 cytoplasmic tail binds to RhoGDI1, a molecule that binds the inactive GDP-bound form of Rac1. Knockdown of RhoGDI1 resulted in increased levels of GTP-bound Rac1 and CDC42 and a reduction in GBM cell invasion in 3D Matrigel assays. Thus β8 promotes GBM invasion by sequestering RhoGDI1 and reducing activated Rho proteins. Guerrero and colleagues (Guerrero et al. 2017) reported that the tumour initiating stem cells from GBM expressed significant levels of β8. When they sorted freshly isolated GBM stem cells (CD133+) into β8-high and β8-low populations, compared with β8-low cells, the β8-high cells formed neurospheres more successfully and survived longer in vitro, and formed well-vascularized invasive tumours after injection into brain parenchyma. Interestingly the β8-high fraction expressed low TGFBR2 levels, whereas β8-low fraction had much greater TGFBR2

expression; both populations responded to exogenous TGFβ1 by reduced proliferation and reduced neurosphere formation. The authors proposed that in vivo that the β8-high stem cell fraction bound latent TGFβ1/3 in the matrix, activating TGFβ that acted on adjacent β8-low cells suppressing their growth and promoting their differentiation. This model explained why the β8-high sub-population were more resistant to TGFβ signals (low TGFBR2) and could remain proliferative, less differentiated, self-renew and promote GBM.

In recent studies Jin et al. (2019) noted that β8 was elevated in pancreatic ductal carcinoma and correlated with significantly poorer survival. Analysis of multiple PDAC cell lines revealed that αvβ8 was located mostly in the Golgi and perinuclear regions, unusual locations for αv integrins. Genetic knockdown of ITGB8 PDAC lines resulted in increased sensitivity to ionizing radiation and the chemotherapeutic gemcitabine.

The GBM and PDAC studies described above reveal that αvβ8 activity is highly variable depending on the cell of origin but also the differentiation state of cells within tumours. As reliable reagents for the detection and blockade of αvβ8 become more widely available, we will see a greater clarity as to which other cancers αvβ8 is actively involved in modulating.

4 Targeting Integrin-Dependent TGFβ Signalling in Cancer

In our earlier review (Brown and Marshall 2019), we highlighted that the biology of αv-mediated activation of TGFβ should be leading to an increase in clinical trials that included such strategies as part of combination therapies against fibrosis and cancer. Over the last 5–10 years an increasing number groups have produced a variety of small molecules, peptides, peptide mimetics and antibodies that inhibit αv integrins at low nM concentrations. Many of these compounds are elegantly described and discussed elsewhere (Ludwig et al. 2021; Slack et al. 2022). Despite the rapidly accumulating evidence that targeting specific αv integrins could significantly enhance therapy in cancer there remains too little clinical activity to see the pre-clinical progress turn into treatment efficacy. However, perhaps small glimmers of light suggest positive changes are ahead.

Table 1 is developed from clinicaltrials.gov and shows all studies targeting integrins in cancer. It can be seen that from 2001 to 2011, 18 studies most used compounds directed against αvβ3, αvβ3/αvβ5 or pan-αv. None of these studies have resulted in the development of effective drugs for cancer treatment. This lack of clinical and commercial success for the sponsors probably explains the 7 year gap before four different companies registered to undertake new anti-cancer trials using anti-integrin targets. In 2018 SFJ Pharmaceuticals planned to use the Merck KGaA humanized pan-αv blocking antibody Abituzumab that targets all αv integrins (NCT03688230). This antibody was used in the earlier Phase 1/2 POSEIDON trial

Table 1 Anti-integrin anti-cancer trials registered in clinicaltrials.gov (till June 2022)

Start year	Sponsor	Target	Drug type	Drug name(s)	Cancer	Phase	Clinicaltrials.gov identifier
2001	Memorial Sloan Kettering Cancer Centre	αvβ3	Antibody	MEDI522/ etaracizumab	Colon	1/2	NCT00027729
2001	MedImmune LLC	αvβ3	Antibody	MEDI522/ etaracizumab	Colon and others	1	NCT00284817
2003	MedImmune LLC	αvβ3	Antibody	MEDI522/ etaracizumab	Prostate	2	NCT00072930
2003	MedImmune LLC	αvβ3	Antibody	MEDI522/ etaracizumab	Melanoma	2	NCT00066196
2005	Centocor, Inc.	Pan-αv	Antibody	Intetumumab (CNTO 95)	Melanoma	1/2	NCT00246012
2005	National Cancer Institute (NCI)	αvβ3/αvβ5	Cyclic-peptide	Cilengitide	Glioblastoma	2	NCT00112866
2005	MedImmune LLC	αvβ3	Antibody	MEDI-522/Abergrin	Melanoma	1	NCT00111696
2006	Merck Sharp & Dohme LLC	Pan-αv/ α5β1	Small molecule	MK-0429	Prostate/bone metastases	1	NCT00302471
2007	Centocor, Inc.	Pan-αv	Antibody	Intetumumab (CNTO 95)	Prostate	2	NCT00537381
2008	Merck KGaA, Darmstadt	αvβ3/αvβ5	Cyclic-peptide	Cilengitide	SCC Head and Neck	1/2	NCT00705016
2008	ImmunoGen,Inc.	Pan-αv	Antibody-drug conjugate	IMGN388 (aka CENTO95-DM4)	Solid tumours	1	NCT00705016
2008	EMD Serono	αvβ3/αvβ5	Cyclic-peptide	Cilengitide	Glioblastoma	3	NCT00689221
2008	National Cancer Institute (NCI)	Pan-αv	Antibody	MEDI522/ etaracizumab	Metastatic Kidney	1/2	NCT00684996
2009	Merck KGaA, Darmstadt,	αvβ3/αvβ5	Cyclic-peptide	Cilengitide	NSCLC	2	NCT00842712

(continued)

Table 1 (continued)

Start year	Sponsor	Target	Drug type	Drug name(s)	Cancer	Phase	Clinicaltrials.gov identifier
2009	Merck KGaA, Darmstadt	Pan-αv	Antibody	Abituzumab (EMD525797)	Ras wild-type, metastatic colon	1/2	NCT01008475
2010	National Cancer Institute (NCI)	αvβ3/αvβ5	Cyclic-peptide	Cilengitide	Glioblastoma	1	NCT01122888
2011	Galapagos NV	Pan-αv	Small molecule		Solid cancers	1	NCT01313598
2011	Merck KGaA, Darmstadt	Pan-αv	Antibody	Abituzumab (EMD525797)	Solid cancers	1	NCT01327313
2018	SFJ Pharmaceuticals X, Ltd	Pan-αv	Antibody	Abituzumab (EMD525797)	High β6, Ras wild-type, metastatic colon,	2	NCT03688230
2019	Pfizer	αvβ8		PF-06940434	Solid cancers	1	NCT04152018
2020	OncoSynergy, Inc.	β1	Antibody	OS2966	Glioblastoma	1	NCT04608812
2021	ProDa BioTech, LLC	αvβ3	Rationally-designed protein[53]	Pro-Agio	Pancreas, other solid	1	NCT05085548

SCC squamous cell carcinoma, *NSCLC* non-small cell lung carcinoma

(NCT01327313), to treat KRas wild-type metastatic colorectal cancer patients with Abituzumab at either of two doses (500 or 1000 mg/every 2 weeks) with standard of care (cetuximab and irinotecan). While the study did not achieve its primary objective (investigator-assessed progression-free survival) post-analysis revealed that patients with high αvβ6 on their tumours did benefit from longer overall survival in both Abituzumab groups (Élez et al. 2015). It is interesting to speculate that as the same antibody would likely have inhibited αvβ8-mediated TGFβ activation on Treg cells, combination with anti-checkpoint drugs could enhance the anti-cancer efficacy of Abituzumab further. The trial design for SFJ Pharmaceuticals also suggested treatment with Abituzumab plus standard of care in Ras wild-type metastatic colorectal cancer patients, this time pre-screened to have left-sided disease with high αvβ6 expression. Such a preselection would likely have seen the earlier POSEIDON study achieve its primary objectives. Thus it was disappointing that the SFJ Pharmaceuticals trial was withdrawn for unknown reasons, as this is a logical clinical study design. In 2019 Pfizer launched their Phase 1 trial (NCT04152018) testing their αvβ8-blocking molecule PF-06940434 in combination with their anti-PD1 drug PF-06801591 in patients bearing metastatic carcinomas. Although this is just a safety/efficacy/dose-determining trial, this is the first anti-integrin trial in cancer that mirrors very recent pre-clinical benefits (Dodagatta-Marri et al. 2021; Takasaka et al. 2018) of αvβ8/PD-1 simultaneous therapy discussed above. The study data will be eagerly awaited. In an interesting study, Oncosynergy Inc. is seeking to test an integrin β1-subunit blocking antibody (OS2966) in glioblastoma patients undergoing surgical resection. The authors intend to pump the antibody directly into the brain tissue through catheters inserted into the tumours (referred to as 'convection-enhanced delivery'). This study is developed from the work of Lee et al. who described the use of the pan-β1 integrin blocking antibody OS2966 enhancing the efficacy of oncolytic herpes simplex virus-mediated therapy of triple-negative breast cancer and glioblastoma (Lee et al. 2019). Finally, in the trial sponsored by ProDa BioTech (NCT05085548), ProAgio, a rationally-designed protein that binds αvβ3 at a site distant from the ligand-binding site (Turaga et al. 2016), will be administered to patients with pancreatic and other solid cancers that have failed other therapy. These authors reported that ProAgio induced apoptosis in αvβ3 expressing cells, including pancreatic stellate cells, and increased survival of transgenic mice with pancreatic cancer (Turaga et al. 2021). Thus, while only one-out of-four of these new recent trials addresses integrin-mediated TGFβ activation directly, it is encouraging that four companies new to integrin targeting entered the race to developing anti-integrin therapies for cancer.

5 Conclusion

The accumulating data on integrin targeting are gradually developing into clearer therapeutic strategies for the treatment of patients diagnosed with cancer. For many cancer patients that present in the clinic, their tumours will already have a highly

immunosuppressive TME rich in Tregs, M2 macrophages and N2 neutrophils (both TGFβ induced immunosuppressive cells-discussed in (Brown and Marshall 2019)), platelets, and be low in functional CD8 and NK effector cells, in part through increased expression of checkpoint inhibitors. As described above, this whole TME pattern can be driven entirely by integrin-activated latent-TGFβ. As the TGFβ may be activated by integrins αvβ8 on Treg cells (Edwards et al. 2014; Worthington et al. 2015), αvβ6 and αvβ8 on cancer cells (Takasaka et al. 2018; Saha et al. 2010), αvβ1 and αvβ5 integrins on CAFs (discussed in (Brown and Marshall 2019)) and thrombin (and probably integrins) from platelets (Metelli et al. 2020), effective therapy requires inhibition of multiple αv integrins, platelets and checkpoint inhibitors, to restore host anti-cancer immune responses. For each cancer patient, it is likely that the balance of sources of activated TGFβ in their cancers is likely to vary (for example more TGFβ from CAFs in desmoplastic tumours (Mariathasan et al. 2018), more TGFβ from platelets if thrombocytosis occurs (Metelli et al. 2020)) and thus therapies may need to be tailored to each patient. By inhibiting the local mechanisms in tumours that generate activated-TGFβ (GARP, αvβ6, αvβ8, platelets) such combination anti-immune therapies, managed in combination with conventional radio- and chemotherapy, offer great promise for the future of cancer therapy.

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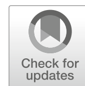
Part III
Human Disease

α11β1: A Mesenchymal Collagen-Binding Integrin with a Central Role in Tissue and Tumor Fibrosis



Cédric Zeltz and Donald Gullberg

Abstract With a current focus on studying fibroblast heterogeneity in different conditions where extracellular matrix (ECM) dynamics are part of the tissue homeostasis and pathology, it is increasingly important to also understand the underlying cellular mechanism of tissue and tumor fibrosis. In this context we predict that collagen-binding integrins will turn out to play an important role in matrix remodeling in fibrotic diseases in specific fibroblast subsets.

Data from experimental cancer models have questioned the exclusive tumor-supportive function of the tumor stroma and suggested that the stroma might also act as a barrier to inhibit tumor metastasis. In the context of tissue fibrosis, a barrier function of the fibrotic ECM affects access of anti-fibrotic therapeutic reagents. In all types of fibrotic diseases, a major focus currently concerns fibroblast heterogeneity largely based on transcriptional profiling and the role of different subpopulations of fibroblasts in the fibrotic process. It is important to remember that current transcriptional RNA-seq profile data, which now appear in many publications, will have to be confirmed at the protein level and at multiple tissue levels. In the current chapter we will focus on the role of the collagen-binding integrin α11β1 in fibrosis based on our own work as well as published datasets with information on α11 mRNA expression.

1 Introduction

Integrin α11β1 (ITGA11) is the last addition to the integrin family and has been characterized as a receptor for fibrillar collagens involved in cell migration, cell proliferation, and collagen reorganization (Tiger et al. 2001a, b; Zeltz and Gullberg 2016a, b; Erusappan et al. 2019; Zeltz et al. 2022). Its expression appears to be specific to mesenchymal non-muscle cells and more specially, in normal tissue development and adult tissue homeostasis, α11β1 is restricted to mesenchymal

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stem cells and subsets of fibroblasts (Tiger et al. 2001a, b; Popova et al. 2004; Popov et al. 2011; Shen et al. 2019).

To summarize some central facts about integrin α11β1: (1) under normal conditions it is expressed on mesenchymal stem cells and fibroblasts in vivo (Popova et al. 2004; Shen et al. 2019), (2) its levels are regulated by TGF-β (Lu et al. 2010), (3) it contributes to collagen remodeling (Tiger et al. 2001a, b; Schulz et al. 2015), and (4) it induces myofibroblast differentiation (Carracedo et al. 2010). All these properties have qualified α11β1 as an important actor during wound healing; a role confirmed in excisional wound healing studies (Schulz et al. 2015). Since wound healing, fibrosis, and tumor stroma interactions share similar features, it is logical to also discuss the role of integrin α11β1 in tissue and tumor fibrosis (Fig. 1). Below we will summarize published mechanistic studies on the role of α11 in these processes and also relate these findings to recent published transcriptional profiling datasets.

2 Re-definition of Fibroblast Heterogeneity

In a landmark paper Buechler et al. have assembled bioinformatic data resulting in an overview of fibroblast heterogeneity, based on transcriptional profiles in steady state and "perturbed" mouse tissues as well as in "perturbed" human tissues (Buechler et al. 2021). In this study a number of genes are expressed in the different fibroblast subsets, and we will not be able to go through the specifics of all these markers, including the corresponding proteins and their characteristics and will just explain abbreviations for some of the major biomarkers. In the steady-state mouse atlas, Buechler and colleagues identified ten fibroblast clusters of which two are pan-tissue fibroblasts (distinguished by Pi16$^+$(peptidase inhibitor 16; a GPI anchored membrane protein) or Col15a1$^+$(collagen XV; a secreted extracellular matrix (ECM) protein) expression, but both also expressing Dpt (dermatopontin; a secreted proteoglycan)) and the eight remaining suggested to be specialized tissue fibroblast subtypes (Ccl19$^+$, Coch$^+$, Comp$^+$, Cxcl12$^+$, Fbln1$^+$, Bmp4$^+$, Npnt$^+$, and Hhip$^+$). To create the perturbed mouse fibroblast atlas, the authors integrated 17 scRNA-seq datasets from 13 tissue-associated disease models. Using their approach, they found that the fibroblast population could also be divided into ten clusters. The universal Pi16$^+$ and Col15a1$^+$ subsets as well as the Comp$^+$, Ccl19$^+$, Cxcl12$^+$, Hhip$^+$, and Npnt$^+$ subpopulations were found to be similar to the analogous steady-state fibroblast subtypes. Pi16$^+$ fibroblasts were found in perivascular niches and Col15a1 expression was noted deeper inside parenchymal tissues. However, three clusters (Lrrc15$^+$, Cxcl5$^+$, and Adamdec1$^+$) appeared to represent activated fibroblasts that are specific to the perturbed state. Thus, this analysis shows the existence of universal-, tissue specific-, and disease-specific subpopulations of fibroblasts (Buckley 2021).

Based on the data from Buechler et al., we have analyzed integrin α11 expression in the different clusters. Integrin α11 displayed some low expression on the universal Dpt$^+$Pi16$^+$ and Dpt$^+$Col15a1$^+$ fibroblast populations in both normal and perturbed

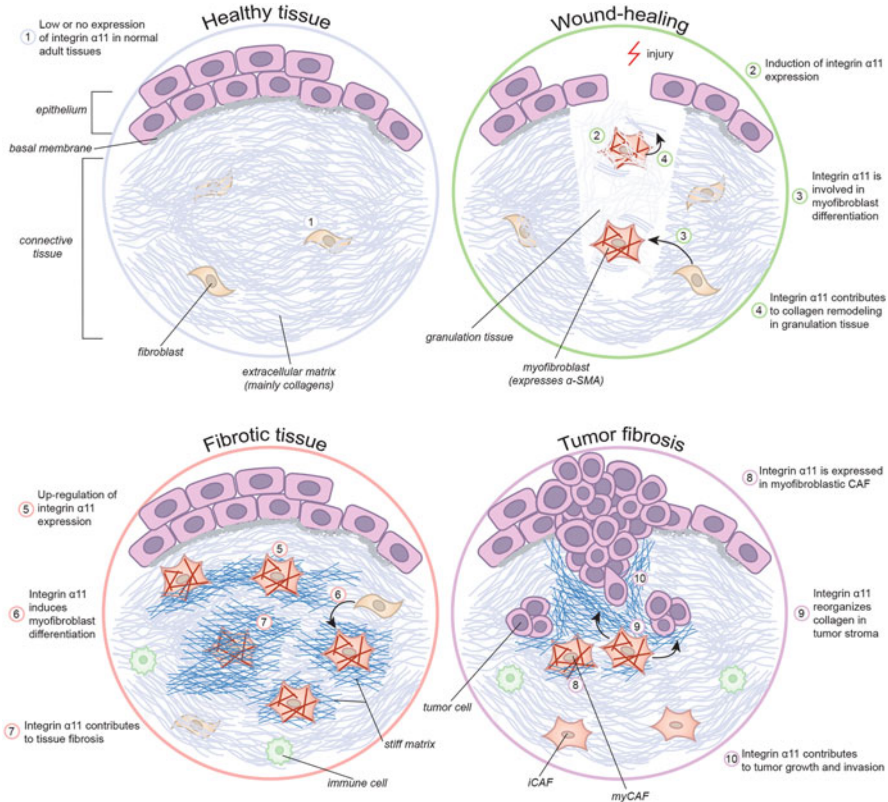

Fig. 1 Role of integrin α11β1 in wound healing and fibrosis**.** In adult healthy tissues, integrin α11β1 is lowly expressed or not detectable at all. During wound healing, the wound is filled with the granulation tissue formed by myofibroblasts. Integrin α11β1 expression is induced in fibroblast after wounding and contributes to myofibroblast differentiation and collagen reorganization in the granulation tissue. Fibrotic tissues are characterized by excessive deposition of extracellular matrix and fibroblast proliferation. During this process, integrin α11β1 expression is upregulated and α11β1 induces myofibroblast differentiation. It contributes to tissue fibrosis presumably by reorganizing the collagen matrix and stiffening the tissue. In tumor tissue, fibrosis is mediated by subsets of cancer-associated fibroblasts (CAF) with myofibroblastic features (myCAFs). Integrin α11β1 expression is mainly induced in myCAF and participates in collagen reorganization in the tumor stroma to promote tumor growth and invasion. iCAFs, inflammatory CAFs

mouse tissues. Buecheler et al. showed that $Dpt^{+}Pi16^{+}$ fibroblasts express high levels of genes associated with stemness and predict that these cells can differentiate into another fibroblast population such as the $Lrrc15^{+}$ cluster that is a “perturbation-specific” cluster found in cancer tissues and not present in the steady-state tissues. Interestingly, our analysis of the published dataset does indicate expression of integrin α11 in the $Lrrc15^{+}$ fibroblast subcluster. This transcriptional profile is present in mouse cells from wounds, fibrotic tissues, and pancreatic ductal

carcinoma (PDAC) and is characterized by α-smooth muscle actin (α-SMA, Acta2) and periostin (Postn) mRNA expression. Buechler et al. suggest that these fibroblasts represent a specific myofibroblast population. Interestingly, the expression of integrin α11 mRNA in the Lrrc15$^+$ fibroblasts in the assembled dataset was more obvious in the perturbed human tissues. Based on data from three human tissues, the Lrrc15+ myofibroblast-like cluster is found to be enriched with cells from lung and pancreatic cancers. In contrast, ITGA11 was not highly expressed in the myofibroblast cluster characterized by COL3A1$^+$ and enriched from patients with COVID-19.

The work of Buechler and colleagues, suggesting the existence of universal fibroblast lineages, requests an update of the current view on the source of activated fibroblasts in different disease conditions. It will be important to determine how these pan fibroblasts and the different specialized tissue subtypes can be related to the fibroblast subtypes already described (something we will discuss in sections below). Since we observed low mRNA expression of integrin α11 in some universal fibroblast datasets, it will in the future be important to determine the role of α11 in these fibroblast types as well as its detailed role in myofibroblast activation in these experimental systems.

3 Integrin α11β1 in Wound Healing

The strong induction of integrin α11 expression 7 days after inflicting excisional wounds on mouse back skin (Zweers et al. 2007) prompted us to investigate its role during dermal wound healing. Using α11-deficient mice, we could show that dermal wounds displayed reduced granulation tissue 7 days after wounding due to a defect in myofibroblast differentiation (Schulz et al. 2015).

This finding is in agreement with previous in vitro studies in which α11β1 was shown to contribute to TGF-β-induced myofibroblast differentiation (Carracedo et al. 2010). The α11$^{-/-}$ wounds displayed poorer tensile strength, indicating that integrin α11 is crucial for collagen remodeling in granulation tissue. We also demonstrated that integrin α11β1 is the main collagen receptor in dermal fibroblasts which contributes to early collagen remodeling in a TGF-β-dependent manner (Schulz et al. 2015). Mechanistically, integrin α11-mediated collagen reorganization involves a cross-talk with TGF-β signaling, through the non-canonical c-Jun N-terminal kinase (JNK) (Fig. 2). Further details of this collaboration between α11β1 and JNK remain to be determined.

Another interesting finding in our study was the increased number of adipocytes observed in the granulation tissue of defective α11$^{-/-}$ wounds (Schulz et al. 2015). It will be interesting to determine the source of these adipocytes.

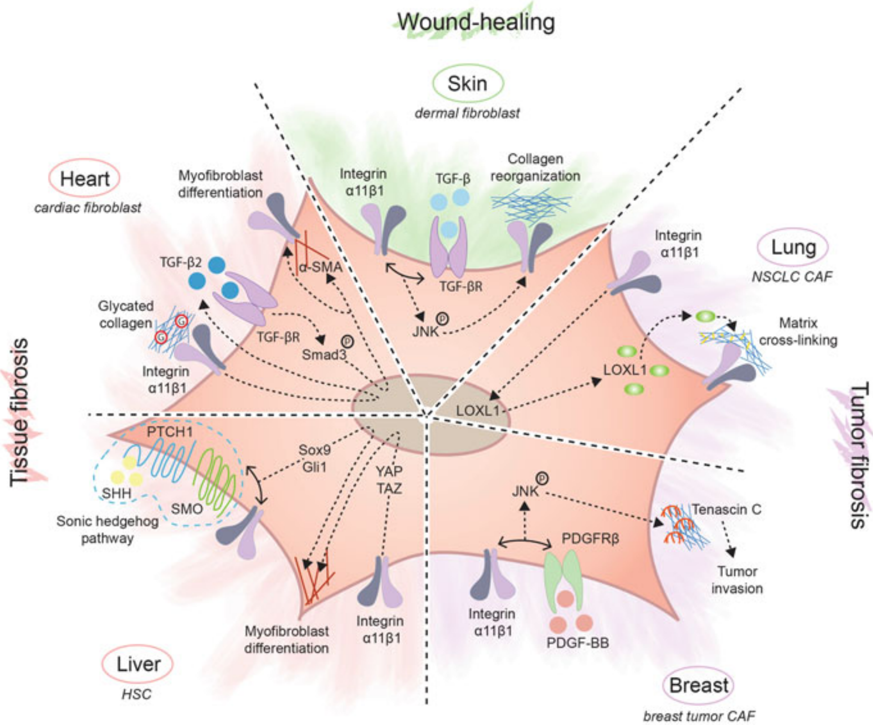

Fig. 2 Integrin α11β1 mechanistic in wound healing and fibrosis. During wound healing, integrin α11β1 in dermal fibroblasts cross-talks with TGF-β signaling to activate c-Jun N-terminal kinase (JNK), leading to collagen reorganization. In cardiac fibrotic tissue, interaction of integrin α11β1 on cardiac fibroblasts with glycated collagen increases expression of TGF-β2, which in turn contributes to myofibroblast differentiation by inducing α-smooth muscle actin (α-SMA) expression via Smad3 activation. In hepatic stellate cells (HSC) in vitro, integrin α11β1 has been suggested to cross-talk with the sonic hedgehog pathway to increase Sox9 and Gli1 expression to contribute to myofibroblast differentiation (SHH: sonic hedgehog ligand; SMO: smoothened; PTCH1: patched-1 receptor). Abrogation of integrin α11β1 in cultured HSC in vitro was found to result in reduced YAP and TAZ expression and to a defect of myofibroblast differentiation. In non-small cell lung cancer (NSCLC), integrin α11β1 on cancer-associated fibroblasts (CAFs) regulates LOXL1 (lysyl oxidase-like 1) expression, which contributes to matrix cross-linking promoting tumor invasion. In breast cancer, integrin α11β1 cross-talks with PDGFR-β in CAFs to activate JNK that leads to deposition of tenascin C in tumor stroma to promote tumor invasion

4 Integrin α11β1 in Tissue Fibrosis

Fibrosis is a condition where tissue repair is dysregulated and which is characterized by excessive deposition of ECM and fibroblast proliferation, which ultimately lead to organ dysfunction or failure (Friedman et al. 2013; Plikus et al. 2021). It is becoming clear that a characterization of fibroblast heterogeneity is essential in order to reach a better understanding of the fibrosis mechanisms and to optimize

adequate therapeutic strategies and regimens. With the emergence of single-cell RNA sequencing (scRNA-seq), an increasing number of studies have analyzed the different subpopulations of fibroblast involved in tissue homeostasis and fibrotic disease (Dobie et al. 2019, 2021; Adams et al. 2020; Deng et al. 2021).

4.1 Integrin α11 in Skin Fibrosis

In a recent study Deng et al. performed scRNA-seq analysis of keloid and normal scar dermis (Deng et al. 2021). They could divide fibroblasts into 13 subclusters which in turn could be grouped to form four subpopulations: secretory-papillary, secretory-reticular, pro-inflammatory, and mesenchymal fibroblasts. The mesenchymal subpopulation, enriched in $CD266^{+}/CD9^{-}$ fibroblasts, was shown to be more important in keloids compared to normal scars. This fibroblast population displayed increased expression of POSTN (periostin) and COL11A1 (collagen type XI), increased activation of TGF-β signaling and is associated with ECM organization. We inspected integrin α11 mRNA levels in this dataset. In normal scar tissue, mRNA data suggest expression of α11 in a secretory-reticular fibroblast population, whereas in fibrotic skin, the expression of integrin α11 mRNA was restricted to the mesenchymal fibroblast cluster. It is interesting to note that in this study only a part of integrin $\alpha11^{+}$ fibroblasts stained positive for α-SMA.

ScRNA-seq performed in the progressive fibroproliferative Dupuytren's disease, composed of Dupuytren's disease tissue, non-pathogenic Skoog's fascia, and healthy dermis, identified four mesenchymal clusters (Dobie et al. 2021). In this dataset a cluster almost exclusively formed by cells isolated from Dupuytren's disease was recognized as $PDPN^{+}$ myofibroblasts, Which express FAP (Fibroblast activation protein) mRNA, ACTA2 (α-smooth muscle actin), and ITGA10 (integrin α10). Surprisingly, integrin α11 mRNA was only noted in a restricted number of cells of the myofibroblast cluster. In this study, more abundant integrin α11 mRNA was observed in cells of the fibroblast cluster Skoog's fascia. It is interesting to note that platelet-derived growth factor receptor A (PDGFRA) expression was noted in fibroblasts of the same subcluster with detected integrin α11 mRNA. The authors suggested in their study that $PDPN^{+}$ myofibroblasts differentiate from resident fascial PDGFRA+ fibroblasts. Since it is known that integrin α11 is involved in myofibroblast differentiation, it is tempting to speculate that integrin α11 might participate in the early stages of the Dupuytren's disease.

In a collaborative work with Schultz et al. at University of Cologne, we have investigated the contribution of integrin α11β1 to dermal fibrosis (Schulz et al. 2018). Using a mouse model of scleroderma based on bleomycin injections, we demonstrated that $\alpha11^{-/-}$ mice were protected from the development of fibrosis in the dermis, while wildtype mice exhibited an increase of dermis thickness and the loss of adipose tissue. In contrast, the integrin α1 and α2 mutants displayed fibrotic features similar to the wildtype mice, indicating that integrin α11 could be a prominent key player in the pathogenesis of scleroderma.

4.2 Integrin α11 in Cardiac Fibrosis

The role of integrin α11 in cardiac fibrosis has been first studied in diabetic cardiomyopathy, a pathology in which the presence of high levels of glucose induces collagen glycation, a non-enzymatically post-translational modification, which may interfere with cellular interactions with the ECM. Together with Talior-Volodarsky et al. we have seen that the interaction of cardiac fibroblasts with glycated collagen via α11β1 integrin in a diabetic rat model (streptozotocin (STZ)-treated) increased TGF-β2 expression, which in turn induced α-SMA expression (Talior-Volodarsky et al. 2012) (Fig. 2). The interaction of integrin α11β1 with glycated collagen is reduced, but is still sufficient to stimulate myofibroblast differentiation. In these conditions, the expression of integrin α11 is increased, possibly occurring in an attempt by the cells to compensate for reduced adhesion. The study by Talior-Volodarsky suggests that TGF-β2 is responsible for the increased levels of α11 expression in a mechanism involving Smad3 binding to an uncharacterized α11 promoter site (distinct from the site previously described to induce TGF-β1-stimulated ITGA11 transcription) (Lu et al. 2010; Talior-Volodarsky et al. 2015). In collaboration with Civitarese and colleagues we have further demonstrated that in an STZ-mouse diabetic model, the depletion of integrin α11 attenuates heart fibrosis, with a reduction of fibrillar collagens (Civitarese et al. 2016). In contrast, STZ-treated $\alpha11^{+/+}$ mice display increased collagen type I and III in the left ventricle. It will be important to also sort out the role of α11 in other cardiac fibrosis models and to establish α11 expression in different cardiac fibroblast subsets.

In an effort to identify the role of α11 in different tissues we generated a transgenic mouse model in which integrin α11 is overexpressed in different tissues and notably in the heart (Romaine et al. 2018). In this transgenic model α11 overexpression mainly occurred in the cardiac myocyte, normally not expressing α11. Interestingly, the overexpression of integrin α11 in cardiomyocytes led to hypertrophy of the left ventricle, with an increase of collagen type I and III expression, denoting a development of fibrosis and supporting a pro-fibrotic role of α11. Gene differential analysis of transgenic fibrotic heart revealed an overexpression of TGF-β2 and TGF-β3, of NF-κB signaling that mediates cardiac hypertrophy, and of genes associated with cardiomyopathy. In this context it will be important to also investigate the role of α11 in mouse models in which heart failure is induced by pressure overload.

Historically the relative number of fibroblasts in the heart has been a controversial issue. The most recent data reports a proportion of cardiac fibroblasts (cFbs) in heart, from approximately 20% to 60% in rats and humans (Vliegen et al. 1991; Banerjee et al. 2007; Ongstad and Kohl 2016; Tucker et al. 2020). cFb numbers increase with development, aging, and disease. Banerjee et al. showed that, in both rats and mice, the number of fibroblasts increases postnatally until adulthood is reached (≈30% to ≈64% in rat and ≈10% to ≈25% in mouse) (Banerjee et al. 2007). An increase in cFbs has also been demonstrated in heart failure and following myocardial infarction (MI) (Sridhar et al. 2017; Nagaraju et al. 2019; Wang et al. 2020).

Regarding fibroblast heterogeneity of cardiac fibroblasts, Litviňuková et al. identified seven fibroblast subpopulations in healthy heart using an adult human heart cell atlas (Litviňuková et al. 2020). Analysis of this dataset demonstrated low ITGA11 expression essentially in three fibroblast clusters: one cluster enriched in ventricles and the other one in atria and which both express canonical fibroblast genes. The third subpopulation expressing ITGA11 was characterized by expressing genes involved in TGF-β signaling and involved in ECM production and organization. We predict that the fibroblast subpopulations that express integrin α11 might be engaged in cardiac fibrosis, especially the cluster associated with TGF-β signaling, which most likely would have an impact on collagen reorganization.

4.3 Integrin α11 in Fibrotic Lung

In a pulmonary fibrosis study, Habermann et al. identified four fibroblast clusters that they named: fibroblast, ACTA2^{+} myofibroblast, PLIN2^{+} lipofibroblast-like, and HAS1^{hi} fibroblast, the last three fibroblast populations being enriched in fibrotic lung (Habermann et al. 2020). Has1^{hi} fibroblasts were found to be restricted to the subpleural region of fibrotic lung and to express genes associated with cellular stress and epithelial-mesenchymal transition. When we analyzed this dataset, we could observe higher expression of integrin α11 mRNA in chronic interstitial lung diseases compared to non-fibrotic tissues. ITGA11 was in this dataset mostly present in the myofibroblast and HAS1^{hi} fibroblast populations, which localize to separate areas in the lung. This observation was confirmed from another study that focused on idiopathic pulmonary fibrosis (IPF) (Adams et al. 2020). Analysis of integrin α11 mRNA in this dataset has identified ITGA11 expression in a myofibroblast subtype and in an invasive fibroblast population characterized by high expression of HAS1 in IPF lung. Interestingly, using lineage reconstruction the authors suggested that IPF myofibroblasts are more likely to arise from myofibroblasts already present in the healthy lung rather than from resident fibroblasts, implying that integrin α11 expressed on invasive fibroblasts would have a role distinct from that of supporting myofibroblast differentiation, such as maintaining the active myofibroblast state.

4.4 Integrin α11 in Fibrotic Liver

Hepatic stellate cells (HSC), which are considered a type of pericyte, are regarded as the main source of myofibroblasts in liver fibrosis (Iwaisako et al. 2014). Depletion of integrin β1 in rat HSC in vitro reduced myofibroblast differentiation, migration, and collagen production (Martin et al. 2016). The authors suggested that these effects might be the result of the loss of integrin α11β1, since they observed an increase of integrin α11 expression after activation of HSC-derived myofibroblasts in vitro and a decrease of cell migration following ITGA11 knockdown in these

cells. In the same study, the authors find that downstream of integrin β1 signaling, the mechanosensitive transcriptional co-activator YAP regulated the myocontractile MYL9, and together with PAK, contributed to the HSC-derived myofibroblast activation. Abrogation of integrin α11 or absence of integrin β1 resulted in decreased PAK, YAP, and MYL9 expressions, and interestingly, YAP seemed to be required for full expression of integrin α11β1. It is important to note that a majority of this study was done on HSC cells cultured in vitro. A separate study using human HSC, commercial α11 antibodies and various fibrosis animal models also observed that integrin α11 was upregulated in mouse CCL_4-treated liver and in human cirrhotic liver (Bansal et al. 2017). Knockdown of integrin α11 in HSC in vitro led to the downregulation of several fibrosis-related genes such as Acta2 and to inhibition of HSC activation. However, Bansal and colleagues suggest that the regulation of integrin α11 expression in fibrotic HSC involves hedgehog signaling (Fig. 2). They show that sonic hedgehog ligand positively regulated integrin α11 and using a selective inhibitor of the hedgehog pathway, Erismodegib, they confirmed the reduced expression of integrin α11 in TGF-β-activated HSC in vitro and in the in vivo mouse fibrotic model. Both fibrotic liver studies are interesting and well performed, but the majority of the data relate to HSC cultured in vitro. We are aware that culture conditions (e.g., stiffness and TGF-β-containing serum) induce expression of integrin α11 in cell type that normally do not express α11 in vivo. It is actually uncertain whether integrin α11 is induced in HSC in vivo at all. Further studies using α11 mAbs in combinations with more biomarkers are required to definitely conclude this. Finally, to be able to unequivocally rule out a role for integrin α11 in liver fibrosis in the mouse, in vivo experiments using $\alpha11^{-/-}$ mice are needed.

In a separate study of CCl_4-induced fibrotic mouse liver, three mesenchymal subpopulations were identified: $CD34^+$ fibroblasts, reelin $(Reln)^+$ HSCs, and calponin 1 $(Cnn1)^+$ vascular smooth muscle cells (Dobie et al. 2019). Examining the dataset, integrin α11 mRNA expression was concentrated in the fibroblast cluster with only few cells expressing α11 in the HSC population that may correspond to the myofibroblast fraction of the central vein-associated HSCs. In human liver cirrhosis (Ramachandran et al. 2019), integrin α11 mRNA expression was noted in the myofibroblast subpopulation that expands in the disease. This data needs to be confirmed at protein level.

Several experimental in vivo models exist to study liver fibrosis (Yanguas et al. 2016), such as the chemical-based models including CCl4-induced fibrosis used in the aforementioned studies, which leads to HSC activation, but in addition genetically modified models and surgery-based models exist. One surgery-based method includes bile duct ligation causing periportal biliary fibrosis. In this model, portal fibroblasts are the source of the myofibroblasts that take part of the portal fibrosis (Iwaisako et al. 2014). Regarding our studies and analyses on integrin α11 expression in liver, it becomes clear that integrin α11 is more likely expressed on fibroblasts rather than HSC and it is thus important to consider which model to choose when studying the role of this collagen-binding integrin in liver fibrosis. Furthermore, at this point it is thus not clear whether α11β1 function in vivo in mouse liver

fibrosis varies with the model used, if the role of α11 in HSC in fibrosis is restricted to in vitro models alone, and if α11 is involved in human liver fibrosis.

In summary, our analyses of scRNA-seq databases in fibrotic tissues mostly identified expression of integrin α11 mRNA in myofibroblast subpopulations, which is an active cell type in fibrotic diseases. In addition, Bansal et al. (Bansal et al. 2017) found induction of integrin α11 expression, which correlates with α-SMA expression, in ex vivo fibrotic lung and kidney. TGF-β is a major growth factor involved in fibrotic disease (Budi et al. 2021), and since it regulates integrin α11 expression in fibroblast, it is not surprising to observe an increase of integrin α11 expression in fibrosis. However, ITGA11 is not only a simple biomarker gene among others, but also part of the α11β1 collagen receptor which actively participates in the fibrotic process (Table 1). Functional studies have indicated the involvement of integrin α11 in cardiac and skin fibrosis and except for the tissues mentioned above, relatively little is still known about the expression and role of integrin α11 protein in other fibrotic tissues. We yet assume that integrin α11 is an important factor in the development of tissue fibrosis and predict that it will be an important target for anti-fibrosis therapeutic strategies.

5 Integrin α11β1 in Tumor Fibrosis

Tumor fibrosis, often termed desmoplasia, is characterized by excessive ECM deposition, reorganization, remodeling, and cross-linking that create a stiff substratum for tumor cells regulating tumor growth, invasion, and metastasis (Piersma et al. 2020; Zeltz et al. 2020). Desmoplasia is mediated by subsets of cancer-associated fibroblast (CAF) and is a prognostic marker of poor survival in several cancer types (Takahashi et al. 2011; Togo et al. 2013; Rudnick et al. 2021). We have shown that integrin α11 is overexpressed in the stroma of desmoplastic tumors such as lung, breast, and PDAC (Zeltz et al. 2019a). In these cancers, integrin α11 colocalizes to different degree with α-SMA expression, suggesting that integrin α11 could be expressed in a subset of myofibroblastic CAFs.

5.1 Integrin α11 in Non-Small Cell Lung Carcinoma (NSCLC)

Together with the group of Ming-Sound Tsao, we first showed that ITGA11 among five other genes including COL11A1 was identified by representation differences analysis as being differentially expressed in lung adenocarcinoma as compared to normal lung (Wang et al. 2002). In further analyses, the Tsao laboratory analyzed the differentially expressed genes in CAFs versus normal lung fibroblast and identified 22 upregulated genes in NSCLC CAFs in which ITGA11 and COL11A1 are also

Table 1 Summary of expression and role of integrin α11 in tissue and tumor fibrosis

	Expression of α11 in fibroblast subtypes	Role of α11
Tissue fibrosis		
Skin	– In keloids (Deng et al. 2021), α11 mRNA[a] detected in mesenchymal fibroblast subpopulation – In Dupuytren's disease (Dobie et al. 2021), α11 mRNA[a] mainly detected in Skoog's fascia fibroblasts, few in myofibroblast cluster	In a mouse model of scleroderma, $\alpha 11^{-/-}$ mice are protected from dermal fibrosis (Schulz et al. 2018)
Heart	In normal heart (Litviňuková et al. 2020), low α11 mRNA[a] detected in: – Canonical fibroblasts enriched in ventricles and atria – A fibroblast subpopulation characterized by ECM production and organization	– Overexpression of α11 in transgenic mice induces cardiac fibrosis (Romaine et al. 2018) – In an STZ-mouse diabetic model, $\alpha 11^{-/-}$ reduces heart fibrosis (Civitarese et al. 2016) – Interaction of α11β1 with glycated collagen promotes myofibroblast differentiation in vitro (Talior-Volodarsky et al. 2012)
Lung	In pulmonary fibrosis (Adams et al. 2020; Habermann et al. 2020), α11 mRNA[a] detected in: – In $ACTA2^{+}$ myofibroblasts – In $HAS1^{hi}$ fibroblasts	Not yet determined
Liver	– In mouse CCL4-fibrotic liver (Dobie et al. 2019), α11 mRNA[a] detected mainly in CD34+ fibroblasts, few in HSC – In human liver cirrhosis (Ramachandran et al. 2019), α11 mRNA[a] detected in myofibroblast subpopulation	Knockdown of α11 in HSC inhibits HSC activation in vitro (Martin et al. 2016; Bansal et al. 2017)
Tumor fibrosis		
Lung	In lung adenocarcinoma (Kim et al. 2020), α11 mRNA[a] detected mainly in the myofibroblast cluster, some in $Col14A1^{+}$ matrix fibroblasts	– $\alpha 11^{-/-}$ SCID mice display growth inhibition of A549 NSCLC cells and reduced metastasis (Navab et al. 2016) – α11 regulates expression of LOXL1, a matrix cross-linking enzyme (Zeltz et al. 2019b)
Breast	– In an MMTV-PyMT mouse model (Bartoschek et al. 2018), α11 mRNA[a] detected mainly in matrix CAF, some in developmental CAF – Using α11 mAb, α11 detected in $PDGFR\beta^{+}$ CAF subset (Primac et al. 2019)	– $\alpha 11^{-/-}$ PyMT mice exhibits inhibition of breast tumor growth and metastasis (Primac et al. 2019) – $\alpha 11^{-/-}$ CAFs deficient in collagen matrix remodeling (Primac et al. 2019)

(continued)

Table 1 (continued)

	Expression of α11 in fibroblast subtypes	Role of α11
Pancreas	– In PDAC, α11 mRNA[a] detected in LRRC15+ CAF subpopulation characterized as myCAF (Dominguez et al. 2020) – Using α11 mAb, α11 detected in PDGFRβ$^+$/α-SMA$^+$ CAF subset (Zeltz et al. 2019a)	Knockdown of α11 in PSC inhibits CAF activation and PDAC cell invasion in vitro (Schnittert et al. 2019)

[a]Observations based on scRNA-seq dataset associated with the citation
ECM extracellular matrix, *HSC* hepatic stellate cell, *NSCLC* non-small cell lung carcinoma, *CAF* cancer-associated fibroblast, *PDAC* pancreatic ductal adenocarcinoma, *mAb* monoclonal antibody, *PSC* pancreatic stellate cell

present (Navab et al. 2011). The overexpression of integrin α11 introduced it as a novel candidate tumor biomarker in NSCLC. Our analysis of the scRNA-seq dataset of lung adenocarcinoma published by Kim and colleagues (Kim et al. 2020) showed higher expression of integrin α11 mRNA in the myofibroblast cluster that is specific to the tumor tissue. We also observed some cells expressing α11 in the Col14A1$^+$ matrix fibroblasts, which formed the main subpopulation in normal lung and early-stage tumor. The depletion of integrin α11 in SCID mice inhibits growth of implanted A549 lung adenocarcinoma cells (Navab et al. 2016), indicating that integrin α11 is an important stromal factor in NSCLC. Expression of α-SMA correlated with expression of integrin α11 in this tumor tissue, confirming a role of integrin α11 in myofibroblast differentiation also observed in fibrotic tissue. In addition, differential gene analysis in mouse NSCLC stroma revealed the downregulation in α11$^{-/-}$ mice of lysyl oxidase-like 1 (Loxl1), a matrix cross-linking enzyme, which later was shown to be regulated by integrin α11 in stromal cells (Zeltz et al. 2019b) (Fig. 2). As a consequence, in the NSCLC context integrin α11 is strongly suggested to regulate collagen reorganization and tissue stiffness to promote metastasis.

5.2 *Integrin α11 in Breast Cancer*

Bartoschek et al. have identified four subpopulations of breast CAFs in an MMTV-PyMT mouse model that they named vascular CAF (vCAF), matrix CAF (mCAF), cell cycle CAF (cCAF), and developmental CAF (dCAF) (Bartoschek et al. 2018). When we analyzed the scRNA-seq data from this study, we observed integrin α11 mRNA expression mainly in the mCAF subclass, which displays the strongest ECM signature and are thought to be derived from resident fibroblasts. Integrin α11$^+$ CAFs are mostly Pdgfrb$^+$ and some are Pdgfra$^+$ and Acta2$^+$. We also noticed some expression of Itga11 mRNA in dCAFs, which share expression patterns with tumor epithelial cells and are suggested to originate from malignant cells. This is interesting because it suggests that integrin α11 could be expressed on spreading

tumor cells that have undergone EMT. In this context, Westcott et al. identified ITGA11 as an invasion promoting gene in the leading invasive "trailblazer" cells observed in a breast cancer cell invasion assay in vitro (Westcott et al. 2015).

In a collaborative study with Primac et al., we confirmed that integrin α11 is strongly associated in breast cancer specimens with a PDGFRβ^{+} CAF subset and to lower degrees with PDGFRα, α-SMA, NG2, and FAP (Primac et al. 2019). Like in NSCLC, we could show that depletion of integrin α11 in mice inhibited breast tumor growth and metastasis and that α11$^{-/-}$ CAFs are deficient in remodeling of a collagen matrix. Interestingly, this study highlighted a cross-talk between α11β1 integrin and PDGFRβ, both receptors interact upon stimulation with PDGF-BB leading to JNK activation and to the deposition of tenascin C, a proinvasive matricellular protein (Fig. 2). As mentioned in Sect. 3., we have previously shown that JNK was downstream of the cross-talk between integrin α11 and TGF-βR during collagen reorganization (Schulz et al. 2015), suggesting that in tissue and tumor fibrosis, integrin α11 can cooperate with growth factor receptors and signals through JNK to regulate the ECM. Thus, targeting JNK might be an alternative option to block the pro-fibrotic activities of integrin α11.

5.3 Integrin α11 in Pancreatic Cancer

In most models of tumor stroma interactions, a majority of published data suggest that the tumor stroma is tumor supportive (Han et al. 2015; Alexander and Cukierman 2016). In molecular terms this corresponds to various types of cross-talk between tumor and stromal cells including paracrine- as well as integrin-mediated signaling. In pancreatic cancer, the stroma has been suggested to support tumor growth, tumor metastasis and to be involved in tumor chemoresistance (Pan et al. 2015). Other studies suggest that stroma might act as a restraining barrier preventing tumor expansion and tumor spread. With the increased awareness about CAF heterogeneity within the tumor microenvironment (TME) many published studies might have to be revisited and the effects of TME re-examined in more detail, keeping in mind the CAF heterogeneity. New data generated in more targeted approaches to CAF subsets support data from widely cited paper from Ozdemir et al. suggesting that conditional deletion of αSMA-expressing fibroblasts in experimental PDAC mouse model worsened tumor outcome (Özdemir et al. 2014). Experimentally the αSMA-thymidine kinase mouse was crossed with two different models of PDAC, namely the LSLS-$^{KrasG12D/+}$;Trp53$^{R172H/+}$; Pdx $^{cre/+}$ (KPC) mouse and the Ptf1a$^{cre/+}$; Kras$^{Gt2D/+}$;TGFbr2 $^{flox/flox}$ (PKT) mouse, and cell depletion of αSMA-expressing cells was induced with ganciclovir. These rather drastic cell depletion protocols with reduced number of myofibroblasts resulted in more invasive and undifferentiated tumors with local areas of necrosis. The cell population targeted in this approach includes the CAFs we now call myCAFs, but importantly also include other cell types. In a more recent publication, an advanced PDAC mouse model was used to analyze the effects of collagen I deletion in αSMA-expressing cells and in the

context of TME (Chen et al. 2021). The results indicate that deletion of collagen I makes tumor more proliferative, supporting the concept that the collagen I-producing myCAFs are really tumor re-restraining. Interestingly, detailed analysis of the PDAC tumor model by Chen et al. suggest that myCAFs lacking collagen I synthesis stimulate the PDAC cells to secrete CXCL5 which in turn attracts neutrophils contributing to an immunosuppressive environment, which further explains this interesting phenotype. These results are in agreement with recent data from a study of tumor collagen content arguing for a correlation between degree of desmoplasia and disease severity (Jiang et al. 2020). PDAC patients with more desmoplasia had a better prognosis which led the authors to inhibit LOXL2 activity in a mouse tumor model. Their data thus also support the concept that a collagen matrix restrains PDAC tumor growth, while this effect is tumor- and context-dependent. In this background, the question is to determine what is the role of integrin α11, whether it is supporting or restraining the pancreatic tumor. In breast and lung cancer, we mentioned above that integrin α11 is expressed in myofibroblastic CAFs and contributes to tumorigenicity. When it comes to its expression in the collagen rich PDAC stroma, integrin α11 mRNA has been identified in an LRRC15^{+} CAF subpopulation in PDAC, that is derived from TGF-β-activated CAFs, characterized as myCAF (Dominguez et al. 2020). Interestingly, ablation of LRRC15^{+} stromal cells in a PDAC mouse model has recently been shown to attenuate tumor growth (Krishnamurty et al. 2022). Using the selected monoclonal antibody mAb 203E3, we have found that integrin α11 protein was restricted to the stromal compartment of PDAC and was localized in PDGFRβ^{+}, α-SMA^{+} and/or FAP^{+} CAF, but not in NG2^{+} CAFs (Zeltz et al. 2019a). The origin of integrin α11-expressing CAFs in PDAC is still uncertain, i.e., whether they are activated from stellate cells or from resident fibroblasts. However, the lack of NG2 colocalization would suggest that α11 expressing CAFs are not pericyte-derived. It is interesting to note that α11β1 is upregulated in human pancreatic stellate cells activated with PDAC conditioned media in vitro (Schnittert et al. 2019). Like in other cancer models, abrogation of integrin α11 in the stromal cells in vitro reduces CAF activation and matrix reorganization. Furthermore, in this study knockdown of integrin α11 reduced paracrine secretion from stellate cells, inhibiting PDAC cell invasion (in vitro). Our previous studies using α11 mAbs need to be repeated with more markers to conclude if α11 is expressed on stellate-derived CAFs in vivo and to sort out the origin and role of PDAC α11^{+} CAFs in PDAC.

Integrin α11 mRNA has also been shown to be strongly expressed in desmoplastic liver, ovary, uterus, and head and neck carcinoma where it colocalizes with α-SMA (Parajuli et al. 2017; Zeltz et al. 2019a). The tumor microenvironment is quite complex and CAF activation might depend on the tumor type and mutations that can modify how tumor cells and CAFs interact. In some cancers, similar CAF populations are found such as myCAFs, which display matrix production and remodeling features, but with some different specific markers and mechanisms of induction. Although integrin α11 seems to be strongly associated with myCAF activation independent of the desmoplastic features, not all α11^{+} CAFs are α-SMA^{+}, suggesting that myofibroblast differentiation in vivo might only occur in

specific CAF subsets of a specific origin. Further studies are indeed required to better characterize the phenotype of CAFs that express integrin α11 and thus determining the function of this collagen-binding integrin in each CAF subpopulation.

6 Integrin α11 Tools for Fibrosis Studies and Therapeutics

6.1 Monoclonal Antibodies Directed Against Human Integrin α11

We have generated integrin α11 monoclonal antibodies (mAbs) for two different purposes: for tissue detection and for use as blocking antibodies. Integrin α11 is barely detectable in human adult normal tissues; however, it is upregulated in tissue and tumor fibrosis, implicating integrin α11 as a potential biomarker that can predict patient outcome. In order to specifically detect integrin α11 in different fibrotic tissues, we used mAb 203E3 in cryosection analysis of α11 expression (Zeltz et al. 2019a) and mAb 210F4B6A4 for paraffin-embedded tissues (Smeland et al. 2020). These immunostaining-designed antibodies will be useful to confirm expression of integrin α11 in the different fibroblast subpopulations that we have mentioned.

In this chapter, we have described the pro-fibrotic functions of integrin α11 in the tissues studied, suggesting that integrin α11 is an interesting target for anti-fibrosis therapy. One candidate for combination treatment and antibody drug conjugate-based treatments is the α11 mAb 203E1, which we have shown to interfere with cell–collagen interactions (Zeltz et al. 2019a).

6.2 ITGA11-Driver Cre Mouse Strain

scRNA-seq data have shown that fibroblasts involved in fibrosis are heterogeneous, but lack of fibroblast-specific markers is an important issue when studying the role of these fibroblasts. Since integrin α11 is specifically expressed on mesenchymal cell, shows low expression on adult mouse tissue, and is upregulated in fibrotic tumors and tissues, we have generated an ITGA11-driver Cre mouse strain (Alam et al. 2020). We showed that expression of ITGA11-driven Cre is induced in granulation tissue during wound healing and in fibrotic heart upon aortic banding. Interestingly, ITGA11-driven Cre was expressed in 60% of isolated mouse embryonic fibroblast, indicating that integrin α11 is expressed on a subset of these fibroblasts. Thus, ITGA11-Cre mouse strain is a useful tool for cell lineage tracing and for gene deletion in subpopulations of fibroblast. It could also be an attractive tool to ablate cells in an ITGA11-specific manner if crossed with ROSA26iDTR mice (Buch et al. 2005) to determine function of fibroblast subsets in fibrosis.

7 Conclusion

Expression of integrin α11 is heterogeneous among mesenchymal cells. In tissue and tumor fibrosis, integrin α11 is mainly found in myofibroblast subpopulations, which are associated with TGF-β signaling and matrix reorganization, where it may mediate myofibroblast differentiation and collagen remodeling (Fig. 1 and Table 1). However, we also observed integrin α11 expression in some other fibroblast clusters in fibrotic tissues that needs to be confirmed and in which its role remains to be defined. For this purpose, we have generated new integrin α11 tools that will be helpful in further studies of the pro-fibrotic integrin α11.

scRNA-Seq Data Used for Integrin α11 Expression

Buechler et al., R Seurat objects of mouse perturbed-state atlas (https://drive.google.com/file/d/15YvdVZpe3uRjx2hjNDkNV4y5bi62QRkn/view) and human perturbed-state atlas (https://drive.google.com/file/d/1n-XLSYacOIXSlFQbe0XZP3BhhW0CBl7Z/view); *Deng* et al., R Seurat object of dataset GSE163973; Dobie et al.(2021), R Seurat object of dataset GSE173252; *Habermann* et al. and *Adams* et al., interactive IPF cell atlas (http://www.ipfcellatlas.com/); Dobie et al. (2019), interactive mouse liver fibrosis cell atlas (https://shiny.igmm.ed.ac.uk/livermesenchyme/); *Ramachandran* et al., interactive human liver fibrosis cell atlas (https://shiny.igmm.ed.ac.uk/livercellatlas/); *Litviňuková* et al., interactive heart cell atlas (https://www.heartcellatlas.org/); *Kim* et al., interactive lung cancer cell atlas (URECA) (https://www.ureca-singlecell.kr/); *Bartoschek* et al., R scripts of scRNA-seq of breast CAF (https://github.com/KPLab/SCS_CAF).

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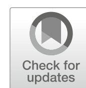
Integrins in Pathological Tissue Remodelling of Joints



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Abstract Osteoarthritis (OA), a primarily degenerative disorder, and rheumatoid arthritis (RA), a chronic autoimmune inflammatory disease, belong to the most prevalent joint pathologies. Both conditions involve the entire joints with their different tissues and may lead to their complex remodelling and eventually to their destruction. The interaction of articular cartilage with the synovial membrane is a hallmark of both OA and RA and characterized by strong communication between their resident cells such as chondrocytes and fibroblasts as well as invading inflammatory cells such as monocytes with the local extracellular matrix (ECM). Integrins have been assigned a key role in this communication and have, thus, been involved strongly in the pathogenesis of OA and RA. Using OA and RA as examples, this chapter summarizes the role of integrins in both joint pathologies and points to potential therapeutic implications.

1 Introduction

Unlike other organs of the human body, synovial joints are not just composed of a single—more or less complex—tissue but of different morphological structures that constitute different tissues by themselves. These include bones that form the articulating ends of diarthrodial joints, tendons that link adjacent muscles to these bones to allow for motion as well as hyaline cartilage that covers the articulating ends of the bones and the synovial membrane that lines the synovial cavity with the synovial fluid inside.

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These tissue structures are structurally and functionally distinct but show a high degree of inter-tissue communication. This is particularly true for the articular cartilage with the subchondral bone underneath and the synovial membrane, all of which are related in their embryonic development and are functionally highly interdependent throughout adult life. Therefore, it has become evident that most joint diseases not only affect one tissue component but the joints as a whole, and numerous recent data have also shown that inter-tissue communication not merely is an epiphenomenon of most joint diseases but a key driving element of their pathogeneses.

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most prevalent joint diseases and as one common feature share the chronic destruction of articular cartilage that along with other characteristics of the diseases leads to progressive disability (Pap and Korb-Pap 2015).

Osteoarthritis is largely considered a degenerative joint disorder that is closely linked to mechanical stress as well as genetic, metabolic and age-related factors. Several lines of evidence support a concept in which the loss of phenotypic stability in chondrocytes as the only cells of the cartilage along with changes in subchondral bone and inflammatory changes in surrounding tissues including the synovial membrane all together lead to a sequence of events that mimic important aspects of embryonic development, particularly endochondral ossification (Singh et al. 2019; Xiao et al. 2018; Ripmeester et al. 2018). As a result, subchondral bone thickens and articular cartilage is degraded or partly remodelled into bone. Inflammatory factors as derived from the cartilage itself as well as from adjacent tissues may promote and accelerate the disease resulting in a vicious circle with chronic pain and disability (Wang et al. 2018).

While rheumatoid arthritis (RA) as one key feature also leads to the progressive destruction of articular cartilage, it is a primary inflammatory joint disease with an autoimmune aetiology (Pap and Korb-Pap 2015). It is now well accepted that pathological immune phenomena such as the occurrence of specific autoantibodies may precede the clinical manifestation of the disease (Tracy et al. 2017). However, the question of why this systemic autoimmunity affects the joints and what the tissue determinants of joint manifestation are remain incompletely understood. In recent studies and with relevance to this chapter, it has been hypothesized that early cartilage damage in the context of alterations in adaptive immunity, particularly decreased functioning of regulatory T cells, may provide an explanation for organ specificity in RA (Korb-Pap et al. 2016; Pap et al. 2020).

In this context, fibroblast-like synoviocytes have been assigned a key role in the pathogenesis of RA and it has been shown that in the course of disease the cells acquire an autonomously aggressive, sometimes also called ‘tumour-like’ phenotype (Korb-Pap et al. 2016). As a result, they attach to and invade into the articular cartilage which is a major reason for the progressive destruction of the joints.

Integrin-mediated cell–matrix interactions are a key element in the pathogenesis of both OA and RA and contribute largely in three different ways. By mediating the interaction of tissue-resident cells, particularly of chondrocyte and fibroblast-like synoviocytes with their surrounding ECM-rich environments, integrins are involved

in the disease-specific response and differentiation of these cells. As chondrocytes and synovial fibroblasts are key drivers of either disease, integrin-mediated changes to their phenotypes greatly impact on tissue remodelling and on maintaining a vicious circle in which alterations to the biology and behaviour of chondrocytes and fibroblast-like synoviocytes affect and promote tissue destruction and composition. This in turn is sensed by integrin receptors on these cells further driving their phenotypic switches.

A second mechanism by which integrins contribute to the pathogenesis of joint diseases like OA and RA is the interaction of resident cells with neighbouring tissues in the highly interconnected structures of the joints. This is particularly evident for RA, where integrins are key adhesion molecules for the attachment and subsequent destruction of the adjacent articular cartilage by the transformed fibroblast-like synoviocytes. However, interaction of different tissues within the joints is also a hallmark of degenerative disorders such as OA and increasing evidence indicates that integrins significantly contribute to these interactions.

Thirdly, integrins have been implicated prominently in the recruitment of immune cells into synovial tissues, particularly the synovial membrane and, thus in chronic inflammation. Again, this is of particular relevance to RA, where chronic, non-resolving synovitis is a key feature of the disease.

Therefore, this chapter looks at synovial joints as complex organs with distinct yet highly interconnected structures. We will try to demonstrate that this concept not only is key to understanding the development and function of articular joints but also becomes relevant particularly under pathological conditions such as arthritis, which not only affects joints as a whole but which is also based on complex pathological interactions between tissue components of the joints such as cartilage and synovium.

2 Integrins in the Pathogenesis of Osteoarthritis

While, as indicated, osteoarthritis is increasingly perceived as a disease that affects the whole joint, degeneration and remodelling of the articular cartilage is a key feature of the disease and (along with changes in subchondral bone) considered as a key pathogenic feature. This is of importance because articular, hyaline cartilage as a tissue has a number of peculiarities that are based on its function.

Together with the synovial fluid that is produced by the synovial membrane, articular cartilage is largely responsible for generating a smooth and low friction surface to enable the gliding of the bony ends of joints. In addition, articular cartilage serves as an elastic and load absorbing tissue and the underlying biomechanical properties of articular cartilage are achieved by its unique structure. Hyaline cartilage is an ECM-rich tissue that is characterized by the presence of only one cell type, the chondrocytes. These cells are loosely embedded into the highly complex and organized ECM which is made up of a type II collagen dominated mesh. This type II collagen network is interconnected and complemented by a variety of other matrix molecules particularly proteoglycans such as the large aggrecan but also small

leucine-rich proteoglycans such as decorin and biglycan as well as glycoproteins such as fibronectin (Heinegard and Saxne 2011). Their distribution varies in different areas of the cartilage. This structural organization along with the fact that the cartilage ECM harbours numerous soluble factors such as cytokines, chemokines and growth factors that are bound to specific components, particularly proteoglycans, makes the hyaline cartilage a highly bioactive and instructive environment for the chondrocytes that reside in it.

Articular cartilage itself can be structured into at least four distinct zones. These differ with respect to the density and orientation of the cartilage matrix fibrils as well as the number and shape of embedded chondrocytes. The superficial zone makes up about 10% of the cartilage thickness and is also called tangential zone. This zone is in direct contact with the joint cavity and contains densely packed cartilage fibrils with long-shaped chondrocytes in between. The middle zone that is also called transitional zone makes up about 40% of the articular cartilage and has more round-shaped chondrocytes, which are embedded into a less densely packed ECM. The deep or radial zone is the thickest layer of articular cartilage, and the chondrocytes here are oriented in perpendicular direction to the cartilage surface. The deep radial zone is separated by the tidemark from the calcified zone that is in direct contact with the subchondral bone.

In all these zones, integrins are important cell surface receptors for chondrocyte ECM interactions. The expression and regulation of the different members of the integrin family in chondrocytes has been studied extensively (for review, see Loeser (2014); Tian et al. (2015); Dieterle et al. (2021)) and it has been understood that β1-integrins are key interaction molecules through which chondrocytes interact with the cartilage ECM to sense its composition and react to mechanical stimuli. Thus, chondrocytes express a number of I-domain collagen-binding motif containing integrins such as α1β1, α2β1 and α10β1 integrin. In this context, integrin α10 has been found to be of particular importance during cartilage development (Camper et al. 2001) and for the differentiation of mesenchymal stem cells (MSCs) into chondrocytes (Varas et al. 2007). In addition, the RGD-binding domain containing integrins α5β1 and αVβ3 as well as integrins α3β1 and α4β1 have been found to be expressed in chondrocytes. Due to their binding of non-collagenous matrix molecules such as fibronectin (mainly integrin α5β1, but also αVβ3) and osteopontin (integrin αVβ3) that have been suggested in mediating pathogenic signals, integrin α5β1 and αVβ3 have gained special attention recently.

2.1 Changes in the Expression of Integrins in OA

Several studies have demonstrated alterations in the expression of integrins in chondrocytes during OA development. While increased expression of some integrins has been described in early studies (for review, see Loeser (2014); Tian et al. (2015)), the current picture seems to be more complex and changes attributable largely to three distinct phenomena: the effects of soluble mediators such as

cytokines, alterations in chondrocyte differentiation and changes in the composition and function of the ECM. All these factors do not merely result in increased expression of integrins in OA chondrocytes but in profound changes in their cell surface expression pattern as well as their sensitivity. Thus, it has been demonstrated that some integrin subunits such as α2, α4 and β2 are hardly found on normal chondrocytes but become expressed during OA development (Ostergaard et al. 1998; Almonte-Becerril et al. 2014). Also, chondrocytes in different zones show different expression levels, for instance, of the αV subunit (Ostergaard et al. 1998), which most likely can be attributed to their shape and differentiation state. This notion is also supported by a study that demonstrated alterations in integrin expression following immortalization of chondrocytes with SV40-Tag (Loeser et al. 2000). While this is an artificial and controversial system that may not be translated directly to the situation in OA cartilage, the observed changes in the expression of collagen-binding integrins α1, α2 and α3 well illustrate how changes to phenotypic properties affect integrin expression and, thus, interaction with the surrounding ECM.

Of note, several studies have linked the altered expression of integrins to changes in the composition of the cartilage ECM and, partly as a result of progressive destruction by matrix-degrading enzymes, to the stage of disease in OA. As human samples are obtained usually from end-stage disease at joint replacement surgery, most of these data originate from animal studies, particularly from murine models of the disease. Examples include changes in expression levels of α1, α2, α4 and α5 (Almonte-Becerril et al. 2014; Zemmyo et al. 2003) but it needs to be noted that matrix composition most likely affects integrin expression not purely through altered availability or conformation of the respective ligands but also through resulting alterations in mechanical forces. This has been suggested particularly for the integrins α4 and α5 that together with β1 bind fibronectin (Almonte-Becerril et al. 2014).

Degradation of cartilage ECM in OA may affect integrin expression and function through yet another mechanism. Ample evidence suggests that cartilage is a bioactive matrix that harbours chemokines. As shown recently, degradation of the cartilage ECM leads to the release of these soluble factors with the chemokine CXCL6 being one prime example (Sherwood et al. 2015). CXCL6 is bound to the cartilage ECM through interactions with heparin-binding domains and its sequestration seems to be of importance for maintaining phenotypic stability of articular cartilage chondrocytes. Degradation of cartilage in the course of OA, thus, disrupts the CXCL6–CXCR2 axis and promotes changes in the chondrocyte phenotype indicated by a loss of the transcription factor and stability marker SOX9. As a consequence, integrin expression and potentially function may change, the latter of which may involve inside-out signalling events and other, yet unidentified co-receptors.

Integrins seem to be regulated directly by cytokines and growth factors both in terms of expression and function. Thus, the inflammatory cytokine interleukin-1 (IL-1) has been identified as a regulator of integrin subunits α2, and α5 in osteosarcoma cells (Milam et al. 1991) and of the β1 subunit in synovial fibroblasts (Pirila and Heino 1996).

A more recent study demonstrated that IL-1β increased the availability and clustering of integrin α5β1 availability on human mesenchymal stem cells (hMSCs) (Maynard et al. 2021). Although such data are largely lacking for chondrocytes in OA, similarities in the signal transduction machinery may suggest that there is a close interplay between IL-1 and integrins in which—as described below—integrins not only modulate IL-1 signals but where IL-1 also affects expression and function of disease-relevant integrins.

Growth factors, particularly of the TGFβ superfamily have been implicated more directly in the expression of integrins in chondrocytes. Thus, it was shown that GDF-5 induces the expression of integrin α5, which in the light of functional data suggests that GDF-5 induced expression of integrin α5β1 may have a homeostasis promoting effect (Garciadiego-Cazares et al. 2015). Interestingly in the same study, BMP-7 induced the expression of integrin αV that has been implicated in chondrocyte hypertrophy (Garciadiego-Cazares et al. 2015) and, thus, affects OA pathogenesis in an even broader manner (Wang et al. 2019).

In addition to transcriptional regulation, regulation of integrin levels through long non-coding RNAs as well as epigenetic mechanisms has been suggested, for example, for integrin α1 (Kim et al. 2013).

2.2 Functional Consequences of Altered Integrin Expression in OA

The majority of functional data on the role of integrins in cartilage development, degeneration and regeneration stem from animal studies, particularly from genetically modified mice, and a great number of mice with constitutive or cell-specific/conditional deletion of integrin subunits have been generated. When studying integrins using knock-out mice it is important to note that these provide far more information on their role in development than they do on a potential contribution of individual integrins to the pathogenesis of a degenerative joint disease in adults such as OA. This is not only based on the increasing awareness that murine models of OA, while providing useful insights into distinct disease mechanisms, are difficult to use as disease models and have low predictive value when it comes to deriving translational implications. More importantly, many of these knock-out mice exhibit developmental effects on cartilage and this fact along with the complexity of integrins and their partial overlap in function complicates the interpretation of OA-related data both when studied through aging and (mostly surgical) interventions.

Nonetheless, important lessons have been learnt about the role of individual members of the integrin family for the homeostasis of articular cartilage. Thus, several studies have shown that integrin α1 deficiency is associated with more severe OA-like changes both upon aging (Zemmyo et al. 2003) and following surgical intervention by removing the medial meniscus (Shin et al. 2016). Zemmyo and colleagues also showed that the loss of integrin α1 increased the expression of

disease-relevant matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-3 and apoptosis (Zemmyo et al. 2003). This suggests an anabolic role of integrin α1β1 and work by Shin and colleagues suggested that this may be due to interaction with the EGF-receptor, as its inhibition reduced the enhanced OA-like changes in integrin α1 deficient mice. Parekh and colleagues, however, did not find differences in cartilage and subchondral bone between integrin α1 deficient and control mice at least up to 6 months of age but demonstrated that α1deficient chondrocytes showed reduced intracellular calcium response to IL-1 and enhanced responses to TGFβ1 (Parekh et al. 2014). How these data may help to explain the aforementioned findings is not entirely clear but they all point to an important matrix sensing and regulatory function of integrin α1β1 in joints.

Regulation of MMPs through integrins along with a regulatory role of IL-1 has been shown for quite some time and these effects do not seem to be specific for collagen-binding integrins. Rather, it has been shown that RGD peptides that amongst others bind to integrin α5β1 can induce the expression of a variety of MMPs such as MMP-3, MMP-2 and MMP-9 that all have been implicated in cartilage degradation in OA (Arner and Tortorella 1995). In this study too, a crosstalk with the IL-1 receptor was observed as the IL-1 receptor antagonist (IL-1Ra) was able to inhibit the RGD-induced MMP production.

While these early data suggested that RGD-motif binding integrin engagement on chondrocytes may promote tissue remodelling and along with inflammatory mediators such as IL-1 have catabolic effects, the functional picture is not as clear as that and more recent evidence suggested that there are differences in the pathogenetic roles particularly between integrin α5β1 and integrin αVβ3.

As clearly worked out in a recent review by Dieterle and colleagues, integrin α5β1 seems to have very distinct functions in normal vs. pathologic, particularly IL-1 driven, environments (Dieterle et al. 2021). They suggest that integrin α5β1 may act in two ways, namely in a cell-protective and anabolic way under physiological loading conditions in a 'normal' joint but that it may also function as an 'overload' integrin. According to their hypothesis, 'overstimulation' of the integrin α5β1 particularly in an inflammatory-prone environment may lead to a shift towards a catabolic chondrocyte metabolism which is seen in OA (Dieterle et al. 2021).

This notion is very much supported also by a study that showed differences in the α5β1-mediated membrane hyperpolarization response to pressure-induced strain between normal and osteoarthritic chondrocytes (Millward-Sadler et al. 2000). An elegant study using mice in which integrin α5 had been conditionally deleted using the Gdf5Cre system also confirms this notion. In this study mice harbouring the cell-specific deletion of integrin α5 were born and developed normally with no obvious phenotype compared to the control mice. However, induction of OA-like changes through surgical intervention in adult mice demonstrated that the lack of integrin α5 in GDF5 lineage cells was associated with less severe osteoarthritic changes (Candela et al. 2016). However, underscoring the complexity of integrin expression during development and adult life, their specific spatiotemporal function and ECM composition, another study used (Col2a-Cre;Fn1RGE/fl) mice, in which articular chondrocytes expressed a mutant fibronectin that through substitution of the aspartic

acid of the RGD-binding motif with a glutamic acid (FN-RGE) was unable to bind to the classic fibronectin receptor α5β1. In these mice, surgical removal of the medial meniscus along with forced exercise led to more severe OA-like changes with increased expression of disease-relevant MMP-3 and -13, underscoring the crucial role of integrin α5β1–fibronectin interaction in cartilage matrix homeostasis (Almonte-Becerril et al. 2018).

These data are of interest for assessing the role of integrin αVβ3 that also binds to the RGD motif. In a recent study Wang and colleagues identified integrin αVβ3 and the integrin-associated receptor CD47 as key drivers of cartilage degradation OA (Wang et al. 2019). Importantly, they also found an abundance of ligands to stimulate the αVβ3/CD47 signalling axis and were able to show upregulated integrin αVβ3 not only in chondrocytes but also in synovial lining cells. Moreover, their data indicate that high availability of factors like COMP together with the interaction of αVβ3 with CD47 also triggers and promotes an inflammatory response (Wang et al. 2019). Based on their data, the authors conclude that αVβ3, CD47, and their signalling pathways may serve as valuable targets for disease-modifying therapies that so far have been missing for OA.

2.3 Therapeutic Strategies Targeting Integrins in OA

Based on the aforementioned data, different therapeutic strategies have been suggested that aim at treating OA by modulating integrin signalling. These not only include αVβ3 and its interplay with CD47 (Wang et al. 2019) but also β1 integrins such as integrin α2β1 (Kanamoto et al. 2021) and α5β1 (Candela et al. 2016). However, evidence that direct modulation of integrins in OA cartilage may translate into an alteration of the disease course has been sparse and to date no robust data have been generated to support that notion, particularly with respect to human disease.

In addition to the direct modulation of target molecules in the diseased joint, transfer of MSCs to regenerate the damaged cartilage has been one approach to treat OA. While this concept faces a number of general challenges such as the homing of MSCs into cartilage defects, the use of MSCs that are selected for their chondrogenic potential based on their integrin expression profiles has been investigated recently. Thus, it has been suggested that fibronectin may promote the chondrogenic differentiation of progenitor cells through integrin α5β1-dependent mechanisms and that fibronectin could be used to activate such progenitors which then could be used for the treatment of cartilage disorders (Tao et al. 2018). More recently, research has focused on integrin α10 which is based on its aforementioned established role in chondrogenesis (Camper et al. 2001; Varas et al. 2007). Preclinical studies in horses have demonstrated that MSCs that had been selected for high expression of integrin α10β1 ameliorated the progression of OA-like cartilage damage (Delco et al. 2020). Based on these data, a Phase 1/2 clinical trial has been initiated most recently to assess the safety, tolerability and preliminary efficacy of such MSCs when

administered as a single intra-articular injection to patients with symptomatic knee OA (Xintela AB (Xindu Pty Ltd) 2022).

3 Integrins in the Pathogenesis of Rheumatoid Arthritis

In contrast to OA, RA is largely considered a primary inflammatory disorder that has a strong autoimmune component. RA most likely starts in the periphery and in a second step affects the joints, particularly the synovial membrane that in the course of disease becomes chronically inflamed, hyperplastic and is transformed into an aggressive tissue invading into adjacent cartilage and bone.

As a result, different tissues are of importance and interact in RA. Like in OA, cartilage is a main target tissue that becomes degraded in the course of disease and the mediators of cartilage destruction seem to be highly overlapping between OA and RA. Moreover, several lines of evidence suggest that cartilage degradation and remodelling is an early event in RA and occurs even before the inflamed pannus tissue attaches to and invades the articular surface. The relevance of this early cartilage damage for the pathogenesis of RA is just beginning to emerge but according to the mechanisms described for OA above, it may have important effects as alterations to cartilage matrix composition would, on the one hand, increase its vulnerability to the invasive growth of the synovial membrane and, on the other hand, result in the release of soluble factors that contribute to chronic inflammation and cellular changes in the synovial membrane itself.

Of note one of the most prominent and lasting changes within the synovial membrane affects the resident mesenchymal cells that are called fibroblast-like synoviocytes. It is now established that these fibroblast-like synoviocytes play a key role both in the local initiation and the perpetuation of RA. In the course of disease, they acquire an autonomously aggressive, sometimes also called 'tumour-like' phenotype that makes them attach to and proteolytically attack the articular cartilage which leads to its progressive destruction.

3.1 Expression and Function of Integrins in RA-Fibroblast-like Synoviocytes

RA-fibroblast-like synoviocytes express various adhesion molecules that mediate their binding to the cartilage ECM and integrins have been investigated most intensively in this context.

Several, already early studies have demonstrated the expression of a variety of integrins, particularly of different β1 receptors on RA-fibroblast-like synoviocytes (el Gabalawy and Wilkins 1993; Nikkari et al. 1993; Rinaldi et al. 1997; Pirila et al. 2001; Nakayamada et al. 2003; Asano et al. 2014) and cytokines such as IL-1 have

been linked to their increased expression in the inflammatory context of RA (Pirila and Heino 1996). As this inflammation-triggered increase in the expression of β1 integrins has been associated with the specific 'tumour-like' phenotype of the cells, it has been hypothesized that they contribute directly to the adhesion of RA-fibroblast-like synoviocytes to adjacent joint structures, particularly articular cartilage and, thus to progressive joint destruction. This notion is supported by recent data, in which the large-conductance calcium-activated potassium channel KCa1.1, a potentially important regulator of RA- fibroblast-like synoviocyte migration and invasion has been shown to exert some of its effects through regulating β1 integrins (Tanner et al. 2017). Studies have also shown that RA fibroblast-like synoviocytes express high levels of integrin α9, which acts as a receptor for tenascin-C, osteopontin, VEGF and thrombospondin-1 (Asano et al. 2014).

Functional evidence for the involvement of individual members of the integrin family has come from early in vitro studies that have used antibody-mediated inhibition of integrins and from more recent work in which integrin deficient mice have been used. Thus, it was shown that the invasion of RA-fibroblast-like synoviocytes into cartilage-slices can be inhibited by antibodies to integrin α4, α5, αV and β1, particularly when RA-fibroblast-like synoviocytes were activated with IL-1 (Wang et al. 1997). Other studies have demonstrated the regulation of proliferation and MMP expression of RA-fibroblast-like synoviocytes by integrins (Sarkissian and Lafyatis 1999) and suggested that growth factor signalling such as the response to PDGF is facilitated by ECM signals (Sarkissian and Lafyatis 1999). Moreover, alterations in FasL/CD95L-induced apoptosis, one important characteristic of RA-fibroblast-like synoviocytes has also been attributed at least in part to integrin β1 signalling (Nakayamada et al. 2003; Kitagawa et al. 2006).

With respect to integrin α9 it was demonstrated that its knockdown in fibroblast-like synoviocytes had significant effects on the pathological tissue structure in a 3D system and decrease in both the spontaneous and TNFα-induced expression key effector molecules such as MMPs (Emori et al. 2017).

While these studies established a role for a variety of integrins in fibroblast-like synoviocyte activation in RA, it is difficult to assess potential therapeutic effects of integrin inhibition to treat RA. On the one hand, there seems to exist a substantial, at least functional, redundancy in the function of these ECM-binding integrins in fibroblast-like synoviocytes, on the other hand, however, some studies indicate that the loss of individual members of the family of collagen-binding integrins, for instance, may already have significant effects on the course of RA-like disease in animal models. Thus, it was shown that the loss of integrin α2 in mice significantly decreases the attachment of fibroblast-like synoviocytes to the cartilage ECM and reduces cartilage destruction in different animal models of RA (Peters et al. 2012).

3.2 *Role of Cartilage Damage in Attachment and Cellular Activation of RA-Fibroblast-like Synoviocytes*

Interestingly, several lines of evidence suggest that attachment of the RA synovial membrane as a whole and of isolated fibroblast-like synoviocytes is particularly strong and the differences to non-diseased fibroblast-like synoviocytes are particularly evident when articular cartilage is already damaged. This suggests that alterations to the cartilage ECM as occurring subsequent to proteolytic damage facilitate the interaction of cell surface integrins on fibroblast-like synoviocytes with the cartilage matrix. Indeed, it was shown in arthritic human TNFα transgenic mice (*hTNFtg* mice) which develop an RA-like destructive disease, that cartilage damage is a very early event in the course of their disease and preceded the attachment of the inflamed synovial tissue to the cartilage (Korb-Pap et al. 2012). Notably, this study clearly showed that the attachment of synovial tissue occurred only after substantial loss of ECM, particularly of proteoglycans, from the articular cartilage had already taken place. Moreover, it was shown in this and other studies that the loss of IL-1, which is a well-established stimulator of proteoglycan loss, prevented cartilage destruction in *hTNFtg* mice and significantly reduced the attachment of synovial tissue and of fibroblast-like synoviocytes to the cartilage (Korb-Pap et al. 2012; Zwerina et al. 2007). These data altogether indicate that damaged cartilage is a potent stimulus for the attachment of fibroblast-like synoviocytes and it may be hypothesized that early cartilage damage, particularly the loss of proteoglycans is a key triggering factor of synovial attachment, fibroblast transformation and the progression of disease.

At present, the nature of the underlying changes is only incompletely understood and the question of what the specific factors and mechanisms are by which cartilage damage facilitates attachment of synovial cells to the cartilage remains a matter of ongoing studies. However, currently available data suggest different scenarios:

It has been hypothesized that components or fragments of the cartilage ECM are released at the degradation of cartilage or are ‘unmasked’ and thereby become accessible as ligands for integrins and other cell surface receptors on fibroblast-like synoviocytes. Indeed, several lines of evidence indicate that ECM components such as fibronectin fragments that originate from (damaged) cartilage may enhance synovial pannus formation and attachment through interaction with integrins but also with other surface molecules such as syndecans or CD44 (Korb-Pap et al. 2012).

More recently, several studies have suggested that citrullination of ECM proteins may constitute another mechanism by which interaction of diseased ECM with integrins on resident cells may be altered. Citrullination is a posttranslational modification of proteins that is found prominently as part of the pathogenesis in RA. Citrullination of different proteins leads to the occurrence of disease-specific antibodies but it may also affect cartilage ECM molecules such as fibronectin and collagen and consequently impact cell-ECM recognition by integrins and alter the behaviour of fibroblast-like synoviocytes. The mechanisms that may play important roles here have been reviewed before (Zeltz and Gullberg 2014). Thus, one study

demonstrated that citrullination of collagen type II decreased the adhesion of fibroblasts mainly through affecting the binding to integrin α10 and α11 (Sipila et al. 2014). More recent work found that citrullination of fibronectin alters integrin clustering and focal adhesion stability. Specifically, it led to a shift in integrin binding preference from αvβ3 to α5β1 most likely involving both a decrease in affinity to αvβ3 and altered ligation of integrin α5β1. This was associated with an increase in force-triggered integrin signalling as seen from enhanced activation of the FAK-Src and ILK-Parvin pathways (Stefanelli et al. 2019). As these have been mainly in vitro findings, the relevance for the pathogenesis and the course of RA remains to be determined.

A third way of how alterations in the cartilage ECM may act on fibroblast-like synoviocytes in RA is the release of factors that provide (co-)stimulatory signals, i.e., either act by themselves or in combination with matrix-sensing receptors such as integrins. Cartilage contains small leucine-rich proteoglycans such as biglycan and decorin that have been suggested to act in a modulatory way on the inflammatory response and invasive behaviour of fibroblasts. Thus, biglycan has been demonstrated to act as an endogenous ligand of TLRs, particularly TLR2 and TLR4 and may induce inflammatory responses through activation of the innate immune system (Babelova et al. 2009; Schaefer et al. 2005). With more relevance to this chapter, biglycan has also been implicated in the regulation of integrin β1 in melanoma microenvironments recently (Andrlova et al. 2017). As shown in that study, not only melanoma cells but also fibroblasts from $bgn^{+/+}$ matrices showed higher integrin β1 levels than $bgn^{-/-}$ fibroblast matrices. In turn, overexpression of integrin β1 in melanoma cells abolished the positive effects on survival seen in $bgn^{-/-}$ mice. Moreover, the expression of biglycan in human melanoma samples correlated with the expression of integrin β1, supporting data from organotypic invasion-assays and in vivo mouse studies (Andrlova et al. 2017). Interestingly, even more evidence suggests that decorin is involved in the regulation of integrin signalling, particularly integrin α2β1 (Fiedler et al. 2008; Jungmann et al. 2012). The question of whether these and other studies in cancer (Zheng et al. 2021) can be translated into the situation in RA remains open and will require additional work.

3.3 Integrins in the Regulation of Joint Inflammation and Autoimmunity in RA

The role of integrins in inflammation is covered elsewhere in this book, but it needs to be mentioned that integrins have also been linked to chronic synovial inflammation in RA, particularly in the recruitment and retention of inflammatory cells in the diseased synovial membrane. So early studies have already demonstrated that both synovial membrane and synovial fluid T cells expressed high levels of integrin α4β1 and were able to adhere to a 38-kD FN proteolytic fragment of fibronectin (Laffon et al. 1991). The notion that integrin-mediated interactions of synovial T cells and

monocytes with the local endothelium and subsequently the synovial ECM regulate their recruitment and cytokine production has been confirmed in a number of subsequent studies (Grober et al. 1993; Miyake et al. 1993; Takahashi et al. 1992). The question if there are disease or tissue-specific factors that may explain particular features of RA has been discussed extensively and it has been suggested that CS1, a 25-amino acid sequence present within the alternatively spliced IIICS region of fibronectin is expressed at high levels in the diseased synovium and contributes to the recruitment of integrin α4β1 expressing leukocytes in RA (Elices et al. 1994).

It has also been shown that patients with active RA had higher numbers of integrin αMβ2 expressing memory T cells than RA patients that were responding to therapy or normal controls suggesting that stimulation outside of the synovial compartment contributes to the stimulation of these cells (Nielsen et al. 1999). The clinical relevance of these findings remains to be determined.

3.4 Therapeutic Approaches Targeting Integrins in RA

Although these data have suggested that inhibition of integrins, for instance, by specific antibodies may provide novel therapeutic options, clinical trials using such antibodies in RA patients have largely failed at an early stage. The reasons for this overlap to some extent with those in the cancer field and these have been summarized extensively most recently (Bergonzini et al. 2022).

One example is integrin α9. Although antibody-mediated blockade of integrin α9 had beneficial effects in 3D in vitro culture systems of fibroblast-like synoviocytes (Emori et al. 2017) and inhibition of integrin α9 was able to prevent synovial inflammation and joint destruction in a mouse model of RA (Kanayama et al. 2009), efforts to translate these data into humans have not been successful so far. In a Phase 2a clinical trial that was aimed at evaluating the safety and efficacy of intravenous ASP5094, a monoclonal antibody against α9 in RA patients, neither the primary endpoint (ACR 50 response) nor the secondary endpoints were met (Astellas Pharma Inc 2019; Takeuchi et al. 2020).

Based on preclinical evidence that integrin αVβ3 may serve as a promising target for RA (summarized in Wilder (2002)), the humanized monoclonal antibody against integrin αVβ3, MEDI-522, has been assessed in a Phase 2 clinical trial (MedImmune LLC 2007). The 6 months' study was set up to compare the effects of subcutaneously administered MEDI-522 on disease activity and progression of structural joint damage in RA patients with active RA despite disease-modifying treatment. Unfortunately, this study too failed to achieve its endpoints (ACR 20 response) showing no clinical benefit (Jose 2021).

Despite these setbacks, there is still considerable interest in integrin-targeting therapies not only in RA but also in disease entities with partly overlapping pathogenic principles such as cancer or fibrosis. Novel developments there, as summarized recently (Slack et al. 2022), may, therefore, also impact on RA.

4 Conclusions

Altogether there is increasing awareness of cell–matrix interactions contributing significantly to the pathogenesis of both degenerative and inflammatory joint pathologies as represented by OA and RA. Integrins are an important part of this interaction and have been identified as key structures by which resident cells, particularly chondrocytes and fibroblast-like cells sense their environment as well as changes therein. As a result, alterations to the phenotypes of these resident cells including their differentiation state, their reaction to soluble factors and eventually their matrix remodelling capacity are regulated by integrins, which makes them interesting candidates for intervention and therapeutic approaches. These are complicated, however, by the redundancy of different integrins, the complexity of their structure and signalling pathways as well as a lack of in-depth understanding of how they contribute specifically to disease phenotypes. The successful introduction of integrin-targeting biologics in other diseases (Bergonzini et al. 2022; Slack et al. 2022; Ley et al. 2016) will certainly promote further research in the field and may eventually lead to novel strategies to interfere with integrins also in OA and RA.

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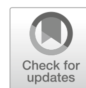
α4 Integrins in Immune Homeostasis and Disease



ChangDong Lin, ShiHui Wang, and JianFeng Chen

Abstract α4 integrins (including α4β1 and α4β7) are primarily expressed on leukocytes. By interacting with their ligands expressed on high endothelial venules (HEVs), α4 integrins mediate the recruitment of leukocytes from blood circulation to lymphoid organs and inflamed tissues, thereby playing essential roles in immune surveillance and host defense. The function of integrins is dynamically regulated by their activation and signaling. Integrin activation is associated with global conformational rearmament from a bent to extend conformation, mainly triggered by intracellular activation signals, termed "inside-out" signaling. The ligand binding to the integrin can also activate multiple intracellular pathways, which is termed "outside-in" signaling. In this chapter, we summarize the major findings regarding α4 integrins over the last decades, including their structures, extracellular ligands, intracellular adaptor proteins, and functions in homeostasis and diseases, including cancer, multiple sclerosis (MS), inflammatory bowel disease (IBD), and other autoimmune diseases.

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1 Introduction

Integrins are a family of α/β heterodimeric cell adhesion molecules, which exist extensively in all multicellular organisms (Hynes 1987; Tamkun et al. 1986). They were named “integrins” because they are integral membrane protein complexes for cell adhesion to the extracellular matrix (ECM) and were first proposed in the 1980s at the molecular level (Hynes 1987; Tamkun et al. 1986). Integrins have been major cell adhesion receptors since the early evolutionary history of the metazoans (Burke 1999; Hughes 2001), and homologous integrin sequences are even found in many prokaryotes (Johnson et al. 2009), suggesting they are a conserved evolutionary process and have pivotal functions in a wide range of scenarios. The vertebrate integrin family originates from homologous domains residing in the protists and prokaryotes, adapting itself to diverse functions within complete immune and closed circulatory systems throughout chordate evolution from lower organisms to individuals of phylum Chordata (Johnson et al. 2009). In vertebrates, 18 integrin α subunits and 8 β subunits form at least 24 different integrins (Hynes 2002). These integrins form a superfamily of cell adhesion receptors that bind to ECM ligands, cell-surface-expressing ligands, and pathogenic ligands and are responsible for mediating cell–matrix attachments, cell–cell and cell–pathogen interactions.

Integrin α4β1, also named very late activation antigen-4 (VLA-4), and CD49d/CD29 were described approximately 30 years ago (Hemler et al. 1987a) as a new member of the VLA antigen proteins that were originally found to appear during late T-cell activation (Hemler et al. 1985). Integrin α4β1 facilitates the recruitment of leukocytes to endothelial cells upon inflammation through the interaction with its ligand, vascular cell adhesion molecule-1 (VCAM-1) (Elices et al. 1990). Furthermore, the α4β1–VCAM-1 axis in different connective tissue cells has fundamental roles in many physiological processes, such as tissue organization, angiogenesis, and heart and skeleton development (Bouvard et al. 2001; Yong and Khwaja 1990). Integrin α4β1 on lymphocytes also recognizes the fibronectin (FN) Type III connecting-segment-1 (CS-1) region during T-cell function execution and immune responses (Wayner et al. 1989b). Soon after the discovery of integrin α4β1, another β integrin subunit named β7 was reported to form heterodimers with the α4 integrin subunit in mice (Holzmann and Weissman 1989; Kilshaw and Murant 1991) and humans (Erle et al. 1991). Integrin α4β7 (lamina propria-associated molecule-1, LPAM-1; CD49d/β7), whose distribution is restricted to subsets of immune cells in peripheral blood, mediates adhesion to mucosal tissues, such as gut-associated lymphoid tissue (GALT) through binding to its primary ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (von Andrian and Mackay 2000; Yu et al. 2012). α4β1 and α4β7 share some ligands, including VCAM-1 and FN. α4β7 exhibits preferential binding to MAdCAM-1 over the other two ligands (Yu et al. 2012). Moreover, integrin α4β7 can bind to human immunodeficiency virus-1 (HIV-1) envelope glycoprotein gp120 to mediate an HIV–T-cell interaction, thereby participating in HIV-1 infection of T cells (Arthos et al. 2008; Liu and Lusso 2020; Wang et al. 2021).

Overall, α4 integrins play critical roles in multiple biological and pathological processes, including leukocyte recruitment from the bloodstream into lymphoid organs and targeted tissues, immune cells, especially T-cell co-stimulation, and embryonic development.

2 Structure of α4 Integrins

Integrins are α/β heterodimeric type I transmembrane proteins. Both α and β subunits consist of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain (Fig. 1a) (Arnaout et al. 2005). In humans, the α4 subunit contains 1032 amino acid residues, and both β1 and β7 subunits contain 798 amino acid residues. Since the first characterization of the integrin α4 subunit in 1989, many studies have shed light on the structure of α4 integrins (Baldini and Cro 1994; Kassner et al. 1992; Wang et al. 2018; Yu et al. 2012).

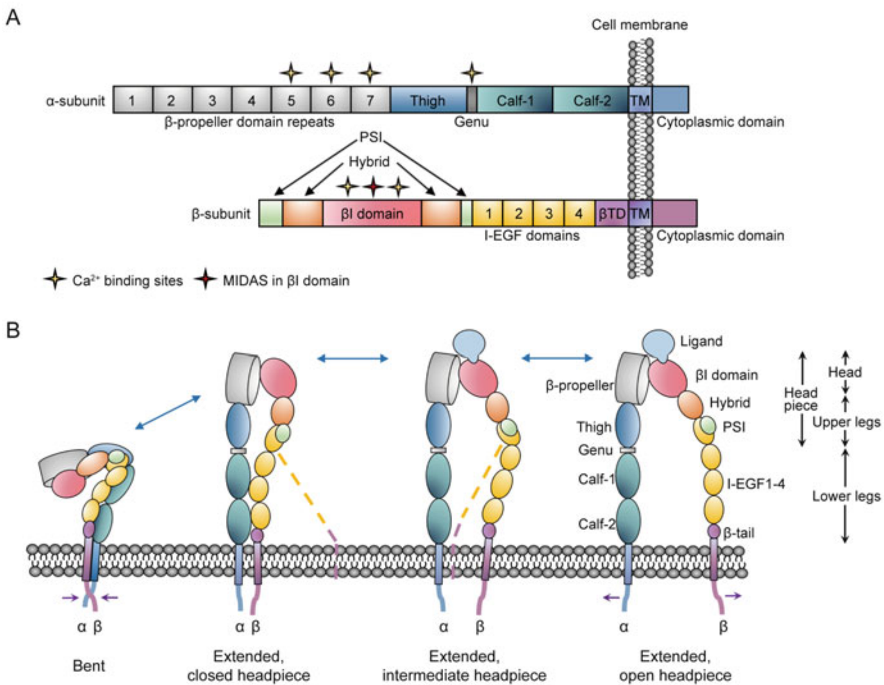

Fig. 1 Schematic of the α4 integrin structure and conformational rearrangements. (**a**) Domains within the primary structure of α- and β-subunits of α4 integrin are shown. Yellow and red asterisks denote Ca^{2+}- and Mg^{2+}-binding sites, respectively. (**b**) Conformational rearrangements of the α4 integrin during activation. Broken lines symbolize lower leg flexibility. Redrawn from Zhang and Chen (Zhang and Chen 2012)

2.1 Extracellular Domain

Integrin α4 subunit, which contains five extracellular domains, including a seven-bladed β-propeller, a thigh, a genu, and two calf domains, belongs to the α-I less integrin family because it lacks an I domain inserted between blades 2 and 3 in the β-propeller domain (Fig. 1a) (Larson et al. 1989). For the β1 and β7 subunits, the extracellular domain consists of a βI (βA), hybrid, plexin-semaphorin-integrin (PSI), cysteine-rich integrin epidermal growth factor (I-EGF) 1–4, and β-tail domains (Fig. 1a) (Xiao et al. 2004; Yu et al. 2012).

The conformations and dimensions of several integrin ectodomains (e.g., αXβ2, αVβ3, and αIIbβ3) have been extensively studied by different research groups (Xie et al. 2010; Xiong et al. 2001; Zhu et al. 2008a). The overall shape of the integrin extracellular domain is a large "head" on two long "legs" with flexible "knees" (Fig. 1b). In 2012, the structure of the integrin α4β7 ectodomain was revealed by electron microscopy (EM) and X-ray crystal structure (Yu et al. 2012). The integrin "head piece" contains a β-propeller domain and βI domain, together with the "upper leg," which contains an α thigh and β hybrid, PSI, and I-EGF1 domains (Yue et al. 2013). The remaining calf-1/2, I-EGFs, and β-tail domains in the ectodomain make up the "lower leg" of the integrin (Fig. 1b) (Yu et al. 2012).

The adhesion between an α4 integrin and its ligand is metal ion-dependent. In the α4 subunit, there are three Ca^{2+}-binding sites located in the loops extending between propeller blades 5 to 7 and one site located at the "genu" (Fig. 1a). The β-propeller Ca^{2+}-binding sites are involved in the regulation of the integrin-ligand binding affinity. Conservative mutations in these sites of the α4 β-propeller significantly decreased the affinity for ligand binding to α4β1 (Masumoto and Hemler 1993).

In the β1 and β7 subunits, the βI domain contains an interlinked linear array of three metal ion-binding sites, with a metal ion-dependent adhesion site (MIDAS) at the center flanked by two other sites: the synergistic metal ion-binding site (SyMBS) and the adjacent to MIDAS (ADMIDAS). Based on the electron density in crystal structures, MIDAS is occupied by Mg^{2+}, but the two flanking sites are loaded with Ca^{2+} (Zhu et al. 2008b). The divalent cation in the MIDAS site forms a critical interaction with a negatively charged residue, usually Asp, in the integrin ligand. Disruption of the MIDAS site by mutations completely abolished the MAdCAM-1 binding to integrin α4β7 (Chen et al. 2003). SyMBS, also termed ligand-associated metal-binding site (LIMBS), exhibits a positive regulatory effect for integrin-ligand binding, which is required for the activation of α4β7 (Chen et al. 2003). The SyMBS metal ion can form a cation-π interaction with a conserved aromatic residue (Tyr or Phe) in the specificity-determining loop (SDL), which may help to stabilize SyMBS metal ion coordination and maintain the proper conformation of SDL for an integrin high-affinity state (Pan et al. 2010; Zhang and Chen 2012) (Fig. 2). When occupied by Ca^{2+}, ADMIDAS functions as a negative regulatory site responsible for keeping the integrin in the low-affinity state by preventing the downward movement of the βI domain C-terminal α7-helix, a critical step in integrin activation. Mn^{2+} can compete with Ca^{2+} to occupy the ADMIDAS site and induce integrin activation by the release

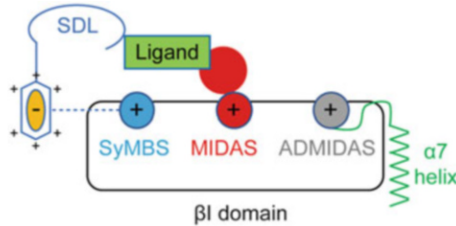

Fig. 2 Metal ion cluster in the βI domain. The linear cluster of the βI domain metal ion-binding sites is shown as SyMBS, MIDAS, and ADMIDAS from left to right. The metal ion-binding sites are colored as follows: blue, SyMBS; red, MIDAS; gray, ADMIDAS. The cation-π interaction between the SyMBS metal ion and the aromatic side chain of Tyr or Phe is shown by a blue dashed line

of the inhibitory effects of Ca^{2+} (Chen et al. 2004; Humphries et al. 2003) (Fig. 2). The downward displacement of the βI domain C-terminal α7-helix allosterically alters the geometry of the metal ion clusters in a way that increases the affinity for ligand binding (Zhang and Chen 2012) (Fig. 2).

2.2 Transmembrane Domains

The integrin α and β subunits anchor themselves within the cell plasma membrane via a single short transmembrane domain (TMD) (Fig. 1a). In addition to connecting the integrin extracellular domain and cytoplasmic domain, TMD has an important role in transducing bidirectional signaling across the plasma membrane (Kim et al. 2003). The linkers between the integrin ectodomain and TMD helices are suggested to be quite flexible during integrin activation, facilitating the β hybrid domain swing-out and overall conformational shift of the integrin molecule to a high-affinity conformation (Lau et al. 2009). Acidic phospholipids in the plasma membrane may ionically interact with the integrin intramembrane basic residue (K/R) close to the TMD and cytoplasmic domain border, which is believed to stabilize the integrin transmembrane dimer and hold the integrin in a resting state (Kim et al. 2011; Lu et al. 2016b). Intriguingly, calcium ions (Ca^{2+}) may disrupt the ionic K/R–lipid interaction through its positive charge, which leads to the separation of the integrin α/β TMDs and the subsequent extracellular domain extension to a high-affinity conformation (Guo et al. 2018).

2.3 Cytoplasmic Domains

The integrin cytoplasmic domain is very short. The α4 and β1/β7 subunits have approximately 30 and 50 amino acid residues, respectively. It serves as a vital bond

connecting its mechano-sensing ectodomain with its cytoplasmic adaptor proteins within the cell (Legate et al. 2006; Wiesner et al. 2006). The binding proteins for the β subunit cytoplasmic domain have been well characterized (Harburger and Calderwood 2009; Zaidel-Bar et al. 2007). For example, talin binds to the membrane-proximal NPxY motif via its canonical phosphotyrosine-binding (PTB) domain, which is critical for integrin activation (Calderwood et al. 2003; Wegener and Campbell 2008). Kindlin binds to a serine/threonine (S/T) rich motif and the membrane-distal NxxY motif, which functions as an integrin co-activator (Calderwood et al. 2003; Wegener and Campbell 2008). Some other PTB-containing proteins, such as docking protein 1 (DOK1) and integrin cytoplasmic domain associated protein 1 (ICAP1), suppress integrin activation by competition with talin (Liu and Boggon 2013; Wegener et al. 2007). Although filamin has no PTB domain, it also competes with talin/kindlin for binding to the β tail because of the overlapping binding sites in the integrin β tail (Calderwood et al. 2013b). The adaptor proteins for the α4 tail are less well studied. Integrin inhibitors, such as mammary-derived growth inhibitor (MDGI) and SHANK-associated RH domain-interacting protein (SHARPIN), interact with the α4 cytoplasmic domain around the conserved juxtamembrane GFFKR motif (Bouvard et al. 2013). The GFFKR motif serves as an important intrinsic structural component in making a salt bridge between the α/β cytoplasmic tails to keep the integrin inactive (Lu et al. 2001). Paxillin specifically binds to the α4 tail and regulates cell spreading and migration (Liu and Ginsberg 2000; Liu et al. 2002; Liu et al. 1999). Moreover, a study using chimeric α2, α4, and α5 integrins demonstrates that the cytoplasmic tails of different α subunits contribute to the determination of integrin-ligand binding specificity (Chan et al. 1992).

3 Extracellular Ligands for α4 Integrins

3.1 VCAM-1

The cell-surface glycoprotein VCAM-1 belongs to the Ig superfamily (IgSF) and contains seven Ig-like extracellular domains or is alternatively spliced into six or eight Ig-like domains (Renz et al. 1994). VCAM-1 is widely expressed on the stimulated endothelial cells of blood vessels, peripheral lymph nodes (PLNs), and bone marrow (BM) (Berlin-Rufenach et al. 1999). Both integrin α4β1 and α4β7 can bind to VCAM-1. Domain 1 (D1) and domain 4 (D4) of VCAM-1 contribute to α4β1-dependent adhesion (Osborn et al. 1992). The linear sequence for QIDSPL, especially the tripeptide IDS (Ile-Asp-Ser), in the D1 and D4 has been demonstrated to be critical for α4β1 recognition (Baiula et al. 2019; Vonderheide et al. 1994). It is noteworthy that both the resting and activated integrin α4β1 can bind to VCAM-1 D1, whereas only the activated α4β1 can bind to VCAM-1 D4 (Kilger et al. 1995). Similarly, integrin α4β7 can also bind to D1 and D4 of VCAM-1 (Ruegg et al. 1992) but with much less affinity than α4β1.

3.2 MAdCAM-1

MAdCAM-1, an IgSF type I transmembrane glycoprotein, is the primary ligand for integrin α4β7. MAdCAM-1 is specifically expressed on the endothelium of HEVs in the gut and GALT, such as Peyer's patches and MLNs, and the venules of the lamina propria (LP) (Berlin et al. 1995; Cox et al. 2011; Springer 1994). The interaction between integrin α4β7 and MAdCAM-1 has an essential role in supporting gut-targeting lymphocyte homing. MAdCAM-1 consists of two Ig-like domains, a mucin-like region, a short TMD, and a cytoplasmic domain (Shyjan et al. 1996). Structural analysis of the MAdCAM-1–α4β7 complex has revealed that α4β7 directly binds to MAdCAM-1 aspartate 42 (Asp42) located on the protruding CD loop in Ig domain 1 through the MIDAS site located in the β7 I domain (Tan et al. 1998; Yu et al. 2012). Moreover, a D strand in the I1 set of MAdCAM-1 Ig domain 2 is also necessary for α4β7 recognition and binding (Newham et al. 1997; Tan et al. 1998). The mucin-like domain, which is a serine/threonine-rich region, leads to a more upright conformation of MAdCAM-1 because of the electrostatic repulsion between negative charges in the extracellular microenvironment and mucin-like region, facilitating α4β7-mediated cell adhesion (Yuan et al. 2020).

A previous study revealed that different chemokines could activate integrin α4β7 in a ligand-specific manner to mediate selective homing of lymphocytes to the gut via its interaction with MAdCAM-1 or to PLNs through binding with VCAM-1 (Sun et al. 2014). In particular, chemokine (C-C motif) ligand 25 (CCL25) enhances the α4β7-mediated lymphocyte adhesion to MAdCAM-1 but suppresses adhesion to VCAM-1, whereas C-X-C motif chemokine 10 (CXCL10) stimulation has the opposite effect. The selective adhesion of α4β7 to MAdCAM-1 and VCAM-1 is determined by the distinct active integrin conformation induced by CCL25 and CXCL10 (Sun et al. 2014; Wang et al. 2018).

3.3 FN

FN is a high-molecular-weight glycoprotein synthesized by hepatocytes and mesenchymal cells (White and Muro 2011). α4 integrins serve as alternative FN receptors in addition to the FN receptor integrin α5β1 (Guan and Hynes 1990; Wayner et al. 1989a). Interestingly, the α4 integrins and α5β1 recognize independent sites in intact FN. Integrin α5β1 recognizes the Arg-Gly-Asp (RGD) containing cell adhesion domain in FN (Pytela et al. 1985), whereas integrin α4β1 recognizes a carboxy-terminal cell adhesion region containing the Heparin II and Type III connecting-segment-1 (CS-1) domains (Guan and Hynes 1990). An acidic motif in CS-1, termed "LDV", is functionally related to RGD and responsible for α4β1 binding. Integrin α4β7 not only recognizes the same site within the alternatively spliced connecting segment of FN as α4β1 (Ruegg et al. 1992) but also directly binds to FN splice variants containing type III repeats III5 (containing a KLDAPT

sequence) and the Extra Domain A (EDA domain, containing an EDGIHEL sequence only in cellular FN) (Pankov and Yamada 2002).

3.4 Gp120

Gp120 is an envelope glycoprotein of HIV-1. Some studies have shown the interaction between integrin α4β7 and gp120 through a conserved tripeptide Leu-Asp-Val/Ile (LDV/I) motif in the V2 loop of gp120 (Peachman et al. 2015; Wang et al. 2021). By mimicking the binding epitopes in MAdCAM-1, gp120 binds to α4β7, which facilitates productive HIV-1 infection of α4β7-expressing $CD4^+$ T cells in GALT (Cicala et al. 2009). However, unlike MAdCAM-1, which can adhere to both inactive and active α4β7, gp120 predominantly binds to α4β7 active conformers with a highly extended conformation (Wang et al. 2021).

3.5 Osteopontin

Osteopontin is a secreted highly acidic glycoprotein originally isolated from bone. Its expression is upregulated at sites of cardiovascular injury, and monocytes and macrophages also synthesized it within injury sites (Bayless et al. 1998). As a ligand for integrin α4β1, osteopontin can promote α4β1-mediated leukocyte adhesion (Bayless et al. 1998). The N-terminal thrombin fragment of osteopontin has been demonstrated to bind to integrin α4β1 (Bayless and Davis 2001).

3.6 Invasin

Another α4β1 ligand is invasin, an outer membrane bacterial protein mediating the attachment of *Yersinia pseudotuberculosis* to human cells. The α4β1 on T cells binds strongly to purified invasin, facilitating a T-cell proliferative response (Ennis et al. 1993). Direct interactions of T cells with bacterial pathogens such as *Yersinia* may be relevant to host immune responses to bacterial infection (Ennis et al. 1993).

3.7 Other α4 Extracellular Domain-Binding Proteins

Several membranal proteins have been demonstrated to interact with integrin α4β1, including junctional adhesion molecule-2 (JAM2), A Disintegrin and Metalloproteinase (ADAMs), CD14, and even the α4 subunit itself. The binding of integrin α4β1 to JAM2 is only enabled following prior adhesion of JAM2 with

JAM3 and is not detectable in cells where JAM3 expression is absent (Cunningham et al. 2002). The disintegrin domains of ADAM7 and ADAM28 are recognized by α4β1 and α4β7, respectively, and the recognition requires integrin activation (Bridges et al. 2005). The lipopolysaccharide (LPS) receptor, CD14, a GPI-linked cell-surface glycoprotein, is a novel ligand for α4β1, exhibiting similar activation-state dependent binding characteristics (Humphries and Humphries 2007). Additionally, the α4 subunit itself can serve as a ligand. There are three conserved LDV motifs in the extracellular sequence of the α4 subunit. α4β1 and α4β7 exhibited homophilic interactions with α4 subunits in vitro, suggesting that α4 integrins may bind to α4 subunits on adjacent cells (Altevogt et al. 1995).

4 Intracellular Adaptor Proteins for α4 Integrins

4.1 β1 and β7 Cytoplasmic Tail-Binding Proteins

4.1.1 Talin

Talin is a 250 kDa cytoskeletal protein that links integrins and the actin cytoskeleton. Talin comprises a 220 kDa rod domain and a 50 kDa N-terminal head domain (THD). THD harbors four subdomains, including the FERM (Band 4.1, ezrin, radixin, moesin) domain made up of F1, F2, and F3 domains, and the N-terminal F0 domain preceding the FERM domain (Calderwood et al. 1999; Critchley 2009). The F3 subdomain binds to integrin β cytoplasmic tails through its canonical PTB domain. β1 and β7 tails bind to talin via their membrane-proximal NPxY motifs (NPIY motif for β1 and NPLY motif for β7), and the α-helical region lying between the membrane and the NPxY motif (Anthis et al. 2009; Wegener et al. 2007). The binding of talin to the β cytoplasmic domain requires the dephosphorylation of the Tyr in the NPxY motif and is critical for α4 integrin activation (Calderwood et al. 2002). Upon talin binding, integrin β cytoplasmic tails form a stable span between the TMD and cytoplasmic membrane-proximal region, which disrupts the salt bridge between the α/β cytoplasmic tails by forming a non-covalent interaction with a conserved aspartate or glutamate residue at the β subunit membrane-proximal region, and consequently, activates integrin (Calderwood et al. 2013a).

4.1.2 Kindlin

Kindlins also contain the conserved FERM domain and bind to a serine/threonine motif and membrane-distal NxxY motifs (Harburger et al. 2009; Malinin et al. 2010; Sun et al. 2014). In mammals, there are three kinds of kindlins (Lu et al. 2016a; Sun et al. 2014; Sun et al. 2019). Kindlin-1 is mainly expressed in epithelial cells. Kindlin-2 is expressed ubiquitously, except for the hematopoietic system. Kindlin-3 is restricted to hematopoietic cells. Kindlin and talin are indispensable for integrin

activation through their crucial functions in connecting surface-expressing integrins with intracellular actomyosin and regulating actin dynamics (Sun et al. 2019).

4.1.3 Filamin

Filamin, a high-molecular-weight actin-cross-linking protein, serves as an important integrin inactivator via interaction with the β subunit membrane-proximal NPxY motif and the subsequent serine/threonine-rich region, which overlaps the talin and kindlin-binding sequences and competes for their association with β tails, thereby inhibiting integrin activation (Calderwood et al. 2001).

4.2 α4 Cytoplasmic Tail-Binding Proteins

4.2.1 SHARPIN

SHARPIN was described as an important inactivator of integrins 10 years ago (Rantala et al. 2011). SHARPIN binds to the α integrin membrane-proximal region that contains the conserved GFFKR motif. SHARPIN binding does not involve the arginine residue in the GFFKR; therefore, it does not interfere with the formation of the clasping salt bridge. Furthermore, SHARPIN bound to the α tail inhibits talin and kindlin binding to the β tail presumably through steric hindrance (Rantala et al. 2011). Therefore, SHARPIN inhibits the activation of both integrin α4β1 and α4β7. Moreover, SHARPIN deficiency has been shown to enhance integrin-mediated cell adhesion and reduce cell migration velocity (Park et al. 2015; Pouwels et al. 2013).

4.2.2 MDGI

Similar to SHARPIN, MDGI also binds directly to the cytoplasmic tail of integrin α subunits, including α4, through a conserved GFFKR sequence. MDGI bound to α tails retains integrin in an inactive conformation attenuating integrin-mediated adhesion, migration, and invasion (Nevo et al. 2010).

4.2.3 Paxillin

Paxillin is the first identified and extensively studied signaling adaptor protein of α4 integrins (Liu et al. 1999). It is widely expressed and comprises five leucine-rich LD motifs in its N-terminus and four LIM domains in its C-terminus (Rose 2006). Paxillin physically associates with α4 integrins, and this association markedly reduces cell spreading, focal adhesions, and stress fiber formation (Liu et al. 1999). A region of nine amino acid residues (Glu983-Tyr991) within the α4

cytoplasmic domain contains a minimal sequence sufficient for paxillin binding. Furthermore, Tyr991 and Glu983 are critical residues, and either a Y991A or E983A substitution disrupts the interaction of α4 integrins with paxillin (Liu and Ginsberg 2000; Liu et al. 1999). In turn, the stretch of amino acid residues Ala176-Asp275 in the LD3 and LD4 repeats of paxillin is sufficient for binding to the α4 tail (Liu et al. 2002). The interactions between LD3 and LD4 of paxillin and the α4 tail have been confirmed by nuclear magnetic resonance (NMR) studies and the docked structures of the α4 tail with these LD repeats, suggesting possible polar and/or salt bridge and non-polar packing interactions (Chua et al. 2013). Paxillin binding to the α4 tail is also regulated by post-translational modification of the latter. Phosphorylation at Ser988 blocks paxillin binding to the α4 tail and reverses the inhibitory effect of α4 on cell spreading (Han et al. 2001). Phosphorylation of α4 is restricted to the leading edge and is absent from the sides and rear of migrating cells (Goldfinger et al. 2003). The binding of paxillin to the α4 integrin subunit in the trailing edge inhibits adhesion-dependent lamellipodium formation by blocking Rac activation (Nishiya et al. 2005).

Transgenic mice homozygous for α4 Y991A have reduced Peyer's patches and impaired mononuclear leukocyte recruitment to sites of inflammation (Feral et al. 2006). A small molecule that inhibits the interaction of paxillin and α4 integrin inhibits the accumulation of mononuclear leukocytes at sites of inflammation, proving inhibition of α4 integrin signaling as a target for the pharmacological reduction of inflammation (Kummer et al. 2010).

4.2.4 Hsp90

Heat shock proteins (Hsps) are a family of proteins that display enhanced expression in response to thermal stress (Schlesinger 1990). Febrile temperatures (38.5 °C) can efficiently enhance the expression of Hsp90 in T cells and increase α4 integrin-mediated T-cell adhesion and transmigration (Lin et al. 2021; Lin and Chen 2019; Lin et al. 2019). Hsp90 binds to the ENRRDSWSY motif of the α4 cytoplasmic tail and induces association of talin and kindlin-3 with integrin β tails, triggering α4 integrin activation via inside-out signaling. Moreover, the N- and C-terminus of one Hsp90 molecule can simultaneously bind to two α4 tails, resulting in dimerization and clustering of α4 integrins on the plasma membrane and subsequent activation of the FAK-RhoA signaling pathway in T lymphocytes, thereby promoting T lymphocyte adhesion and transmigration. This regulation of α4 integrin function does not require the ATPase activity of Hsp90, suggesting that this function is distinct from the chaperone function of Hsp90, which requires the energy released from ATP hydrolysis (Lin et al. 2019). Abolishing Hsp90-α4 interaction in vivo inhibits whole-body hyperthermia (WBH)-induced T-cell trafficking to draining lymph nodes. Moreover, in a *Salmonella typhimurium* infection-induced mouse fever model, disruption of the Hsp90-α4 interaction in the mice markedly decreased the number of infiltrated T cells and increased bacterial dissemination in the small intestine, resulting in a significantly increased death rate (Lin et al. 2019). Thus, the

fever-induced Hsp90-α4 integrin axis is crucial for promoting immune cell trafficking to inflamed tissues to facilitate the clearance of bacterial infection.

Because other cellular stressors can induce Hsp90 expression, the Hsp90-α4 integrin pathway may also play a role in pathological conditions in allergies, autoimmune diseases, and even cancer. Whether this pathway can be targeted therapeutically to enhance or temper immune trafficking awaits further study (Bird 2019). This pathway might be exploited to make T cells "super-homers" by raising Hsp90 levels in efforts to combat cancer during the administration of cancer immunotherapy (Hampton 2019).

5 Activation of α4 Integrins

5.1 Affinity Regulation

Integrin activation is accompanied by bidirectional signal transduction across the plasma membrane. Both "inside-out" and "outside-in" integrin signaling play important roles in integrin-mediated biological processes. When a cell receives an extracellular stimulus through its cell-surface receptors, such as chemokine receptors or T-cell receptor tyrosine kinases (RTKs), the transduced signals will promote the binding of intracellular integrin activators including talin and kindlins to the integrin β tail, leading to an integrin global conformational change into the active state with enhanced adhesiveness for extracellular ligands. This integrin activation process is termed "inside-out" activation (Kim et al. 2003; Shattil et al. 2010). In addition, integrin can also serve as a classical cell-surface signaling receptor, which transmits signals into cells upon association with its ligands via "outside-in" signaling, thereby mediating cell proliferation, survival, and differentiation (Takagi et al. 2002).

Both of these events lead to integrin affinity regulation along with striking conformational changes in the integrin molecule (Beglova et al. 2002; Luo et al. 2007; Takagi et al. 2001; Takagi et al. 2002). α4 integrins consist of a globular ligand-binding head domain involving an α and β subunit interface localized within the α4-β propeller and β1/β7-βI domains, two long legs connected to the TMD and the cytoplasmic tail of each subunit (Yu et al. 2012). It behaves in at least three overall conformational states, including the bent conformation low-affinity state, the extended conformation with a closed or intermediate headpiece intermediate affinity state, and the extended conformation with an open headpiece high-affinity state (Fig. 1b) (Carman and Springer 2003; Luo et al. 2007).

The activation of α4 integrins is associated with an integrin molecule extension coupled with a β subunit hybrid domain swing-out and separation of the α/β leg domains (Fig. 1b) (Chigaev et al. 2009; Takagi et al. 2002). Separation of the α/β subunit transmembrane domains and cytoplasmic tails has been defined as the crucial initiator of the inside-out signaling cascade, which has been widely verified using fluorescence resonance energy transfer (FRET)-based studies (Kim et al. 2003; Wang et al. 2018). A ~ 62° hybrid domain swing-out with respect to the βI domain is

thought to drive the downward movement of the α7 helix within the I domain and shift the ligand-binding MIDAS site into the open conformation, thereby facilitating high ligand-binding affinity (Liu et al. 2014; Wang et al. 2018; Xiao et al. 2004). This high-affinity integrin conformation can exist independently of ligand binding (Carman and Springer 2003). In the presence of its binding ligand, integrin rearranges its headpiece conformation to an open orientation with an ~ 80° β subunit hybrid domain swing-out (Takagi et al. 2002; Takagi et al. 2003), followed by conformational changes in the tailpiece, which lead to "outside-in" signal transduction.

It is noteworthy that α4β7 can be activated into distinct intermediate- and high-affinity states, which exhibit selective ligand-binding preferences for MAdCAM-1 and VCAM-1 (Sun et al. 2014; Wang et al. 2018). Chemokines CCL25 and CXCL10 can stabilize the α4β7 integrin in two intermediate open conformations via triggering the p38α MAPK/PKCα and c-Src/Syk pathways, respectively, leading to different phosphorylation states of the β7 tail and distinct talin and kindlin-3 binding patterns. The two intermediate open conformers of α4β7 show selective adhesion to either MAdCAM-1 or VCAM-1, which results in selective homing of lymphocytes to distinct tissues in mice.

5.2 Avidity Regulation

Cells treated with a certain stimulus represent an obvious adhesive phenotype to the ligand-coated surface while no high-affinity soluble ligand binding is observed (Stewart et al. 1998; van Kooyk and Figdor 2000), suggesting that another regulatory pathway than "affinity regulation" contributes to integrin activation. Indeed, an integrin binding to its ligands not only changes its global conformation but also leads to clustering or patching of integrin-receptor complexes on the cell surface where cell–cell or cell–matrix contact exists, which is termed "avidity regulation" or "valency regulation" (Carman and Springer 2003; Schurpf and Springer 2011). Diverse patterns of α4 integrins clustering on living cells have been reported (Carman and Springer 2003; Shamri et al. 2002; Sun et al. 2014; Zhang et al. 2013) and can be classified into two types based on the presence of extrinsic ligands or not. In the absence of ligands, integrins redistribute to form active clusters via intrinsic signals and are transported to the cell–matrix contact surface or cell leading edge, facilitating cell adhesion and migration (Sun et al. 2014; Wang et al. 2021; Zhang et al. 2013). The cytoplasmic tail of α4 integrins makes strong positive contributions to integrin-mediated cell adhesion by upregulating integrin clustering and increasing the overall avidity of integrins (Yauch et al. 1997). Hsp90 has recently been identified as a novel α4 intracellular binding protein (Lin et al. 2019). The N-terminal domain (NTD) and C-terminal domain (CTD) of Hsp90 can directly bind to the α4 tail. Thus, one Hsp90 molecule simultaneously associates with two α4 subunits, which induces α4 integrin dimerization and clustering on the cell membrane and subsequent activation of integrin downstream signaling.

Redistribution of α4 integrins on the cell surface also occurs in a ligand-dependent manner. This is probably caused by the multivalent property of integrin-binding ligands, such as FN.

6 Expression and Physiological Functions of α4 Integrins

6.1 Expression of Integrin α4β1 and α4β7

Integrin α4β1 is constitutively expressed on most leukocytes (Luster et al. 2005). In general, neutrophils are assumed not to express α4 integrins. However, α4 integrins have been demonstrated to play a role in mediating neutrophil adhesion and migration (Johnston and Kubes 1999), suggesting that low levels of α4 integrins on neutrophils suffice for functional activity. Moreover, α4β1 expression occurs in hematopoietic stem cells (HSCs) (Williams et al. 1991) and non-hematopoietic cells in multiple embryonic tissues, including somites, heart, vascular smooth muscle and skeletal muscle, the neuroepithelium of the embryonic retina, and neural crest-derived cells, such as melanoblasts (Sheppard et al. 1994; Stepp et al. 1994; Yang et al. 1995). α4β1 is also expressed in various tumor cells, including metastatic melanoma and gastric cancer cells (Mould et al. 1994; Park et al. 2004; Rebhun et al. 2010), indicating vital roles in both physiological and pathological processes.

The expression of integrin α4β7 is restricted on hematopoietic cells. A group of immune cells has been reported to express integrin α4β7 constitutively, including lymphocytes, NK cells, mast cells, basophils, eosinophils, macrophages, and monocytes (von Andrian and Mackay 2000). Under a steady-state, naïve lymphocytes exhibit a relatively low expression level of integrin α4β7 (Erle et al. 1994). Among them, most B lymphocytes show relatively high expression of integrin α4β7 compared with most T lymphocytes in the peripheral lymphoid organs, including the spleen, Peyer's patches, and lymph nodes (Andrew et al. 1996). Intestinal dendritic cells (DCs)-derived retinoic acid (RA) has been shown to upregulate α4β7 expression on memory or activated gut-tropic lymphocytes, promoting lymphocyte homing to mucosal tissues or GALT effectively during intestinal immune responses (Hao et al. 2021; Iwata et al. 2004; Mora et al. 2006). In contrast, the majority of naïve or memory T cells, which lack α4β7 expression in circulation, express α4β1 instead (DeNucci et al. 2010). These cells prefer to home to non-mucosal tissues via binding to VCAM-1 (Rott et al. 1996; Rott et al. 1997). Interleukin-7 (IL-7) can potently induce α4β7 expression on naïve T cells and is closely related to α4β7 activation, resulting in intestinal homing of lymphocytes and immunologic reconstitution in lymphopenic hosts (Cimbro et al. 2012). As observed in hypereosinophilic patients, eosinophils characterized with high α4β7 expression exhibit preferential gut homing rather than trafficking to the lungs (Brandt et al. 2006). Moreover, integrin α4β7 is significantly upregulated on both peripheral naïve and memory T-cell subsets in intestinal graft-versus-host disease (GvHD) patients (Chen et al. 2009; Chen et al.

2013), suggesting integrin α4β7 serves as a crucial regulator in both homeostatic and pathologic conditions.

6.2 Integrin α4β1 and α4β7 in Leukocyte Homing

The recruitment of leukocytes from blood circulation to lymphoid organs and inflamed tissues is regulated by the sequential interactions of adhesion and signaling molecules on leukocytes and blood vessels, essential to immune surveillance and host defense (Butcher and Picker 1996). This recruitment process consists of a highly ordered adhesion cascade that includes tethering and rolling leukocytes along vessel walls of HEVs, chemokine-induced activation, firm arrest, and transendothelial migration (von Andrian and Mempel 2003). The initial transient contact of the circulating leukocytes with the vascular endothelium leads to leukocytes rolling along the vascular wall with greatly reduced velocity. The interaction mainly mediates this process between selectins and their ligands. In addition, inactive α4β1 and α4β7 integrins are also able to support lymphocyte rolling via binding to their endothelial ligands, VCAM-1 and MAdCAM-1 (Ley et al. 2007), respectively. After chemokine-induced activation of lymphocytes on the endothelium, α4 and β2 integrins are activated to mediate cell arrest. During this process, chemokines activate integrins through the rapid triggering of the signaling network that regulates the binding of intracellular effector proteins (e.g., talin or kindlin) to the cytoplasmic domains of integrins, which induces integrin activation (Hogg et al. 2011). The final transmigration step across HEVs involves multiple adhesion molecules, including α4β1, αLβ2, VCAM-1, intercellular adhesion molecule-1 (ICAM-1), ICAM-2, platelet endothelial cell adhesion molecule-1 (PECAM-1), junctional adhesion molecule-1 (JAM-1), and JAM-2 (Carman and Springer 2004). Notably, α4 integrins are involved in each step of leukocyte homing, and thus, have essential roles in regulating lymphocyte trafficking to lymphoid organs and inflamed tissues (Lin et al. 2019).

6.3 Integrin α4β7 in Lymphocyte Gut-Tropic Trafficking and GALT Formation

Integrin α4β7 is the most important gut-homing adhesion molecule in targeting lymphocytes trafficking to mucosal lymphoid tissues, such as Peyer's patches and mesenteric lymph nodes (MLNs) (Habtezion et al. 2016). Naïve and central memory lymphocytes in circulation are recruited to enter the mucosal lymphoid tissues via interaction between surface-expressing α4β7 and its ligand MAdCAM-1 expressed on the HEVs localized in these tissues (Laudanna et al. 2002). Along with priming in the GALT, gut-homing lymphocytes undergo α4β7 and CCR9 upregulation by RA

produced by DCs and/or stromal cells in the MLN, which imprints them with gut tropism and directs these cells to the LP of the small intestine (Agace 2010; Agace and Persson 2012). The conventional intraepithelial lymphocytes (IELs) derived from circulating T cells work similarly as LP lymphocytes (LPLs) (Habtezion et al. 2016), whereas the unconventional IELs originate from $CD8^+$ thymocytes, which already express α4β7 and CCR9 in the thymus, bypassing the imprinting process in GALT (Guy-Grand et al. 2013; Staton et al. 2006).

Integrin α4β7 deficiency shows no defect in T and B cell development in mice, suggesting it is not required for lymphocyte development. However, in $\beta 7^{-/-}$ mice, the failure of α4β7-induced lymphocyte homing results in a large reduction in gut-tropic lymphocytes within the intestinal tissue, which eventually leads to abnormal development of GALT (Wagner et al. 1996). In addition, an integrin α4β7 mutation, which fails to transmit talin-induced TMD topology and blocks lymphocyte gut homing, results in GALT development disruption (Sun et al. 2018). These data suggest a vital role of α4β7 in the development of GALT architecture.

6.4 Role of Integrin α4β7 in Innate Immune Cells

Integrin α4β7 has also been reported to regulate innate immune cell tolerogenic functions in the mucosal immune system beyond lymphocyte trafficking (Kempster and Kaser 2014; Villablanca et al. 2014). A subset of α4β7-expressing $Lin^{neg}Ly6C^{low}$ BM cell progenitors give rise to RA-producing DCs, which are required to imprint gut-homing T cells. Moreover, integrin α4β7 is indispensable for the localization and reconstitution of these tolerogenic gut mononuclear phagocytes with the ability to induce $Foxp3^+$ regulatory T cells and IL-10-producing T cells (Villablanca et al. 2014).

6.5 Integrin α4β1 in T-Cell Proliferation, Activation, and Survival

Integrin α4β1 can influence T cells in different ways. Anti-CD3 plus the integrin α4β1 ligand (CS1) can induce significant proliferation of $CD4^+$ cells (Engelhardt et al. 1998; Nojima et al. 1990). Certain antibodies against integrin α4β1 block the effector activity of class I- and II-specific cytotoxic T lymphocytes (CTLs) in vitro (Clayberger et al. 1987; Takada et al. 1989). Integrin α4β1 also plays a regulatory role for T lymphocyte-antigen presenting cell cognate immune interactions by concentrating at the peripheral supramolecular activation complex (pSMAC) of the immune synapse and driving Th1 responses (Mittelbrunn et al. 2004). Furthermore, adhesion mediated by α4β1–VCAM-1 interaction promotes both T and B cell

survival. Blockade of the interaction via monoclonal antibodies (mAbs) causes increased cell apoptosis (Koopman et al. 1994; Leussink et al. 2002).

6.6 Integrin α4β1 in Hematopoietic Stem Cell Maintenance and Hematopoiesis

Integrin α4β1 also plays an important role in developing and maintaining HSCs and hematopoiesis. Disturbing progenitor cell interaction with stromal cells by blocking α4 integrin inhibits lymphopoiesis in vitro (Miyake et al. 1991). Analyzing hematopoiesis in integrin α4- and β1-deficient somatic chimeric mice demonstrated a requirement of α4β1 in HSC homing to the fetal liver (Arroyo et al. 1999). α4 integrins are also required for hematopoietic precursor cell interactions with BM stromal cells and act as a retention signal for hematopoietic progenitors to remain in the BM compartment (Arroyo et al. 1996). Lack of α4 integrins leads to premature detachment of hematopoietic progenitors and low yields of mature cells in peripheral tissues (Arroyo et al. 1999). It is noteworthy that all detected requirements for α4 integrins in hematopoiesis result from α4-deficient mice. However, the lack of α4 integrins solely on hematopoietic cells in the adult does not hamper hematopoiesis, suggesting α4 integrins in non-hematopoietic cells may involve hematopoiesis (Brakebusch et al. 2002; Bungartz et al. 2006; Scott et al. 2003).

6.7 Integrin α4β1 and Embryonic Development

α4β1 is crucial in embryonic development. Deficiency of the α4 integrin subunit leads to embryonic lethality because of a failure in cell–cell adhesion events in placental and cardiac development (Yang et al. 1995). The early defect in α4-null embryos is characterized by a failure of the allantois to fuse with the chorion during placental development, and these embryos die by approximately embryonic day 11 (Yang et al. 1995). Although in a few cases, the fusion of the allantois and the chorion is successful, those embryos still die at approximately embryonic day 11.5 because of severe hemorrhage in the heart region caused by a failure in the epicardium-myocardium attachment during cardiac development (Yang et al. 1995). As a major counter-receptor of the α4β1 integrin, VCAM-1–deficient embryos show the same placental and cardiac defects, suggesting that these defects are probably caused by α4β1/VCAM-1–mediated adhesive interactions during embryonic development (Gurtner et al. 1995; Kwee et al. 1995). Whether α4β1 and VCAM-1 are involved in skeletal muscle development is controversial (Rosen et al. 1992; Yang et al. 1996).

7 α4 Integrins in Disease

7.1 *α4 Integrins in Cancer*

Altered integrin expression and function have been comprehensively reported in many types of human cancer and are tightly correlated with different stages of tumor progression, patient responses to antineoplastic therapy, and survival rates of patients with cancer (Table 1) (Hamidi and Ivaska 2019). Integrin α4β1 has been

Table 1 α4 integrin-related diseases

Disease	Integrin	Function of integrins and the targeting drugs	References
Neuroblastoma	α4β1	P1H4 (targeting the α4 subunit) blocks neuroblastoma metastasis.	Gazitt and Akay (2004), Young et al. (2015)
Multiple myeloma (MM)	α4β7	MMG49 (targeting highly-activated integrin β7)-derived chimeric antigen receptor (CAR)-T cells significantly dampen MM progression.	Hosen (2020), Hosen et al. (2017)
Thymic lymphoma	α4β7	Integrin α4β7 is involved in abnormal lymphocyte trafficking to the hyperplastic thymus, leading to thymic lymphoma development.	Michie et al. (1995)
Colorectal cancer (CRC)	α4β7	α4β7 mediates the recruitment of IFN-γ-producing $CD4^+$ T cells, cytotoxic $CD8^+$ T cells, and NK cells to the CRC tissue where they exert effective anti-tumor immune responses.	West et al. (2015), Zhang et al. (2021)
Inflammatory bowel disease (IBD)	α4β7	Natalizumab (targeting the α4 subunit); Vedolizumab (targeting the α4β7 heterodimer); Abrilumab (AMG-181, targeting the α4β7 heterodimer); Etrolizumab (targeting the β7 subunit).	Mezu-Ndubuisi and Maheshwari (2021), Sandborn et al. (2013), Pan et al. (2013) Selinger et al. (2018)
Multiple sclerosis (MS)	α4β1	Natalizumab can reduce the risk of the sustained progression of disability and the rate of clinical relapse in patients with relapsing MS.	Polman et al. (2006), Radue et al. (2010), Rudick et al. (2006)
Acquired immunodeficiency syndrome (AIDS)	α4β7	Vedolizumab and etrolizumab have shown efficient inhibition of gp120 binding to α4β7.	Wang et al. (2021), Burnie and Guzzo (2019), Sneller et al. (2019)
Graft-versus-host disease (GvHD)	α4β7	Vedolizumab has now been evaluated to treat steroid-refractory (SR) GI aGvHD.	Chen et al. (2019), Danylesko et al. (2019)

detected on both benign and malignant cells of the melanocytic lineage and suppresses metastasis formation in some experimental tumor models (Gazitt and Akay 2004). However, in the MYCN^{low} neuroblastoma, integrin α4 expression enhances experimental metastasis in a syngeneic tumor model, reconstituting a pattern of organ involvement similar to that seen in patients. Accordingly, antagonism of α4β1 (P1H4, antibody against integrins α4) blocks metastasis, suggesting the adhesive function of α4β1 is required during neuroblastoma metastasis (Young et al. 2015).

A previous study documented the crucial role of integrin α4β7 in the pathogenesis and development of multiple myeloma (MM), also called Kahler's disease, a blood cancer that forms from the abnormal aggregation of antibody-producing plasma cells (Hosen 2020). In MM patients, cancerous plasma cells preferentially accumulate in the BM microenvironment, which crowds out healthy leukocytes and generates superfluous invalid immunoglobulins into blood circulation and bones, thereby causing severe complications. MM cells selectively adhere to either the BM microenvironment ECM components or multiple cell types residing in the BM, including hematopoietic cells, stromal cells, endothelial cells, and osteocytes, through interactions between adhesion molecules, such as integrins α4β1, α4β7, αLβ2, and their ligands VCAM-1, FN, and ICAM-1 (Shishido et al. 2014). In particular, integrin α4β7 is a central mediator in orchestrating MM cell adhesion, migration, aggregation, and extraversion during cancer progression (Neri et al. 2011). The expression of integrin β7 subunit could be upregulated by overexpression of *c-maf* gene (Hurt et al. 2004). The upregulated expression of integrin β7 in MM is inversely correlated with patient survival outcomes (Neri et al. 2011). Depletion of β7 expression in MM cells impairs the adhesion between cancer cells and ECM ligands, such as FN. It weakens its transwell migration induced by the BM-homing chemokine, CXCL12 (stromal cell-derived factor-1, SDF-1), reducing MM cells assembling in the BM microenvironment and subsequently alleviating cancer progression in the mouse model (Hosen 2020; Neri et al. 2011). Moreover, the integrin β7 subunit is primarily associated with the α4 subunit on MM cells rather than the αE subunit (Hosen et al. 2017), underlining the dominating effects of α4β7 in regulating MM pathogenesis. It is noteworthy that integrin β7 on MM cells has been unexpectedly detected to adopt a constitutively active conformation in the process of MM-specific cell-surface antigen screening (Hosen 2020; Hosen et al. 2017). The MM-specific mAb MMG49, which only recognizes the highly-activated conformation of the integrin β7 chain, specifically targets constitutively activated β7 expressed on MM cells instead of other CD45^{+} normal leukocytes in the BM. Usage of MMG49-derived chimeric antigen receptor (CAR)-T cells significantly dampens MM progression and prolongs the survival of tumor-bearing mice without damaging normal hematopoietic cells in the mouse model (Hosen et al. 2017). This suggests promising results from targeting a specific active conformer of α4β7 in anti-MM therapy in the future.

Integrin α4β7 is involved in abnormal lymphocyte trafficking to the hyperplastic thymus, characterized by an increased number of medullary blood vessels showing prominent HEV morphology and MAdCAM-1 expression, leading to thymic lymphoma development (Michie et al. 1995). Importantly, as a gut-tropic molecule,

integrin α4β7 plays a profound role in regulating the progression of colorectal cancer (CRC). α4β7 mediates the recruitment of IFN-γ-producing $CD4^+$ T cells, cytotoxic $CD8^+$ T cells, and NK cells to the CRC tissue where they exert effective anti-tumor immune responses (West et al. 2015). A recent study showed that β7 expression decreased in tumor-derived compared to normal tissue-derived $CD8^+$ T cells (Zhang et al. 2021). Higher β7 expression levels are correlated with longer patient survival, higher cytotoxic immune cell infiltration, lower somatic copy number alterations, decreased mutation frequency of *APC* and *TP53*, and better response to immunotherapy. β7 deficiency led to exaggerated tumorigenesis and progression in both $Apc^{min/+}$ spontaneous and MC38 orthotopic models of CRC, which could have been caused by the reduced infiltration of activated $CD8^+$ T cells, effector memory $CD8^+$ T cells, $IFN\gamma^+$ $CD8^+$ T cells, and other immune cell subsets that are essential players in anti-tumor immunity.

7.2 Integrin α4β1 in Multiple Sclerosis (MS)

The integrin α4β1/VCAM-1 axis plays an essential role in the homing of inflammatory leukocyte cells into the central nervous system (CNS) in different experimental autoimmune encephalomyelitis (EAE) models (Table 1) (Engelhardt et al. 1998; Kent et al. 1995a; Kerfoot and Kubes 2002; Yednock et al. 1992). Although encephalitogenic T cells express both α4β1 and α4β7 at similar levels, neutralizing α4β7 with antibodies does not inhibit EAE development (Engelhardt et al. 1998). It has been reported that antibodies to VCAM-1 inhibit the pathogenicity of encephalitogenic Th1 cells in vivo (Baron et al. 1993). The mouse mAb AN100226m, a potent antibody blocking α4 integrin-mediated binding to FN and VCAM-1, has been demonstrated to inhibit lymphocyte adhesion to tumor necrosis factor (TNF)-stimulated brain endothelium and is shown to reverse disease progression in the EAE model (Kent et al. 1995b). The humanized antibody AN100226, natalizumab, can reduce the risk of the sustained progression of disability and the rate of clinical relapse in patients with relapsing MS (Polman et al. 2006). It is noteworthy that few patients undergoing natalizumab therapy in combination with other immunoregulatory and immunosuppressive agents were diagnosed with progressive multifocal leukoencephalopathy (PML) after its initial approval, therefore the usage of the agent is restricted to monotherapy in patients with relapsing forms of MS (Warnke et al. 2010). Furthermore, the combination of natalizumab with interferon-β 1α (IFN-β 1α) is significantly more effective than IFN-β 1α alone in patients with relapsing MS (Radue et al. 2010; Rudick et al. 2006). Thus, adhesion-molecule inhibitors hold promise as an effective treatment for relapsing MS.

7.3 Integrin α4β7 in Inflammatory Bowel Disease (IBD)

IBD is a chronic and prejudicial inflammation of the gastrointestinal (GI) tract, including Crohn's disease (CD) and ulcerative colitis (UC). The former is diagnosed with intermittent transmural inflammation that occurs mostly on the terminal ileum of the small intestine before the large intestine (colon) but can also affect other sites in the GI tract from the mouth to the anus. Unlike CD, UC primarily occurs in the large intestine (colon) and the rectum, with pathological features characterized by continuous inflammation in the superficial mucosa starting from the rectum and extending further into the large intestine (Gorfu et al. 2009). CD and UC share common clinical symptoms, including persistent diarrhea, abdominal pain, rectal bleeding, bloody stools, body weight loss, and fatigue, seriously hindering the quality of life of the patient (Waljee et al. 2009).

Activation of the immune response generally occurs in IBD patients, manifested by various alterations in inflammatory cytokines and Ig family protein production (de Souza and Fiocchi 2016). During the progression of IBD, the chronic inflammation is accompanied by the accumulation of macrophages, $CD4^+$ and $CD8^+$ T cells, B cells, and other leukocytes in the LP, and crypt abscesses with a high production of cytokines, including TNF-α, IFN-γ, IL-12, IL-13, and IL-5 (de Souza and Fiocchi 2016; Strober et al. 2007; Xavier and Podolsky 2007). Of note, integrin α4β7-mediated lymphocyte trafficking to intestinal tissues is deemed the most significant inducement in IBD etiopathogenesis (Table 1) (Neurath 2014; Zhang et al. 2016), whose inhibitors show promising therapeutic effects in clinical implications of IBD patients. Natalizumab, which targets the integrin α4 subunit to block α4β7 binding to VCAM-1/MAdCAM-1 and α4β1–VCAM-1 binding, provides favorable effects in treating CD patients who are refractory to standard therapy (Mezu-Ndubuisi and Maheshwari 2021). Unfortunately, PML, a rare and often fatal side effect of natalizumab in IBD patients, restricts the use of this drug during treatment (Li et al. 2018b). Vedolizumab, an integrin α4β7 blocking mAb, came on the market for IBD treatment in 2014 from Takeda. It specifically inhibits the interaction between α4β7 and MAdCAM-1 without affecting α4β1 function; thus, it avoids the PML-associated side effects of natalizumab and other systemic immunosuppression influences (Sandborn et al. 2013).

The successful use of vedolizumab in IBD highlights the research and development of therapeutic targets aiming at the gut-tropic molecule α4β7. Recently, abrilumab (Amgen), also called AMG-181, targeting the α4β7 integrin heterodimer, shows encouraging results in a phase II study on moderate to severe CD and UC (Pan et al. 2013). In addition, etrolizumab from Genentech, mAb specifically targets the β7 subunit to block both α4β7- and αEβ7-mediated adhesion, is now in an ongoing robust phase II study on UC and phase III study on CD (Selinger et al. 2018).

7.4 Integrin α4β7 in Acquired Immunodeficiency Syndrome (AIDS)

Acute HIV infection features high virus replication within the GALT, accompanied by a remarkable reduction in $CD4^+$ T cells, especially the gut-homing integrin α4β7-expressing $CD4^+$ T cells (Table 1) (Cicala et al. 2009; Guadalupe et al. 2003). It has been demonstrated that the HIV-1 envelope glycoprotein gp120 can bind α4β7 through its key residues localized in the V2 domain (V2-loop) (Richardson et al. 2015), facilitating the HIV virion-$α4β7^+CD4^+$ T-cell complex recruitment to intestinal tissues via an α4β7 interaction with MAdCAM-1 (Liu and Lusso 2020). During acute HIV infection, the $α4β7^{high}$ memory $CD4^+$ T cells are thought to be the early and primary targets of HIV (Cicala et al. 2011; Pena-Cruz et al. 2013).

Integrin α4β7-gp120 binding and further incorporation of α4β7 in HIV virions exist in a selective manner (Guzzo et al. 2017; Wang et al. 2021). It was recently reported that gp120 only binds to α4β7 active conformers with highly extended conformations, which can be induced by particular chemokines, such as CCL19 and CCL25, and the binding of gp120 to α4β7 induces the activation of multiple signal pathways in T cells (Wang et al. 2021). The α4β7-directed virus gut-tropic distribution subsequently leads to HIV colonization and replication in gut-related tissues in the early stage of HIV infection, further resulting in massive depletion of $CD4^+$ T cells and structural damage of the GI tract (Brenchley et al. 2004; Cicala et al. 2009).

The α4β7 blocking mAb vedolizumab and β7 blocking mAb etrolizumab have shown efficient inhibition of gp120 binding to α4β7 (Wang et al. 2021), supporting the inhibitory effects of α4β7 neutralization in HIV infection (Li et al. 2018a). To date, several open-label, single-arm clinical trials with vedolizumab are being conducted with HIV-infected individuals in the USA, Spain, Canada, and France to better evaluate the effects of the α4β7 inhibitor in anti-HIV therapy (Burnie and Guzzo 2019; Sneller et al. 2019).

7.5 Integrin α4β7 in Graft-Versus-Host Disease

GvHD is identified as two distinct entities: acute and chronic GvHD (Martinez-Cibrian et al. 2021). Acute GvHD (aGvHD) is an immune syndrome induced by activated donor effector T cells, which are CTLs, recognizing host antigens and initiating immune responses to attack host organs, such as the GI tract, skin, and liver (Table 1) (Welniak et al. 2007). It frequently occurs in patients adopting allogeneic HSCT, whose grade of aGvHD is negatively correlated with prognosis and is considered the major cause of mortality post-HSCT surgery (Deeg 2007; Martin et al. 2012).

During aGvHD, donor T cells migrate to intestinal tissues, such as MLN and Peyer's patches utilizing the integrin α4β7–MAdCAM-1 interaction (Dutt et al. 2007). It has been shown that depletion of α4β7 (Petrovic et al. 2004) or β7

(Waldman et al. 2006) on donor T cells and interception of the α4β7–MAdCAM-1 interaction (Ueha et al. 2007) can alleviate aGvHD-induced gut damage in murine allogeneic BM transplantation (BMT) models. Promising data obtained from murine transplant models suggests the usage of integrin α4β7 blocking mAb that prevents α4β7–MAdCAM-1 binding in the treatment of intestinal aGvHD. Integrin α4β7 mAb vedolizumab has now been evaluated to treat steroid-refractory (SR) GI aGvHD in phase I trials and showed a reduced incidence of high-grade aGvHD without generating adverse effects (Chen et al. 2019; Danylesko et al. 2019). However, these results need to be further validated in prospective clinical trials.

8 Conclusion and Future Perspectives

Integrin α4β1 and α4β7 are constitutively expressed on some leukocyte populations. Upon stimulation, α4 integrin expression can be induced on the surface of some leukocytes (Hemler et al. 1987b; Kirveskari et al. 2000). The α4 integrin-mediated aberrant homing of leukocytes to the CNS or gut plays a critical role in MS or IBD, respectively. Blocking antibodies targeting α4 integrins, including natalizumab and vedolizumab, have been used clinically and show efficacy in treating MS and IBD. It is noteworthy that natalizumab treatment may induce PML. Moreover, some UC patients exhibited aggravated colitis after vedolizumab, which the aberrant innate immune response may cause after fully blocking α4β7. Therefore, instead of fully blocking α4 integrins, more precise regulation of α4 integrin function, such as blocking the aberrant integrin activation, could be a better strategy for managing MS and IBD. Screening small molecule compounds or mAbs for blocking the activation of α4 integrins could lead to discovering next-generation MS/IBD drugs with fewer adverse effects (Baiula et al. 2019).

The interaction between integrins and different ligands enables the targeting of lymphocytes to different tissues. Each leukocyte integrin can recognize multiple ligands, which makes the tissue-specific leukocyte homing complicated. Some studies have revealed that different chemokines can induce ligand-specific activation of integrin α4β7, which allows the selective binding of α4β7 to either MAdCAM-1 or VCAM-1 to ensure a tissue-specific homing of lymphocytes (Sun et al. 2014; Wang et al. 2018). Notably, when chemokines promote α4β7 to one ligand, it inhibits α4β7 binding to the other ligand. The chemokine-induced inhibition of α4β7 binding to its ligands may be involved in the selective recruitment of different immune cells into tissues under pathological conditions, which deserves further investigation.

Although integrin α4β7 has been characterized in-depth as a gut-homing molecule expressed on the surface of lymphocytes, it also plays a role in several innate immune cell subtypes, including the BM precursor cells, which differentiate into tolerogenic, RA-secreting DCs, and NK cells and monocytes (Bouchentouf et al. 2010; Picarella et al. 1997; Schleier et al. 2020; Villablanca et al. 2014). The regulatory function of α4β7 appears disparate among different cell types and may

be more complex than originally speculated. More precise studies need to be conducted to investigate the distinct functions in specific immune cell subsets during both homeostatic and pathologic processes.

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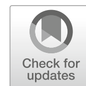
β2-integrins in Immunity: New Roles for Old Players



Liisa M. Uotila, Heidi Harjunpää, and Susanna C. Fagerholm

Abstract β2-integrins are adhesion- and mechanoreceptors expressed exclusively on leukocytes. They mediate diverse functions in immunity and are of fundamental importance in processes such as leukocyte trafficking, phagocytosis, T cell and B cell activation and differentiation and T cell-mediated killing. Furthermore, they can mediate immunosuppressive signaling in cells such as macrophages and dendritic cells. Their crucial importance for immune system function is illustrated by the rare but devastating genetic immunodeficiency disorders Leukocyte adhesion deficiency type I and type III, where β2-integrin expression or function is reduced or lost. Here, we discuss the structure of β2-integrins and their various ligand-binding properties. Furthermore, we describe their regulation, signaling, and mechanotransduction properties in immune cells. In addition, we describe their various functional roles in different immune cells, in host defense, infection, inflammatory diseases, and cancer.

1 Introduction

1.1 β2-integrins

β2-integrins belong to the integrin superfamily of molecules. Integrins are essential adhesion and mechanoreceptors found on the surface of cells, which connect the

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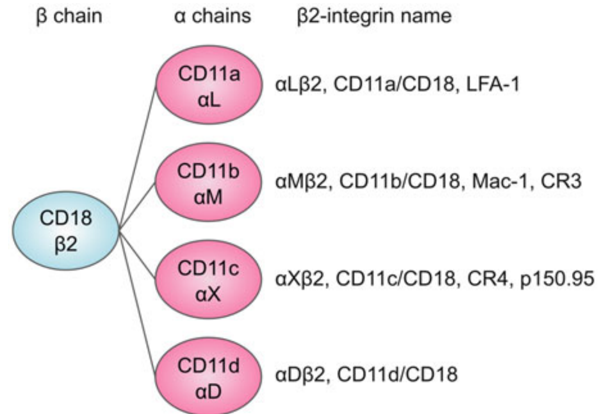

Fig. 1 Four members of the β2 integrin/CD18 family. α/β combinations of β2 family members and their alternative names

outside of the cell to the cytoskeleton inside the cell. Integrins are heterodimeric membrane proteins consisting of non-covalently linked α- and β subunits. To date, 24 different integrin heterodimers have been described, with different cell/tissue distributions, ligands, and biological roles. Integrins have very large extracellular domains, where ligand binding occurs. They span the plasma membrane and have short cytoplasmic domains, devoid of any enzymatic activity, but with extremely important roles in integrin regulation. Integrins do not bind actin directly but connect to the actin cytoskeleton through other proteins, and can form large protein complexes such as focal adhesions and focal complexes.

β2-integrins comprise of a family of four adhesion receptors exclusively expressed in leukocytes. β2-integrins share a common β-chain (β2-integrin or CD18), which can pair with four different α-chains (αL, αM, αX, and αD) to form four different heterodimers with different expression patterns, ligands, and functions in immunity (Fagerholm et al. 2019) (Fig. 1). Integrin names are quite confusing, because there are many alternative names for the same receptors. Thus, αLβ2, is also called CD11a/CD18 or LFA-1, αMβ2 is CD11b/CD18 or Mac-1 or CR3, αXβ2 is equivalent to CD11c/CD18, p150.95 or CR4, and αDβ2 is CD11d/CD18. LFA-1 is expressed in all leukocytes while the other three receptors show higher expression in myeloid cells (neutrophils, monocytes, macrophages, dendritic cells), and are more closely related to each other. This is also reflected in their ligand binding characteristics, described further below.

The first three members of the "LFA-1, Mac-1, p150.95 family" were discovered in the early 1980s. Mac-1 and p150.95 were discovered separately as being part of this family, and as functioning as complement receptors CR3 and CR4 (Davignon et al. 1981; Sanchez-Madrid et al. 1982; Springer et al. 1979; Ross and Lambris 1982; Sanchez-Madrid et al. 1983). After this, their expression patterns, functions and ligands started to be revealed, and the first reported ligands included complement component iC3b and the intercellular adhesion molecule-1 (ICAM-1) (Patarroyo et al. 1985; Rothlein et al. 1986). The most recently discovered member of the β2-integrin family is αDβ2 or CD11d/CD18 (Danilenko et al. 1995; Van der

Vieren et al. 1995). Soon after the discovery of the LFA-1, Mac-1, p150.95 family, mutations in their common β-subunit were found to be the cause of Leukocyte Adhesion Deficiency (LAD), an inherited life-threatening disease causing a variety of defects in immune functions (Kishimoto et al. 1987a).

Sequencing of the β2-integrins has shown that the amino acid sequences of the four α-chains are homologous but that the αL-chain differs from the three others in sequence and in ligand repertoire (30% similarity between αL and other α-chains), whereas αM, αX and αD show approximately 60% sequence homology (Larson et al. 1989; Corbi et al. 1988; Corbi et al. 1987; Wong et al. 1996). The β2-subunit was sequenced simultaneously by several researchers (Kishimoto et al. 1987b; Law et al. 1987). Chromosomal localization of the α-chains reflects the same grouping: all α-chains are localized in chromosome 16, but αM (gene name: *ITGAM*), αX (*ITGAX*), and αD (*ITGAD*) are clustered together, separated from αL (*ITGAL*). *ITGB2* is localized in chromosome 21.

2 β2-integrin Structure

The general structure of β2-integrins is similar to other integrins. They consist of two non-covalently linked transmembrane polypeptide chains with the β2-chain being 95 kDa and α-chains varying in size between 150 and 170 kDa. The schematic structure of β2-integrins is presented in Fig. 2.

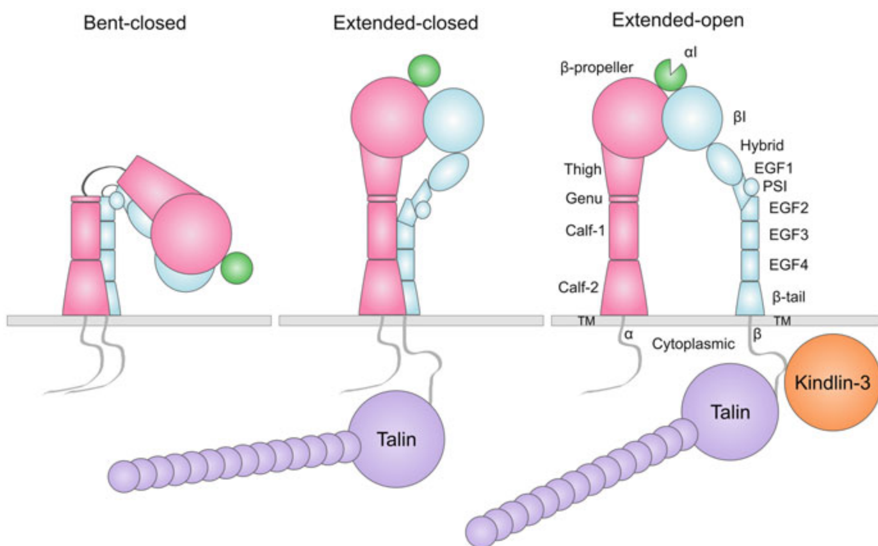

Fig. 2 β2 integrin extracellular part structure and major conformational states. Talin and kindlin binding to the integrin β2-chain intracellular domain (tail) play a major role in integrin conformational changes leading to activation. For details, see text

The extracellular domain (ectodomain) of all four α-chains consists of a seven-blade β-propeller domain, an Ig-domain-like thigh domain, and two calf domains (Kadry and Calderwood 2020). All four α-chains contain an I domain (Inserted domain, also known as A domain as it resembles the A domain of von Willebrand factor) that is approximately 200 amino acids long and serves as the ligand binding site. The I domain is inserted between the second and third blades of the β-propeller.

In the β2-chain, the most membrane-distal part is a PSI (plexin-semaphorin-integrin) domain, which is followed by a hybrid domain. The β2-chain includes an I-like domain, similar to the α-chain I domain, which is inserted in the hybrid domain and is vital for the regulation of α I domain accessibility to ligands. The hybrid domain is followed by four cysteine-rich EGF domains and a membrane-proximal β-tail domain (Kadry and Calderwood 2020).

The helical α and β transmembrane domains span the cell membrane once. They associate non-covalently with each other, and the association/dissociation status dictates the activation of the ectodomain. Dissociation of the helices is the outcome of intracellular signaling, in which the interaction of the integrin cytoplasmic domains with certain cytoplasmic signaling and scaffolding molecules leads to integrin activation and ligand binding, whereas in an inactive integrin, the transmembrane helices stay closely associated. Integrin structure has been recently reviewed in, e.g., Campbell and Humphries (2011) and Kadry and Calderwood (2020).

3 Structural Changes Associated with β2-integrin Activation

The integrins in circulating and tissue-resident hematopoietic cells (platelets, leukocytes, even erythrocytes) should remain in an inactive state when necessary, but they need to be rapidly and accurately activated to bind their ligands and again "turned off" when their activity is no longer needed. There are at least two ways to control the ligand binding capacity of a cell: regulation of integrin affinity and integrin avidity. Affinity means the capability of a single molecule to bind its ligand and is controlled by the conformation of the α/β dimer. Avidity, on the other hand, is the measure of integrins clustering on the cell surface (Dustin et al. 2004).

Crystallization studies and negative-stain electron microscopy images of integrins have revealed dramatic changes in integrin structure upon activation. The ectodomain of β2-integrins can adopt three conformations, where the major conformational changes occur in the so-called headpiece that consists of α chain thigh, β-propeller and I domains together with β chain PSI, hybrid, I-like, and EGF1 domains (see Fig. 2). First a bent conformation with a closed headpiece, second, an extended conformation with a closed headpiece, and third, an extended conformation with an open headpiece. Cytoplasmic signals lead to the separation of the α and β cytoplasmic domains from each other, further transducing the signal to the

extracellular part of the integrin, extending the conformation and offering a site for ligand binding (Nishida et al. 2006; Hogg et al. 2011; Springer and Dustin 2012). In addition to these three well-characterized integrin conformations, an unexpected bent but high-affinity conformation has been detected on human neutrophils. In this conformation, the integrin binds ICAM molecules in cis, thus inhibiting neutrophil adhesion and other functions (Fan et al. 2019).

The opening of the headpiece that consist of the α-chain I domain, the β I-like domain, and their adjacent domains is vital for ligand binding. Opening of the headpiece and initial ligand binding are further strengthened by integrin cytoplasmic interactions with the actin cytoskeleton, and the force transmission between the cytoskeleton and the ligand outside the cell (this is further discussed in the Mechanosignaling section, below).

The I-domain 3D structure of β2-integrins shows an α/β Rossmann-fold-type structure, including seven α-helices and six β-sheets. All I domains contain a coordination site for divalent cations (which is required for ligand binding), called a metal-ion dependent adhesion site (MIDAS). The structures of β2-integrin I domains together with their ligands or peptides derived from them, in the presence of divalent cations (Mg^{2+} or Mn^{2+}), have been published, and the CR4 ectodomain was the first reported extracellular domain structure of an integrin with an α-chain with I domain. (Qu and Leahy 1995; Lee et al. 1995b; Lee et al. 1995a; Vorup-Jensen et al. 2003; Xie et al. 2010).

These and other studies on integrin structure and conformational changes, which have been conducted using X-ray crystallography and negative-stain EM microscopy, have revealed the molecular mechanisms of β2-integrin activation. The α-chain I domain can adopt three different conformations that can flexibly be combined with two conformations of the β2-chain I-like domain. Headpiece opening of integrins requires the interplay between the α-chain I domain and the β-chain I-like domain. A conformational change occurs when the α7 helix of the α I domain is pulled downwards to contact the β I-like domain. An interaction between a glutamate (in the α chain) and the β-chain β propeller/I domain interface exposes the ligand-binding site in the I domain of the α-chain at the same time as the β-chain hybrid domain swings out to create a conformation with open legs (Xie et al. 2010). The mechanisms on integrin activation have been reviewed in excellent reviews on the mechanisms of headpiece opening and integrin action in general (Hynes 2002; Sun et al. 2019; Kechagia et al. 2019).

4 β2-integrin Cytoplasmic Domains and Phosphorylation

Integrin functions are regulated through the interactions of their cytoplasmic tails with other cytoplasmic molecules. The intracellular domains serve as docking sites for various adaptor, signaling and scaffolding proteins and they contain serine and threonine residues that function as sites for phosphorylation. Multiple proteins can bind to these areas at a given time (Calderwood et al. 2003; Takala et al. 2008).

A

1140
CD11a human 1113 KVGFFKRNLKEKMEAGRGVPNGIPAEDSEQLASGQEAGDPGCLKPLHEKDSESGGGKD 1170

CD11a mouse 1109 KVGFFKRNLKEKMEADGGVPNGSPPEDTDPLAVPGEETKDMGCLEPSGRVTRTKA 1163

1142
CD11b human 1129 KLGFFKRQYKDMMSEGGPPGAEPQ 1152

CD11b mouse 1130 KLGFFKRQYKDMMNEAAPQDAPPQ 1153

1158
CD11c human 1129 KVGFFKRQYKEMMEEANGQIAPENGTQTPSPPSEK 1163

CD11c mouse 1138 KAGFFKRQYKEMLEEANGQFVSDGTPTPQVAQ 1169

CD11d human 1124 KLGFFKRHYKEMLEDKPEDTATFGSGDDFSCVAPNVPLS 1161

CD11d mouse 1127 KLGFFKRQYKEMLDLPSADPDPAGQADSNHETPPHLTS 1168

735 745 756 758
CD18 human 724 KALIHLSDLREYRRFEKLKSQWNNDNPLFKSATTTVMNPKFAES 769

CD18 mouse 726 KALTHLTDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES 771

B

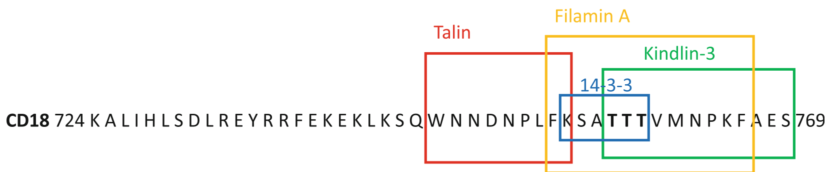

Fig. 3 β2 integrin cytoplasmic parts. (**a**). The cytoplasmic amino acid sequences of β2 integrin α and β chains in human and mouse. Known phosphorylation sites in human α and β chains in **red**. The conserved membrane proximal NPxY/F and membrane distal NxxY/F sequences in β2 chain are in **blue**. (**b**). Interactions of β2 cytoplasmic tail. The amino acid sequence of human β2 tail and the most relevant integrin regulator binding sites are shown. The T^{758}TT motif important for many interactions is in **bold**

Integrin β-chain cytoplasmic domains are structurally conserved between different integrins, and closely related to each other. In contrast, integrin α-chain sequences, including αL, αM, αX, and αD, are for the most part very diverse, except for the membrane-proximal GFFKR-sequence. Indeed, integrin β-chain cytoplasmic domains function as "binding hubs" for various cytoplasmic proteins, while in general, integrin α-chain interactions are poorly known, although there are some exceptions, such as paxillin binding to the α4-integrin cytoplasmic domain.

The β2-chain (like other integrin β-chains) has been shown to interact with numerous intracellular molecules, the most important ones being talin, kindlin-3, filamin A (FlnA) and the phosphopeptide-binding 14–3-3 proteins. In stark contrast, very little is known about the molecular interactions of β2-integrin α-chains. Factors controlling the interactions and thus signal transmission to/from integrins include the phosphorylation of integrin cytoplasmic domains and the overall conformation of the α-β dimer. Changes are transduced through transmembrane helices to the ectodomain.

All integrin intracellular parts have some common traits, such as the α-chain GFFKR sequence in the membrane proximal part, and the NPxY/F membrane proximal and NxxY/F membrane distal sequences in the β-chains, as well as the serine/threonine rich sequence between the two (Ylanne et al. 1995) (Fig. 3a).

Structures of β2-integrin cytoplasmic parts have been studied using the NMR technique (Bhunia et al. 2009; Chua et al. 2011; Chua et al. 2012). According to these studies, the C-termini of the cytoplasmic domains show free-moving and labile structures, whereas the membrane-proximal parts form similar conserved helices in all α-chains. The α and β cytoplasmic domains contact each other through ionic and hydrogen bonding in the membrane-proximal helix area. The αL tail is the longest cytoplasmic tail of the β2-integrins. It makes an exception in the family, as it is packed in three helices held together by salt bridges and/or hydrogen bonds. The helices form a large negatively charged surface that can bind metal ions (Bhunia et al. 2009).

Due to their loose secondary structure, the α-chain cytoplasmic parts offer a lot of variety to the integrin regulation mechanisms and may determine specificity of signaling for the different integrins. All the phosphorylation sites in αL, αM, and αX-chains as well as in β2-chain are outside the membrane-proximal helices and thus available for phosphorylation and de-phosphorylation, and for molecular interactions with signaling and other cytoplasmic proteins (Fig. 3).

Phosphorylation of the β2-integrin cytoplasmic tails has been reported already over 30 years ago, and more specific studies have shown that the β2-chain is phosphorylated upon cell activation, whereas the α chains (αL, αM, αX) are constitutively phosphorylated (Chatila and Geha 1988; Chatila et al. 1989; Buyon et al. 1990). In the initial studies, serine was found to be the predominant phosphorylation target in all polypeptide chains studied, in addition to weak threonine phosphorylation in αX and β2-chains, and tyrosine phosphorylation in the β2-integrin-chain. Later, strong threonine phosphorylation has been detected using phosphatase inhibitors (Valmu and Gahmberg 1995).

In the β2-chain tyrosine 735 and serines in positions 745 and 756 have been shown to be phosphorylated upon cell activation and these phosphorylations regulate e.g. Rap1 interactions (Hibbs et al. 1991; Tohyama et al. 2003; Lim et al. 2011). In addition, the Thr758-Thr-Thr760 sequence was early shown to be important for LFA-1-mediated adhesion (Hibbs et al. 1991; Peter and O'Toole 1995; Valmu and Gahmberg 1995; Valmu et al. 1999; Hilden et al. 2003). The main responsible kinases for Ser745 and Thr758 phosphorylation are protein kinase C delta (PKCδ) and PKCβI/II, whereas Ser756 is not directly phosphorylated by any PKC isoform (Fagerholm et al. 2002).

Binding of cytoplasmic proteins to β2-integrin tail (Fig. 3b) is largely regulated by phosphorylation: 14–3-3 proteins can only interact with β2-tail when the Thr758 is phosphorylated, but the talin head domain is able to activate LFA-1 even when T758 is mutated to non-phosphorylatable Ala (Fagerholm et al. 2005). However, Filamin A cannot bind to the Thr758-phosphorylated β2-integrin (Takala et al. 2008). The functions of β2-integrin cytoplasmic interactions are discussed further in the following sections.

The α-chain phosphorylation sites in three out of four β2-integrin family members have been determined (Fig. 3a). α-chain phosphorylation regulates important integrin functions such as adhesion, cell trafficking, and phagocytosis. αL is phosphorylated on Ser1140, and this phosphorylation regulates ligand binding and

adoption of the active conformation of the integrin (Fagerholm et al. 2005). αM is phosphorylated on Ser1142, which controls binding of certain ligands, expression of activation epitopes and, importantly, leukocyte homing in vivo (Fagerholm et al. 2006). The main phosphorylation site in αX is Ser1158, and the mutation of this phosphorylation site abolishes integrin activation by Rap1 (inside-out activation of the integrin) and reduces adhesion to iC3b and phagocytosis, while outside-in signaling through the integrin is not affected (Uotila et al. 2013).

Recent studies on integrin β1 cytoplasmic part have shown the vital role of the PPM1F phosphatase (Protein Phosphatase, Mg2+/Mn2+ Dependent 1F) in integrin activation. Threonine dephosphorylation by PPM1F releases Filamin A, a negative regulator, from β1, leaving space for talin to bind and to activate the integrin. Whether dephosphorylation of β2-integrin by PPM1F regulates also this integrin, remains to be discovered (Grimm et al. 2020).

5 β2-integrin Ligands

All β2-integrins bind to one or more members of the ICAM (intercellular adhesion molecule) family. Interaction with cellular ligands is extremely important for β2-integrin functions as discussed below (see section β2-integrin roles in immune cells). Mac-1, CR4, and CD11d/CD18 are homologous in sequence and share many ligands, including a panel of extracellular matrix (ECM) proteins and their modifications. In contrast, LFA-1 is more distantly related to the other β2-integrins and therefore has a distinct panel of ligands.

The very first ligand of LFA-1 that was identified was ICAM-1 (Rothlein et al. 1986; Patarroyo et al. 1987; Marlin and Springer 1987), followed by the other members of the ICAM-family: ICAM-2 (Staunton et al. 1989), ICAM-3 (de Fougerolles and Springer 1992), ICAM-4 (Bailly et al. 1995) and ICAM-5 (Tian et al. 1997). Other reported LFA-1 ligands are, e.g., junctional adhesion molecule-1 (JAM-1) (Ostermann et al. 2002), E-selectin (Kotovuori et al. 1993), and collagen (Lahti et al. 2013).

Mac-1 is the most promiscuous member of the β2-integrin family, as its ligand repertoire includes over 40 proteins, varying from cell adhesion molecules to ECM molecules. In addition to complement receptor functions and binding to iC3b (Beller et al. 1982), Mac-1 was reported soon after its discovery to mediate granulocyte cell-cell and cell-substrate adhesion, unrelated to iC3b binding function, which led to the discovery of various other ligands (Patarroyo et al. 1985). Mac-1 binds to a variety of ECM proteins, such as fibronectin and collagens (Davis 1992; Thompson and Matsushima 1992; Monboisse et al. 1991; Walzog et al. 1995)(Lammermann et al. 2008). Other Mac-1 ligands include a variety of proteins and molecules, e.g. fibrinogen (Wright et al. 1988), low-density lipoprotein (LDL) receptor (Spijkers et al. 2005), MMP9 (Stefanidakis et al. 2003) and JAM-3 (Santoso et al. 2002), among others. Mac-1 may also serve as a binding site for some pathogens, like *Candida albicans* (Forsyth and Mathews 1996). The extensive variety of Mac-1

ligands in the extracellular matrix and other ligands has raised questions on their physiological relevance, especially as integrins are not directly required for leukocyte migration in 3D environment (Lammermann et al. 2008). Podolnikova et al. have suggested that the broad list of αMβ2 ligands might simply reflect the fact that Mac-1 favors sequences exposed by protein denaturation. This further suggests that these sequences, released from damaged or dead cells, may function as danger signals to alarm the immune system (Podolnikova et al. 2015).

The panel of CR4 ligands is very similar to that of Mac-1, including cellular, soluble, and ECM ligands, including ICAM-1 (Diamond et al. 1993; Blackford et al. 1996; Frick et al. 2005). CR4 also binds to ICAM-2 and VCAM-1 (Sadhu et al. 2007) and ICAM-4 (Ihanus et al. 2007), but not to ICAM-3 (de Fougerolles et al. 1995). Recognition of iC3b by CR4, and this interaction mediating the phagocytosis of complement-opsonized particles, was initially reported already in the mid-1980s. This interaction, its regulation and the mechanisms of interaction have since been studied extensively (Micklem and Sim 1985; Chen et al. 2012b; Jensen et al. 2021). Both CR4 and Mac-1, but not LFA-1, on monocytes, bind to denatured proteins (Davis 1992), and negatively charged residues in disintegrated proteins probably serve as a pattern recognition motifs for these molecules (Vorup-Jensen et al. 2005). CR4 is also a receptor for some pathogens such as Herpes simplex virus 1 (HSV-1) (Allen et al. 2011), *Candida albicans* filamentous hyphae (Jawhara et al. 2012) and rotaviruses (Graham et al. 2003).

CD11d/CD18 binds ICAM-3 and VCAM-1 through the αD I domain (Grayson et al. 1998; Van der Vieren et al. 1999; Van der Vieren et al. 1995). As the high homology with Mac-1 suggests, CD11d/CD18 has been reported to bind to many of Mac-1 ligands, such as vitronectin, fibronectin, and plasminogen (Yakubenko et al. 2006). It also interacts with protein modifications on ECM proteins, such as 2-(-ω-carboxyethyl)-pyrrole (CEP), that is generated during inflammation-mediated lipid peroxidation (Yakubenko et al. 2018; Cui et al. 2019). Interestingly, despite the high homology with two other β2-integrin complement receptors, CD11d/CD18 has not been shown to function as a complement receptor nor to take part in phagocytosis (Blythe et al. 2021).

6 β2-integrin Regulation by Cytoplasmic Proteins

As already mentioned, β2-integrin function is regulated by cytoplasmic molecules that bind to integrin cytoplasmic domains. These interactions can regulate integrins in various ways, including their conformational changes (activity), clustering, signaling, and mechanotransduction. Below, we describe some of the most important β2-integrin cytoplasmic binding partners and their roles in β2-integrin regulation.

6.1 Talin

Talin is a pivotal protein in integrin regulation in general. Talin is required for β2-integrin-mediated lymphocyte adhesion and trafficking (Lefort et al. 2012; Manevich-Mendelson et al. 2010), and for T cell activation (Wernimont et al. 2011). Talin is a large cytoplasmic protein, consisting of an N-terminal "head" domain and a C-terminal rod-domain. It regulates integrin activation by binding to the membrane-proximal NPXY/F-motif in the β-chain tail (Fig. 3b). Binding of the talin head domain to integrins causes the integrin tails to separate, and allows for large conformational changes in the integrin extracellular domain, and thereby causes integrin activation (Kim et al. 2003). Talin recruitment to integrins is regulated by the small GTPase Rap1 and by membrane lipids (Bromberger et al. 2019). Also, other proteins like RIAM (Rap1-GTP-interacting adapter molecule), vinculin, and KANK (Kidney Ankyrin Repeat Domain Protein) may be involved in the process (Bouti et al. 2020).

6.2 Kindlin-3

The crucial role of kindlins in regulating integrins and cell adhesion was discovered much later than that of talin (Moser et al. 2009; Moser et al. 2008; Malinin et al. 2009). Kindlin-3 is expressed in leukocytes and platelets, while other cell types express Kindlin-1 or Kindlin-2. Kindlins, as talin, also binds integrin β-tails, but at the membrane distal NPXY/F site, and also to an intervening serine/threonine-rich site (Morrison et al. 2013) (Fig. 3b). The integrin-kindlin-3 interaction is required for firm adhesion of T cells both under shear flow and shear free conditions (Morrison et al. 2013), and also for T cell activation and B cell antibody production (Morrison et al. 2015). Kindlins has been reported to interact with other proteins such as actin, ILK, and paxillin to stabilize integrin-mediated adhesion (Bledzka et al. 2016; Huet-Calderwood et al. 2014; Gao et al. 2017; Margraf et al. 2020). Interestingly, it has been shown that kindlin-3 recruitment to the plasma membrane, through its PH-domain, precedes the integrin conformational change to the active, high-affinity conformation that is required for the arrest of leukocytes on the endothelial cells, and that full-length kindlin-3 is completely essential for leukocyte adhesion (Wen et al. 2021). These results show not only the requirement of kindlin-3 in leukocyte adhesion but demonstrate the spatiotemporal sequence of the events. Kindlin also seems to promote talin-mediated integrin activation (Kondo et al. 2021; Haydari et al. 2020). Kindlin and talin can assemble together at (active) integrin adhesion sites in a ternary complex (Fischer et al. 2021). However, the signaling pathways and mechanisms that regulate kindlin-mediated integrin activation remain to be elucidated.

6.3 Filamin A

The role of filamin in integrin regulation is still controversial, with contradictory findings from both in vitro and in vivo studies. Filamin is a very large (280 kDa) actin-binding protein, which crosslinks actin filaments. It also binds integrin β-tails, at a membrane distal site at least partially overlapping with the binding sites of both talin and kindlin (Fig. 3b), but in addition to integrins has a wide variety of other reported binding partners in cells. In vitro, it has been reported that Filamin A is a negative regulator of integrins. It appears to compete for the β-tail with talin (Kiema et al. 2006) and 14–3-3 proteins (Takala et al. 2008) and presumably also with kindlin-3 due to overlapping binding sites, although this has not been officially tested. Filamin binding to the β2-integrin tail is negatively regulated by phosphorylation of the integrin cytoplasmic domain at Thr758 (Takala et al. 2008). Structural studies have also reported that filamin can clasp the integrin α- and β-tails together, thereby inhibiting integrins.

However, when it comes to in vivo studies, these conclusions of an inhibitory role for filamin are not fully supported by the published data. In T cells, deletion of Filamin A does not lead to increased T cell adhesion under static or shear flow conditions. Rather, under shear flow conditions, Filamin A deletion leads to weakened adhesion, and according to homing studies, reduced T cell trafficking into lymph nodes and even into sites of inflammation (Savinko et al. 2018). T cell activation, in contrast, is not affected. These results implicate a role for the integrin-filamin-actin linkage (or other function of filamin-actin crosslinking) in stabilizing adhesion under shear flow conditions, and also in generating traction forces by integrins, e.g., mechanotransduction through the integrin (see section below).

In contrast to the situation in T cells, Filamin A deletion in neutrophils does lead to a slight increase in integrin-mediated adhesion under static conditions, indicating that the filamin indeed would play a negative regulatory role of integrins in these cells (Uotila et al. 2017). Other β2-integrin-dependent functions in neutrophils are differently affected. Whether these differences between T cells and neutrophils are due to differences in β2-integrin expression between these cells (T cells express mainly LFA-1) or due to differences in Filamin expression (A/B/C), is currently not known. However, it seems that in T cells, Filamin A plays a role in mechanotransduction through LFA-1, rather than acts as an integrin inhibitor.

6.4 Others

14–3-3 proteins bind to β-integrin tails in a phosphorylation-dependent manner (Thr758) at a site overlapping with kindlin and filamin (Fagerholm et al. 2002; Fagerholm et al. 2005; Takala et al. 2008). 14–3-3 binding to the threonine-phosphorylated β2-integrin regulates integrin outside-in signaling as it is upstream of Rac-1 activation (Gronholm et al. 2011; Nurmi et al. 2007). Also, many other

proteins have been reported to bind directly to integrin β-tails (Morse et al. 2014), while α-tail interacting proteins are still poorly defined.

7 Inside-out Signaling and Integrin Activation

The term "inside-out" signaling for integrins implies that signaling cascades initiated by other surface receptors ultimately lead to changes in the recruitment of intracellular factors that bind to integrin cytoplasmic domains, and (probably together with mechanical forces, depicted in the chapter below) lead to integrin activation, ligand binding, and cell adhesion. Inside-out signaling can be initiated for example by the engagement of the T cell receptor in T cells, or by chemokine receptors in many types of immune cells that traffic from the bloodstream into lymph nodes or tissues, or by Toll-like receptors in myeloid cells (Bouti et al. 2020). A classic example of inside-out signaling of integrins is the activation of integrins by chemokine signaling in neutrophils, which precedes neutrophil firm adhesion and trafficking into sites of inflammation (Fagerholm et al. 2019; Bouti et al. 2020). During this process, the neutrophil, initially engaging with the endothelium through selectins, which mediate rolling, comes into contact with chemokines (CXCL1, CXCL8) presented on the endothelium. Binding of the chemokine to its 7-TM G-protein coupled receptor results in an intracellular signaling cascade, which ultimately leads to recruitment of talin and kindlin to the intracellular tail of the β2-integrin, and, together with mechanical forces (described below) allows for integrin activation. The G-protein coupled receptor activates phospholipase C (PLC), which in turn activates downstream signaling, leading to the activation of Rap1. Rap1 in turn interacts with RIAM, which allows for talin recruitment to the plasma membrane and binding to the β2-cytoplasmic tail. PLC activation also leads to activation of PKC, which in turn causes the activation of Phospholipase D and Arf6, and subsequent activation of phosphatidylinositol phosphate kinase type Iγ (PIPKIγ), leading to increased Phosphatidylinositol 4,5-bisphosphate (PIP2) levels at the plasma membrane. This recruits talin via is PIP2 binding F2 domain, allowing for β2-integrin binding. In contrast to talin recruitment, the inside-out signaling cascade that recruits kindlin-3 to the β2-integrin is less clear, although ILK may be involved in this process (Margraf et al. 2020).

8 Mechanosignaling by β2-integrins

Integrins are important mechanoreceptors in cells. Mechanical cues that cells can sense consist of forces, stress, and strain, but also substrate rigidity, adhesiveness, and topology (Chen et al. 2017b). In immunity, mechanical forces are extremely important in many processes. Mechanical cues impact immune cells in multiple settings, for example during migration in complex mechanical environments in

tissues, during T cell activation, during phagocytosis of coated particles, and during leukocyte adhesion to endothelial cells (Rosetti et al. 2015). In mechanosignaling, mechanical signals are transmitted into cells, where they are converted to biochemical signals, which in turn change cell behavior. Integrins, including β2-integrins, form so-called "catch bonds" with their ligands, e.g., bonds that strengthen under increasing force, until a limit is reached, breaking the bonds (Rosetti et al. 2015; Chen et al. 2017b). A mechanical force transmitted through ligand binding to the integrin can induce changes in the hinge region of the integrin, allowing the conformational change from the bent to the open conformation and therefore integrin activation (by "pulling" the integrin open) (Nordenfelt et al. 2016; Chen et al. 2012a; Zhu et al. 2008). In addition to sensing extracellular forces, cells can also generate forces from within, for example through acto-myosin contraction and actin polymerization; these forces are further transmitted to integrins, which can therefore sense forces bi-directionally across the plasma membrane. Indeed, the mechanical forces from within the cell (cytoskeletal forces) impact on integrin activity. Actin forces are transmitted to integrins to achieve large conformational changes and integrin activation (Comrie et al. 2015).

Integrins transmit forces from the outside of the cell to the cytoskeleton inside the cell, to which they are connected to, and the "catch bond" property of integrin-ligand bonds is important for mechanotransduction (Chen et al. 2017b). There are different models of how this mechanical transduction takes place. Force that stabilizes the integrin-ligand bond from the outside, by allowing the bent-to-extended conformational change, may lead to separation of the α- and β- cytoplasmic tail, and enable talin binding to the integrin β-tail followed by downstream signaling. There is, however, also evidence that integrin activation may occur without integrin extension (Fan et al. 2016). Alternatively, or in addition, talin head-and-rod domain and/or vinculin unfolding (or other mechanosensitive proteins) may be involved in the propagation of the signal into the cell.

Mechanotransduction through integrins occurs, for example, in the large integrin and actin-containing multiprotein assemblies called focal adhesions that exist in fibroblasts; here, initial integrin ligand binding leads to the assembly of the focal adhesion, which in turn strengthens the adhesion of the cell. Integrins function as mechanoreceptors also during cell spreading, migration, and contraction, processes that are all influenced by the stiffness of the environment.

Although focal adhesions are not present in leukocytes, T cells, macrophages, and dendritic cells have all been reported to respond to mechanical signals such as the stiffness of the environment, which modulates immune cell function (Saitakis et al. 2017; McWhorter et al. 2015; Blumenthal et al. 2020; Chakraborty et al. 2021; Mennens et al. 2017). It is likely that β2-integrins participate in (at least some of) these events, by mediating adhesion to ligand-coated surfaces, thus sensing the stiffness of the environment and transmitting these signals into cells. β2-integrin-mediated phagocytosis of coated particles also involves mechanotransduction (Jaumouille et al. 2019; Jaumouille and Waterman 2020). Interestingly, stiffer targets are more easily phagocytosed than softer targets (Jaumouille et al. 2019). β2-integrin-mediated adhesion and sensing of extracellular stiffness can further

regulate epigenetic programming and gene transcription in immune cells, by providing actin links to and changes in the cell nucleus and chromatin accessibility (Guenther et al. 2021).

Cells can also generate forces on their environment that can be measured e.g. through traction force microscopy, where traction forces generated by adherent cells on ligand-coated hydrogels can be assessed, by measuring the deformation of the gels. β2-integrin-mediated traction forces in immune cells require talin- and kindlin-mediated linkage to the actin cytoskeleton (Jaumouille et al. 2019; Guenther et al. 2019). As mentioned above, also filamin A has been shown to be important for β2-integrin generated traction forces in T cells (Savinko et al. 2018). Interestingly, β2-integrins also transmit forces from the cytoskeleton outwards, to contribute to the folding and damaging of fungal hyphae (Bain et al. 2021). Mechanical forces, generated by actin polymerization and myosin contractility, regulate T cell signaling, and LFA-1 participate in these events, by enhancing actomyosin contractility downstream of the T cell receptor (Tabdanov et al. 2015).

There is therefore extensive crosstalk between the extracellular environment of the cell and its interior, with each responding to mechanical signals, and β2-integrins in immune cells playing essential roles in these mechanical responses. In addition, mechanotransduction e.g. mechanical forces acting on the integrin both from the inside and the outside of the cell probably plays a very large role in integrin activation, that has been a previously unrecognized factor when examining these events. The concept of "integrin activation" therefore may have to be revisited to involve these mechanical factors, rather than by just discussing factors inside cells such as talin and kindlin inducing integrin activation. The binding of the intracellular factors without exerting forces from the inside and/or the outside is probably not enough to induce cell adhesion.

9 β2-integrin Roles in Immune Cells

β2-integrins are expressed in leukocytes and have numerous roles in immunity. Here, we briefly describe the role of β2-integrins in some of the most important immune cell types (neutrophils, macrophages, dendritic cells, T cells, and B cells) (Fig. 4).

9.1 Neutrophils; Trafficking and Effector Functions

The best-known and most extensively studied role of β2-integrins is in leukocyte trafficking. β2-integrins are essential for firm adhesion between the leukocyte and the endothelial cell that precedes extravasation and entry into tissues. This is especially important for neutrophils, which do not express significant amounts of other integrins that could compensate for the lack of β2-integrins (Mizgerd et al.

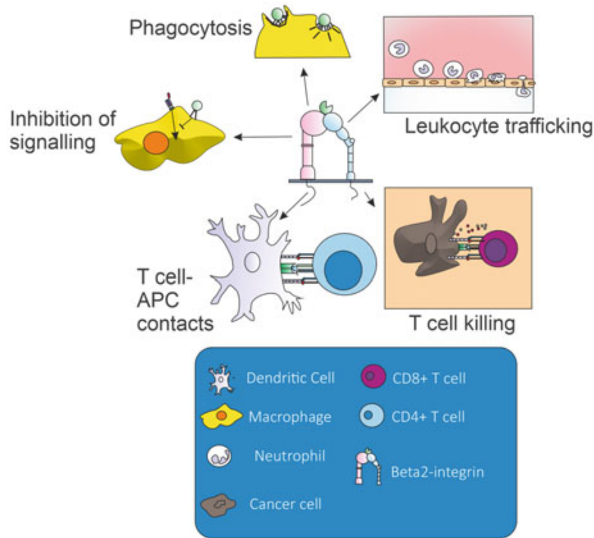

Fig. 4 Functions of β2 integrins in immunity. β2-integrins play a wealth of different roles in immunity. For example, they mediate trafficking of leukocytes into lymph nodes and tissues by mediating the firm adhesion between the leukocyte and endothelial cells, T cell-APC contacts and formation of the immunological synapse, cytotoxic T cell killing of target cells (virus-infected cells, tumor cells), phagocytosis of complement-coated particles, but can also suppress myeloid immune cell and B cell signaling and therefore can also play anti-inflammatory roles in immunity. For details, see text

1997; Scharffetter-Kochanek et al. 1998). This is why a very prominent phenotype of LAD-I and LAD-III diseases is recurrent bacterial and fungal infections, because neutrophils, which are the most important early defense system to these infections, fail to traffic into the infected sites. However, not all sites of infections are affected; lung trafficking can occur without β2-integrins, while trafficking of neutrophils into the inflamed skin is again dependent on β2-integrins (Mizgerd et al. 1997).

Trafficking of neutrophils (and other circulating leukocytes such as T cells) into tissues is a multistep process consisting of rolling, slow rolling, firm adhesion, crawling, and extravasation steps (Fagerholm et al. 2019; Bouti et al. 2020). Selectins mediate rolling adhesion, while both selectins and chemokines presented on the endothelium trigger inside-out signaling cascades that mediate integrin activation. Integrins then mediate slow rolling, firm adhesion and crawling of leukocytes on the endothelium, followed by extravasation either paracellularly or transcellularly. Especially LFA-1 and Mac-1 are important for neutrophil trafficking, although they appear to play different roles in the recruitment cascade. LFA-1 is most important for firm adhesion (Ding et al. 1999), while Mac-1 is used in neutrophils mainly for crawling on the endothelial layer before extravasation (Phillipson et al. 2006). β2-integrins are also important for homeostatic trafficking of monocytes (Schittenhelm et al. 2017), although not under inflammatory conditions (Schittenhelm et al. 2017).

In neutrophils, β2-integrins are also important for killing mechanisms such as for the production of reactive oxygen species (ROS) (Anderson et al. 2008; Zhou and Brown 1994) and for phagocytosis (Bouti et al. 2020).

9.2 Macrophages; Phagocytosis and Signaling

β2-integrins are also essential for the function of other types of myeloid cells. Macrophages are tissue-resident cells, and thus, β2-integrins do not play a major role in their trafficking (Lammermann et al. 2008). However, β2-integrins are important for the process of phagocytosis (Patel and Harrison 2008; Le Cabec et al. 2002; Dupuy and Caron 2008). Mac-1-mediated phagocytosis in macrophages resembles the formation of focal adhesions in other types of cells. A phagocytic cup is formed containing Mac-1, talin, vinculin, α-actinin, and phosphorylated paxillin, FAK and Syk (resembling the composition of focal adhesions). Phagocytosis involves the coupling of this complex to actin and actin polymerization/re-organization driven by Arp2/3 and diaphanous-related formin-1 (mDia) (Jaumouille et al. 2019).

Interestingly, β2-integrins have been reported to play both positive and negative roles in signaling and cytokine responses in macrophages (Rezzonico et al. 2001; Ling et al. 2014; Han et al. 2010; Wang et al. 2010; Schittenhelm et al. 2017). For instance, direct engagement of αM results in expression of pro-inflammatory chemokines MIP-1α (CCL3) and MIP-1β (CCL4). However, αM has also been reported to restrict LPS signaling in macrophages, by both indirect effects, through IL-10 expression, but also by direct effects of integrin-regulated Syk signaling impacting negatively on TLR-signaling and macrophage cytokine responses.

9.3 Dendritic Cells; Restriction of Maturation, Migration, and APC Function

β2-integrins are highly expressed also in dendritic cells, and αX (CD11c) is one of the best-known dendritic cell markers. However, in dendritic cells, β2-integrins are surprisingly not involved in adhesion to the T cell and in the formation of the immunological synapse (while they are crucial for this process on the T cell side, as described below). In addition, β2-integrins are not critically important for trafficking of these cells from tissues to lymph nodes, although they do play a role in the process (Lammermann et al. 2008). Instead, β2-integrins regulate signaling in dendritic cells in various ways. Interestingly, β2-integrins appear to play mostly negative regulatory roles in dendritic cell function (Morrison et al. 2014; Varga et al. 2007; Balkow et al. 2010), although also positive roles in dendritic cell signaling have been reported (Ling et al. 2014). Inhibiting β2-integrins on dendritic cells

increases dendritic cell-mediated T cell activation (Varga et al. 2007; Balkow et al. 2010). Dendritic cells with dysfunctional β2-integrins display a changed gene expression program, and increased maturation with increased costimulatory marker expression, cytokine production (IL-12, IL-10) and chemokine receptor expression (CCR7) and display increased migration to lymph nodes in vivo and increased T cell activation (Morrison et al. 2014; Guenther et al. 2021). Enhancement of migration is thus not a direct consequence of changes in integrin-dependent cell adhesion, but due to re-programming of these cells to a more mature and mobile phenotype.

9.4 T cells; Trafficking, Activation and Differentiation

In lymphocytes, including T cells, β2-integrins are very important (Walling and Kim 2018). They play a fundamental role in lymphocyte adhesion and homing to lymph nodes, as well as in trafficking into sites of inflammation (but not to all organs) (Gerard et al. 2021). Especially LFA-1 is important here (LFA-1 is the major β2-integrin expressed in lymphocytes). LFA-1 on T cells can be activated very quickly by chemokines presented on the endothelium (Shamri et al. 2005). In effector T cells, LFA-1 is highly expressed and appears to be constitutively active, which contributes to effector T cell recruitment into tissues (Shulman et al. 2011; Lek et al. 2013).

Inside lymph nodes, T cells contact dendritic cells presenting cognate antigen. Initially, the interactions are short-lived, but then these short-lived interactions mature to more stable immunological synapses between T cell and antigen-presenting cell (APC) (dendritic cell), allowing for T cell activation (Gerard et al. 2021). LFA-1 is important for the stabilization of the immunological synapse. Also, more short-lived contacts between APC and T cells (so-called "kinapses") have been described, and these can also lead to efficient T cell activation (Dustin 2007).

In addition, there are also other roles described for LFA-1 in T cells, such as in homotypic T-T cell adhesion (a process that has actually been described already a long time ago and has been used as a measurement of cell adhesion in early studies). These interactions allow for efficient sharing of IL-2, a cytokine necessary for T cell activation (Sabatos et al. 2008). LFA-1-ICAM-1 interactions are important for the generation of T cell memory (Parameswaran et al. 2005).

LFA-1 also plays a major role in the interaction between a $CD8^+$ T cell or cytotoxic T cell and a target cell (Anikeeva et al. 2005). It is important for the formation of the immunological synapse between the $CD8^+$ T cell and the target cell and for efficient killing. Interestingly, a role for a significant intracellular pool of LFA-1 in $CD8^+$ T cells has been described, which is necessary for asymmetric T cell activation and differentiation (Capece et al. 2017).

LFA-1 has also been reported to be important for regulatory T cell (Treg) development and function (Marski et al. 2005). More specifically, β2-integrins have been demonstrated to promote the thymic and peripheral Treg development and mice deficient for CD18 or LFA-1, or with decreased expression of CD18, show

reduced Treg numbers in peripheral lymphoid organs (Marski et al. 2005; Wohler et al. 2009; Singh et al. 2013). In addition, Tregs deficient for or with diminished β2-integrin expression have less interaction with dendritic cells and show decreased suppressive function (Marski et al. 2005; Wang et al. 2008; Wohler et al. 2009; Singh et al. 2013). LFA-1 in Tregs is in a highly active state, and engages with its ligands on dendritic cells, causing strong fascin polarization toward the Treg-dendritic cell contact area. This causes a temporary decrease in dendritic cell-mediated T cell priming (Chen et al. 2017a). Gammadelta T cells reside at mucosal sites and are important cells for protection against infections and cancer. Interestingly, β2-integrins have recently been described to be important in the regulation of the development and maintenance of different subtypes of these cells in vivo (McIntyre et al. 2020).

9.5 B cells: Homing, Immunological Synapse Formation, and Tolerance

LFA-1 in B cells is equally important as in T cells. LFA-1 is essential for B cell homing into lymphoid organs, and for the formation of the immunological synapse (Carrasco et al. 2004). Mac-1 has been reported to regulate antibody class switching (Park et al. 2017) and CR4 may induce memory B cell proliferation and migration (Nagy-Balo et al. 2020). In addition to their adhesion-inducing roles, β2-integrins may negatively regulate B cell receptor signaling in order to maintain tolerance toward self (Ding et al. 2013). β2-integrins have also been considered as markers for certain B cell populations, such as the $CD11b^+CD11c^+$ ($\alpha M^+\alpha X^+$) Age-associated B Cells (ABCs) that enhance auto-antibody production (Rubtsov et al. 2011). A very small subgroup of B1 cells, the $CD11b^+$ (αM^+) B1 cells, have been proposed to function as “orchestrator cells,” due to their ability to control T cells using both CD86 expression and IL-10 secretion (Griffin and Rothstein 2012). However, the direct role of β2-integrins in these cells remains unclear.

10 Leukocyte Adhesion Deficiency: When β2-integrins Go Wrong

Because of their many and varied roles in immunity, it is clear that when β2-integrin expression or function is reduced or lost, this causes major effects on the immune system and thus severe immunodeficiency. This is indeed the case, as shown both in murine models and in human patients. Leukocyte adhesion deficiency type I (LAD-I) is caused by reduced or lost expression of the β2-chain of the β2-integrins, while LAD-III is caused by kindlin-3 mutations.

10.1 LAD-I

When β2-integrin expression is reduced or lost, the result is LAD-I or Leukocyte Adhesion Deficiency type I (Etzioni 2009; Kishimoto et al. 1987a), a rare but devastating immunodeficiency disease. The patients suffer from recurrent bacterial and fungal infections of their skin, mucosal sites, delayed umbilical cord separation, and defective wound healing. The severity of the disease correlates with integrin expression levels, and the disorder is usually lethal unless a hematopoietic stem cell transplantation (HSCT) is performed. In a recent multicenter study, it was reported that the overall three-year survival rate for patients following an HSCT is 84% for LAD-I and 75% for LAD-III, with event-free survival even lower, showing that novel treatment options are urgently needed (Bakhtiar et al. 2021).

The increased infections in LAD-I patients are mainly due to a deficiency of neutrophils to traffic into sites of infections and thus reduced infection clearance. In addition, LAD-I is associated with aberrant T cell function. However, viral infections are not abundant in these patients. The murine model of LAD-I with β2-integrin deficiency has a similar phenotype (Scharffetter-Kochanek et al. 1998), while deletion of a single α-chain does not cause such severe phenotype. Paradoxically, in addition to the severe immunodeficiency phenotype of LAD-I patients, they also suffer from increased tissue inflammation, e.g., severe periodontitis with associated bone loss, as well as rare cases of colitis. This may be, at least in part, due to the defective neutrophil trafficking in these patients, which indirectly induces T cells and innate lymphoid cells to produce excessive amounts of IL-17, and drives tissue inflammation in these patients. In physiological conditions, the local IL-17 expression in tissues is controlled by neutrophil rheostat model, where apoptotic neutrophils are phagocytosed, causing downregulation of IL-23 production in tissue phagocytes, which in turn leads to suppression of local IL-17 production by T cells (Moutsopoulos et al. 2014).

10.2 LAD-III

LAD-III was long an enigma but eventually led to the finding of an important new integrin regulator (Etzioni 2009). In LAD-III, which is less common than LAD-I, β2-integrins are normally expressed, but dysfunctional, due to a mutation in kindlin-3 (Malinin et al. 2009; Kuijpers et al. 2009). Kindlin-3 deficient animals show the same phenotype as LAD-III patients (Moser et al. 2009; Svensson et al. 2009). LAD-III patients not only suffer from immunodeficiency, but also from a bleeding disorder, because kindlin-3 is also required for β3-integrin function in platelets (Moser et al. 2008). In addition, the patients may suffer from osteopetrosis. Utilizing kindlin-3 deficient mice, it was found that kindlin-3 also regulates osteoclast function (Schmidt et al. 2011). Indeed, kindlin-3 is required for activation of β1-, β2- and

β3-integrins in osteoclasts, without which osteoclasts fail to form podosomes and sealing zones required for bone resorption (Schmidt et al. 2011).

11 β2-integrins and Infections: Evidence from Mice and Men

β2-integrins (LFA-1, Mac-1) contribute to neutrophil recruitment into tissue, but also to phagocytosis and oxidative burst. Consequently, as mentioned above, LAD-I and LAD-III patients suffer from recurrent bacterial and fungal infections of skin and mucous membranes. Thus infections such as omphalitis, pneumonia, gingivitis are common. Pathogens causing recurrent infections are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. As examples of bacterial infections, intra-tracheal administration of *Escherichia coli* or *Pseudomonas* in CD18 deficient mice led to reduced polymorphonuclear (PMN) cell infiltration into tissues (Rijneveld et al. 2005). In contrast, *Staphylococcus pneumoniae* infection led to increased PMN infiltration but yet increased bacterial load and increased inflammation, showing that the infiltrating β2-integrin-deficient PMN are not necessarily able to fight infection even if they can reach the infected sites (Mizgerd et al. 1999). However, this does not hold true in all infection models such as in *Mycobacterium tuberculosis*-infected αM deficient mice (Hu et al. 2000). Some pathogens appear to even use the immune suppressive roles of αM to evade immune attack (*Porphyromonas gingivalis*) (Hajishengallis et al. 2007).

LAD-I patients often also suffer from Aspergillus infections of the lung (Gazendam et al. 2016). In addition, αM deficient mice have been reported to have increased fungal burden of the lungs and lower phagocytosis of fungi, although neutrophil recruitment was not affected (Teschner et al. 2019). Also, Candida infections are more severe in αM deficient mice (Soloviev et al. 2011), showing that Mac-1 plays an essential role in protection against fungal infections. In contrast to bacterial and fungal infections, LAD-I patients have not been reported to suffer exceedingly from viral infections.

In conclusion, different β2-integrins can compensate for each other to some degree, and other adhesion receptors can compensate for the lack of β2-integrins for example during immune response to viral infections. In addition, the immunosuppressive roles of these receptors also affect the outcome of the infections, such as in periodontitis in LAD patients.

12 Inflammatory Diseases

12.1 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an inflammatory disease that is characterized by hyperactive immune cells, production of auto-antibodies and deposition of immune complexes, causing end-organ damage of different organs. An *ITGAM* (the gene that encodes for αM) genetic variant is strongly associated with SLE (Nath et al. 2008). The variant results in an amino acid substitution in the extracellular part of the β2-integrin (R77H), which leads to the receptor being dysfunctional, e.g. it cannot bind normally to ligands iC3b or ICAM-1, is deficient in catch-bond formation and is deficient in mediating phagocytosis of complement-coated particles (MacPherson et al. 2011; Rosetti et al. 2015). It is possible that the reduced R77H-Mac-1-mediated phagocytosis is associated with the development of the disease, because this may result in reduced clearance of apoptotic cells or immune complexes. However, given that Mac-1 is also associated with negative regulation of myeloid cell signaling, also other disease mechanisms may be possible. Indeed, it has recently been shown that deficient Mac-1 function in SLE is associated with an increased type I interferon signature, which is a major driver of SLE, and increasing R77H-Mac-1 activity with a synthetic small-molecule CD11b agonist LA1 can reduce interferon levels and end-organ damage in SLE mouse models (Faridi et al. 2017). Interestingly, different αM and αX genetic variants have recently been shown to be associated with pre-eclampsia, an inflammatory syndrome of pregnancy (Lokki et al. 2021), which, similar to SLE, is also thought to be associated with deficient phagocytosis of endogenous particles.

12.2 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system where recruited T cells (Th1 and Th17 cells) attack myelin sheaths of neurons. The result is neuro-inflammation and recruitment of other types of immune cells. Leukocyte recruitment across the blood–brain barrier is of critical importance in disease development. β2-integrins have partly overlapping but distinct expression patterns and roles in the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. CD11d/CD18 is not required for the development of the disease, but both Mac-1 and CR4 expression on T cells and on phagocytic cells is essential for the development of EAE. Interestingly, LFA-1 clearly has both pro- and anti-inflammatory functions in EAE (Dugger et al. 2009; Bullard et al. 2005; Bullard et al. 2007; Adams et al. 2007). For instance, αL deficient T cells cause less severe EAE than wild-type (WT) cells (Dugger et al. 2009) and LFA-1 mediates the recruitment of pathological Th17 cells into the brain parenchyma (Rothhammer et al. 2011), showing that LFA-1 on T cells is important for EAE development,

probably by affecting T cell recruitment. Contrarily, transfer of WT encephalitogenic T cells into αL deficient mice caused massive EAE, showing that LFA-1 on other types of cells have a suppressive role in EAE development (Dugger et al. 2009).

12.3 Obesity and Atherosclerosis

β2-integrins are also involved in obesity and regulation of adipose tissue cellularity, metabolism, and inflammation (Zheng et al. 2015; Meakin et al. 2015). In atherosclerotic plaque formation, both CR4 and CD11d/CD18 have been shown to be upregulated and to play an active role, controlling also other integrins' activity (Gower et al. 2011; Aziz et al. 2017; Hernandez et al. 2020). Interestingly, constitutive activation of Mac-1 seems to protect from the development of atherosclerosis (Martinez et al. 2020).

12.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease, causing tissue damage and pain in joints. Study on β2-integrin expression in the synovial fluid and peripheral blood of RA patients showed striking differences in the expression of αL and αM on the dendritic cells in the two compartments. These findings suggest that in tolerogenic steady-state dendritic cells, the expression of total and active αL is higher, whereas in mature inflammatory DCs, the expression of αM is higher. Moreover, Mac-1 blockade in the activated cells leads to decreased stimulation of T cells (Schittenhelm et al. 2021). RA patients have elevated risk of cardiovascular diseases. In RA-related atherogenesis, the L5 LDL, a subfraction of low-density lipoprotein–cholesterol that is needed in the pathogenesis of atherosclerosis, induces αX expression on monocytes. This is considered one of the factors for vascular plaque formation (Chang et al. 2020).

13 β2-integrins in Cancer

β2-integrins have been studied extensively in the context of inflammation and autoimmunity. However, due to the various roles in the immune system, β2-integrins also have an impact on tumor development (Fig. 5).

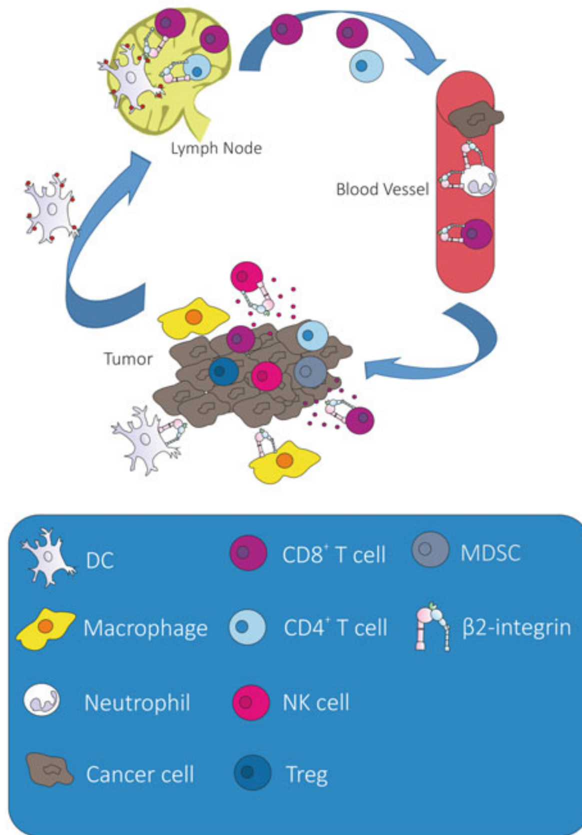

Fig. 5 β2-integrins mediate various immunological functions in a cancer setting. In the tumor tissue, various immune cell populations can be found, which interact with the malignant cells via β2-integrins. For instance, natural killer (NK) cells kill tumor cells by releasing cytotoxic granules in an LFA-1-dependent manner. High expression of Mac-1 on macrophages mediates cancer cell phagocytosis. In addition, dendritic cells (DCs) interact with opsonized tumor cells and pick up tumor antigens in a process partly mediated by Mac-1 and CR4. DCs then enter lymphatic vessels possibly in an LFA-1-dependent manner and migrate to the draining lymph node. In the lymph node, LFA-1/ICAM-1 interaction enhance T cell activation by stabilizing the immunological synapse between the T cell and the antigen-presenting DC, and by providing an additional co-stimulatory signal. The activated T cells next migrate via the bloodstream to the tumor site where they exit the blood vessel following LFA-1/ICAM-1-dependent interaction with the endothelial cells. In the tumor, $CD8^+$ T cells then kill malignant cells by releasing cytotoxic granules after LFA-1/ICAM-1-dependent immunological synapse formation. β2-integrins may also promote tumor growth by suppressing the function of DCs and by enhancing the function of immune suppressive cells such as Tregs and myeloid-derived suppressor cells (MDSCs). Finally, neutrophils can promote cancer metastasis by mediating the interaction between the cancer cell and endothelial cells in the blood vessel in LFA-1/Mac-1-dependent manner

13.1 β2-integrins Affect Immune Cell Recruitment to Tumors

The immune system has a dual role in cancer development. It can both protect the host from malignant cells but it can also occasionally promote the development of a tumor. However, a prerequisite for any type of interaction between immune cells and cancer cells is the access of leukocytes to the tumor site and into the tumor mass. Given that β2-integrins mediate leukocyte adhesion to endothelial cells and extravasation into tissues, they also play a role in mediating immune cell trafficking to the tumor site. Indeed, more lymphocytes have been shown to infiltrate tumors expressing β2-integrin ligand, ICAM-1 compared to ICAM-1 negative tumors (Fujihara et al. 1999; Maeda et al. 2002). Immune cells have also been shown to interact more with ICAM-1^{+} compared to ICAM-1^{-} tumor cells (Tachimori et al. 2005). Importantly, high expression of ICAM-1 in the tumor microenvironment (TME) has been associated with favorable prognosis among cancer patients, suggesting an enhancement in immune responses against malignant cells (Ogawa et al. 1998; Fujihara et al. 1999; Maeda et al. 2002). However, a decrease in the expression of adhesion molecules, including the β2-integrin ligands ICAM-1 and ICAM-2 has been observed on the endothelial cells lining tumor-associated blood vessels in human malignancies (Griffioen et al. 1996; Yoong et al. 1998). Further, low expression of adhesion molecules on endothelial cells has been shown to decrease the interaction between immune cells and endothelial cells and to be associated with lower T cell infiltration in the tumor (Griffioen et al. 1998; Yoong et al. 1998). Thus, downregulation of adhesion molecules in the TME may serve as a powerful mechanism utilized by tumors to restrict leukocyte trafficking into the tumor site.

13.2 β2-integrins Enhance Effector Function of Cytotoxic Cells in the TME

CD8^{+} T cells are considered to be particularly important in protecting the host from cancer development. Indeed, CD8^{+} T cell infiltration and increased ratio of CD8^{+} T cells to Tregs in the TME has been associated with favorable prognosis in patients with various malignancies (Sato et al. 2005; Ino et al. 2013; Shang et al. 2015). Thus, efficient CD8^{+} T cell infiltration in the tumor and proper activation are essential for effective anti-tumor responses and for successful immunotherapy.

Expression of adhesion molecules in the TME, particularly ICAM-1, has been shown to correlate with CD8^{+} T cell infiltration and anti-tumor cytotoxicity in various malignancies, and importantly, also with better prognosis in cancer patients (Yoong et al. 1998; Weishaupt et al. 2007; Mlecnik et al. 2010; Fisher et al. 2011; Lohr et al. 2011). In addition, an increased proportion of T cells has been shown to express LFA-1 in tumors compared to peripheral blood (Yoong et al. 1998),

suggesting that both ICAM-1 expression in the TME, and LFA-1 expression on T cells, affect T cell recruitment into tumors.

Given that LFA-1 interaction with its ligands is required to stabilize the immunological synapse between the T cell and the APC or target cell such as a cancer cell, LFA-1 has an impact on T cell priming and effector function and thus target cell killing also in the TME. Indeed, mice deficient for LFA-1 have been shown to have decreased ability to suppress tumor growth due to inefficient $CD8^+$ T cell priming (Schmits et al. 1996). Further, LFA-1 blockade has been shown to decrease $CD8^+$ T cell-mediated tumor-specific IFNγ secretion and ICAM-1^+ tumor cell killing, and also to abolish the anti-tumor efficacy of adoptively transferred T cells in tumor-bearing mice (Mukai et al. 1999; Davignon et al. 1981; Petit et al. 2016). Reduced T cell priming and thus delayed tumor rejection has also been observed in tumor-bearing mice deficient for ICAM-1 (Blank et al. 2005). Further, it has been shown to be possible to improve tumor cell killing by enhancing LFA-1-mediated immunological synapse formation between $CD8^+$ T cells and ICAM-1 expressing tumor cells (Jeon et al. 2018). However, even though the expression of LFA-1 seems to be essential for T cell-mediated target cell killing, the timing of the expression has to be tightly regulated for optimal cancer cell elimination. Indeed, enhanced LFA-1-mediated adhesion has been demonstrated to prevent $CD8^+$ T cells from moving from one target cell to the next and thus to prevent serial cancer cell killing (Wabnitz et al. 2016).

Natural killer (NK) cells have also been shown to be able to directly kill cancer cells and they are considered to be especially important in preventing metastasis (Myers and Miller 2021). Various studies have demonstrated the LFA-1/ICAM-1 axis being important in mediating NK cell activation and cytotoxicity and thus in enhancing NK cell-mediated tumor cell killing (Barber et al. 2004; Jeong et al. 2018; Saga et al. 2019; Shi et al. 2021). However, on the contrary to T cells, recruitment of NK cells seems to be independent of ICAM-1 expression in the tissues (Fogler et al. 1998). Interestingly, Mac-1 and CR4 have recently also been demonstrated to mediate NK cell-mediated cytotoxicity toward cancer cells via complement-dependent cell cytotoxicity (CDCC) (Lee et al. 2017).

13.3 *β2-integrins Suppress Dendritic Cell-mediated Anti-tumor Responses*

Dendritic cells are in a crucial position in the initiation of immune responses toward tumor cells. For this, dendritic cells must first pick-up tumor antigens in the TME and then migrate to the draining lymph node to interact with and to activate T cells. Mac-1 and CR4 expressed on dendritic cells promote the pick-up of tumor antigens by mediating the phagocytosis of complement opsonized tumor cells (Lukácsi et al. 2017; Vorup-Jensen and Jensen 2018). In addition, Mac-1 has been shown to interact with Fc receptors (FcR) thus promoting also FcR-mediated phagocytosis

of antibody-opsonized tumor particles (van Spriel et al. 2001). However, instead of enhancing anti-tumor immunity, Mac-1 and CR4-mediated phagocytosis of dying tumor cells has in fact been demonstrated to induce tolerance in dendritic cells and hence to suppress anti-tumor T cell responses (Skoberne et al. 2006). The role of β2-integrins in mediating the access of dendritic cells in the lymphatic vessels and migration to the draining lymph node is currently unresolved. There is evidence suggesting that ICAM-1 and LFA-1 expression (on lymphatic endothelium and dendritic cells, respectively) can promote dendritic cell trafficking into lymph nodes (Ma et al. 1994; Johnson et al. 2006). However, other studies have shown dendritic cell migration to lymph nodes to be normal in mice deficient for either ICAM-1 or β2-integrins or even enhanced in mice deficient for functional β2-integrins (Grabbe et al. 2002; Podgrabinska et al. 2009; Morrison et al. 2014). Yet, as described above, the function of β2-integrins has generally been associated with decrease in dendritic cell activation and maturation status (Morrison et al. 2014) suggesting an overall inhibitory role in dendritic cell-mediated anti-tumor responses. Indeed, according to a recent study, a dendritic cell vaccination containing dendritic cells with dysfunctional β2-integrins caused enhanced tumor suppression and increased intratumoral $CD8^+$ T cell response compared to dendritic cells with functional β2-integrins in mouse melanoma models (Guenther et al. 2021).

13.4 β2-integrins Promote Suppressive Immune Cell Populations in the TME

Regulatory T cells (Tregs) are capable of suppressing the function of various immune cells including T and B cells, NK cells and dendritic cells and are thus crucial in maintaining peripheral tolerance (Sakaguchi et al. 2020). However, their presence in the TME is generally considered to be detrimental (Shang et al. 2015; Plitas and Rudensky 2020). As described above, β2-integrins play a role in Treg development and function, which may have effects also on tumors. In addition to Tregs, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) are also able to effectively suppress anti-tumor immune responses. Indeed, high tumor infiltration by TAMs or elevated numbers of MDSCs in the peripheral blood has generally been associated with poor prognosis in cancer patients (Zhang et al. 2012; Xiang et al. 2021; Ai et al. 2018; Wang et al. 2018). TAMs constitute a major component of the immune cell infiltrate in many cancers and can promote tumor growth in various ways including by secreting growth, angiogenic and immunomodulatory factors to promote cancer cell proliferation, tumor angiogenesis and effector immune cell suppression and induction of MDSCs, respectively (Mantovani et al. 2017). MDSCs, which comprise of a heterogeneous population of immature myeloid cells and myeloid progenitor cells with highly suppressive phenotype, also contribute in tumor-associated immune cell suppression with various mechanisms such as by depleting T cell nutrients including L-arginine, by

secreting reactive oxygen species (ROS) and nitric oxide (NO), and by enhancing Treg development (Gabrilovich and Nagaraj 2009). ICAM-1 expression in the TME has been shown to promote macrophage infiltration into the tumor (Usami et al. 2013). In addition, both TAMs and MDSCs express high levels of Mac-1, which contributes to several important functions such as migration, chemotaxis, and phagocytosis (Arnaout 1990). Accordingly, suppressed tumor intestinal growth has been observed in mice deficient for αM with decreased myeloid cell infiltration in the TME (Zhang et al. 2015). αM blockade has also been reported to reduce myeloid cell tumor infiltration thus improving the anti-tumor effects of radiation therapy (Ahn et al. 2010). However, it has also been reported that activation of αM can in fact promote macrophage polarization toward pro-inflammatory phenotype and thus suppress tumor growth (Schmid et al. 2018).

Until recently, the role of neutrophils in tumor development has largely been overshadowed by other leukocytes such as macrophages. However, it is now being appreciated that they have a prominent role in both suppressing and promoting tumor growth by affecting the function of the malignant cells and other immune cells (Coffelt et al. 2016). β2-integrins have a dual role in neutrophils in the tumor setting. Mac-1 has been demonstrated to be vital for the formation of intercellular synapses between neutrophils and tumor cells and for neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC) toward tumor cells (van Spriel et al. 2001). Further, Mac-1 has been shown to mediate neutrophil-mediated tumor growth suppression in a mouse melanoma model (van Spriel et al. 2003). However, LFA-1 and Mac-1 expressed on neutrophils have also been observed to promote cancer cell metastasis by mediating the interaction between the cancer cells and endothelial cells (Liang et al. 2005; Fu et al. 2011). In addition, recruitment to the tumor site and promotion of tumor angiogenesis by VEGF secretion has been demonstrated to be at least partly dependent on Mac-1 expressed on neutrophils and macrophages (Soloviev et al. 2014).

13.5 β2-integrins Also Have Cell Intrinsic Effects on Cancer Cells

Given the specific expression on leukocytes, β2-integrins play a relevant role in the development of hematological malignancies affecting particularly the migration of malignant cells into the lymphoid organs and bone marrow. Chronic lymphocytic leukemia (CLL) is a malignancy of mature B cells and the most prevalent leukemia in Western countries (Fabbri and Dalla-Favera 2016). The expression of β2-integrins has been observed to be decreased in CLL cells compared to normal B cells and a variant of the ITGB2 gene causing reduced expression of β2-integrin, CD18 on B cells has been associated with CLL susceptibility (Lúcio et al. 1998; Goldin et al. 2016). In addition, CLL cells have been reported to express significantly lower levels of LFA-1 compared to healthy B cells, causing impaired migration of CLL cells to

lymph nodes leading to their accumulation in spleen and blood (Hartmann et al. 2009). However, certain CLL patient groups with unfavorable cytogenic anomalies such as trisomy 12 show significantly increased expression of LFA-1 on CLL cells leading to enhanced ligand binding and cell migration (Riches et al. 2014; Hutterer et al. 2015). This may then lead to increased migration of CLL cells in lymph nodes where they would be provided with more survival and proliferation signals possibly leading to disease progression (Hartmann et al. 2009; Riches et al. 2014). Indeed, an association has been observed between an elevated αL expression and increased tumor burden in CLL (Lúcio et al. 1998). Interestingly, in addition to β2-integrin expression levels, differences have also been detected in the regulation of LFA-1 conformational changes leading to aberrant activation of LFA-1 on CLL cells (Till et al. 2008; Montresor et al. 2009).

Multiple myeloma (MM) is the second most common hematological malignancy in Western countries, caused by malignant monoclonal plasma cells, which accumulate in the bone marrow (van de Donk et al. 2021). An upregulation of various adhesion molecules including LFA-1 has been observed in human myeloma cells (Ahsmann et al. 1992; Tatsumi et al. 1996). Further, LFA-1 expression on myeloma cells has been associated with active and aggressive disease and with drug resistance against MM treatments (Ahsmann et al. 1992; Tatsumi et al. 1996; Di Marzo et al. 2016). Studies conducted with a mouse model of MM have also demonstrated that LFA-1 expressed on myeloma cells affect myeloma cell proliferation and disease-causing abilities (Asosingh et al. 2003).

Interestingly, in addition to hematological cancers, β2-integrin expression can also be induced in solid tumor cells. Indeed, expression of LFA-1 has been observed on various human cancer cells and cancer cell lines and has been associated with increased proliferation, metastatic potential, and recruitment of suppressive immune cell populations (Fujisaki et al. 1999; Ghislin et al. 2012; Benedicto et al. 2017).

14 Therapy Targeting β2-integrins

Targeting β2-integrins in the treatment of inflammatory and autoimmune diseases such as multiple sclerosis, psoriasis, rheumatoid arthritis and inflammatory bowel disease have been studied extensively in animal models (Mitroulis et al. 2015). The results from the pre-clinical models demonstrated that targeting β2-integrins may indeed significantly alter disease commencement and/or progression and thus resulted in the development of multiple therapeutic antibodies and antagonizing small molecules aimed to be used in clinical practise. Efalizumab, a humanized monoclonal antibody targeting αL, was the first drug targeting β2-integrins that was approved in 2003 to be used to treat patients with plaque psoriasis (Slack et al. 2021). However, the drug was soon associated with rare but severe systemic adverse events such as thrombocytopenia and hemolytic anemia, and in 2009, efalizumab was withdrawn from the market after three out of 45,000 patients developed fatal viral-based multifocal leukoencephalopathy (PML) (Scheinfeld 2006; Seminara and

Gelfand 2010). Following this major drawback, only one drug targeting β2-integrins, lifitegrast, a small antagonizing molecule targeting LFA-1, has since (in 2016) been approved to be used in the clinic to treat dry ice disease (Slack et al. 2021).

The detrimental adverse events associated with efalizumab therapy highlighted the importance to fully understand all the various roles β2-integrins play in the immune system. Given that LFA-1 has a wide expression pattern on different leukocyte populations and an essential role in immune regulation, targeting other β2-integrins, namely αM, αX or αD, with more restricted expression patterns could provide more suitable options as therapeutic targets to treat inflammatory and autoimmune diseases.

14.1 Cancer Therapy Targeting β2-integrins

The development of cancer therapies targeting β2-integrins has also been hindered by the safety issues associated with efalizumab therapy. However, β2-integrins, particularly LFA-1, could be an appealing target to treat hematological malignancies or solid tumors that have aberrantly upregulated its expression. Indeed, it has been reported that a small molecule antagonist targeting LFA-1 has demonstrated anti-tumor efficacy in a preclinical cancer model (San Sebastián et al. 2013).

One option to avoid non-target effects could be to aim to target β2-integrins in individual cell types. LFA-1 has been shown to be important for the function of Tregs (Singh et al. 2013; Wang et al. 2008) and thus targeting LFA-1 on Tregs could provide a means to diminish immune suppression in the TME. Targeting β2-integrins in dendritic cells also seems as an attractive option since the approach has already been shown to enhance the efficacy of dendritic cell vaccine and thus to suppress tumor growth in mouse models of melanoma (Guenther et al. 2021). Another option could be to target LFA-1 directly on tumor cells. Indeed, an engineered antibody, specific for both, LFA-1 and a tumor antigen, demonstrated anti-metastatic efficacy in a mouse model of B cell lymphoma (Cohen et al. 2003). In addition, leukotoxin, a protein produced by the oral bacterium *Aggregatibacter actinomycetemcomitans*, has been demonstrated to cause LFA-1-dependent malignant leukocyte apoptosis with minor effects on healthy leukocytes (Kachlany et al. 2010). Further, leukotoxin was also associated with increased survival in leukemia and lymphoma mouse models (Kachlany et al. 2010; DiFranco et al. 2015). Yet another possibility could be to promote LFA-1 activity on T cells. Interestingly, by blocking a certain phosphorylation site in VLA-4 belonging to β1-integrins, it has been shown to be possible to increase LFA-1 function in T cells via integrin trans-regulation and to suppress tumor growth in mice (Cantor et al. 2015). The reduced tumor growth was associated with an increase in tumor infiltration by T cells but not with myeloid cells. However, permanent activation of LFA-1 in T cells should still be avoided since it has been shown to lead to defective T cell migration (Semmrich et al. 2005; Yanguas et al. 2018). Interfering with the function of extracellular

vesicles or exosomes carrying β2-integrins or their ligands could also provide a means to affect anti-tumor immunity. Indeed, certain cancer cells have been shown to produce exosomes carrying ICAM-1, which then are able to prevent leukocyte adhesion to endothelial cells (Lee et al. 2010) and may thus interfere with leukocyte trafficking to the tumor site. In addition, leukocytes can also produce exosomes, which carry LFA-1 or ICAM-1, and can thus have an impact on the function of other immune cells either by activating or suppressing their effector functions (Reina and Espel 2017). Finally, a small molecule, 7HP349, which functions as an allosteric activator of both LFA-1 and VLA-4 and has demonstrated anti-tumor efficacy in mouse tumor models is currently on phase I clinical trial (NCT04508179) assessing the safety, tolerability, and pharmacokinetics of the drug (Slack et al. 2021).

Taken together, β2-integrins have both immune activating and suppressing roles during tumor development, and targeting the function of β2-integrins may provide a powerful tool to interfere with cancer development in the future.

15 Concluding Remarks and Future Outlook

β2-integrins are fascinating receptors with a wealth of tasks in the immune system, and can clearly play both pro- and anti-inflammatory roles in immunity and disease. Several of their functions, such as in mechanoregulation of immune cell function, and their role in immune cell reprogramming, are only now starting to be elucidated. Because of their crucial role in immunity, infections, and cancer, they remain attractive potential therapeutic targets in various disorders. However, because of their numerous and varied roles in immune cell functions, it is clear that a careful further delineation of the role of these receptors in different immune cell populations is required to achieve a specific response without unwanted side effects.

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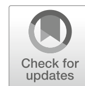
Structure and Function of the Leukocyte Integrin αMβ2



Gregers Rom Andersen and Jonas Emsley

Abstract The integrin αMβ2 (also known as CD11b/CD18, Mac-1, complement receptor 3) is expressed on the surface of leukocytes and mediates numerous responses of these cells critical to innate immunity. The αMβ2 receptor contributes to the recruitment, firm adhesion, and transendothelial migration of leukocytes at sites of vascular injury and facilitates tissue inflammation. Biochemical and cell-based studies have characterized the interactions of the αMβ2 integrin with diverse ligands including plasma protein fibrinogen, complement protein fragment iC3b, and the cell surface receptors platelet glycoprotein Ib (GPIb) and intercellular adhesion molecule 1 (ICAM-1). The αMβ2 integrin exists in an inactive conformation and when activated by a variety of stimulae undergoes a structural change to an active form capable of binding to ligands with high affinity. Concurrently, allosteric changes occur upon ligand binding that result in "outside-in" cell signaling. Here we describe the αMβ2 protein structures and biophysical measurements that underpin the current understanding of diverse ligand recognition through the metal ion-dependent adhesion site (MIDAS).

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1 The Multifunctional Integrin Receptor αMβ2

Integrins are integral membrane proteins, which mediate cell-cell, cell-extracellular matrix, and cell-pathogen interactions. αMβ2 is one such receptor and is also known as complement receptor 3 (CR3), CD11bCD18, and Macrophage-1 antigen (Mac-1). The two subunits αM (CD11b) and β2 (CD18) both consist of a large N-terminal ectodomain, a single transmembrane helix, and a C-terminal cytoplasmic tail. The two subunits associate through extensive non-covalent interactions (Carman and Springer 2003). The ectodomain is divided into a headpiece consisting of the N-terminal domains, and a tailpiece consisting of the membrane-proximal C-terminal domains (Fig. 1a). Integrin ectodomains adopt at least three major conformational states with a distinct affinity for the receptor ligands (Vorup-Jensen and Jensen 2018).

One primary function attributed to αMβ2 is phagocytosis of complement-opsonized cells and immune complexes. Proteolytic cleavage of complement C3 deposits the opsonin C3b on the activator that is converted to iC3b (Zipfel and Skerka 2009). The physiological response to iC3b recognition by αMβ2 depends on cell type and activation state of the expressing cell, but phagocytosis of dying host cells or pathogens is the canonical response of phagocytes (Vorup-Jensen and Jensen 2018; Erdei et al. 2019). αMβ2 is highly expressed on the plasma membrane of myeloid cells including macrophages, monocytes, dendritic cells, and neutrophil granulocytes (Vorup-Jensen and Jensen 2018; Erdei et al. 2019). The receptor is also highly expressed in microglia, the mononuclear phagocytes of the central nervous system (CNS), where αMβ2-mediated phagocytosis of iC3b opsonized presynaptic termini of neurons is important for neural development and homeostasis (Stevens et al. 2007; Schafer et al. 2012; Wakselman et al. 2008; Jiang et al. 2015). Mice deficient in αMβ2 are less efficient in microglial synaptic engulfment during developmental synaptic pruning (Schafer et al. 2012). The defect in pruning caused by αMβ2 deficiency was also observed in a mouse model of Alzheimer's disease (Hong et al. 2016) and CNS injury (Norris et al. 2018).

Monocytes and neutrophils crawl inside the blood vessel toward a preferential site of transmigration in an αMβ2 and Intercellular Adhesion Molecule 1 (ICAM-1) dependent manner (Frommhold et al. 2010; Phillipson et al. 2006; Schenkel et al. 2004). Leukocytes of myeloid origin, especially monocytes and neutrophil granulocytes, uses αMβ2 for diapedesis through the blood vessels to enter zones of inflammation. Furthermore, the ability of αMβ2 to bind extracellular matrix constituents such as fibrinogen and fibronectin are probably also important as step stones in the migration after leaving the blood vessel (Cui et al. 2018), and fibrinogen/fibrin as a substrate for αMβ2 is recognized as important for leukocyte patrolling of wounds (Flick et al. 2004).

Integrin αMβ2 is critical to mediating heterotypic cell-cell interaction between leukocytes, endothelial cells, and platelets (via glycoprotein Ib). The close coordination between these blood cell types is important to innate immune responses and thrombotic disease processes (von Bruhl et al. 2012; Wang et al. 2017). Endothelial

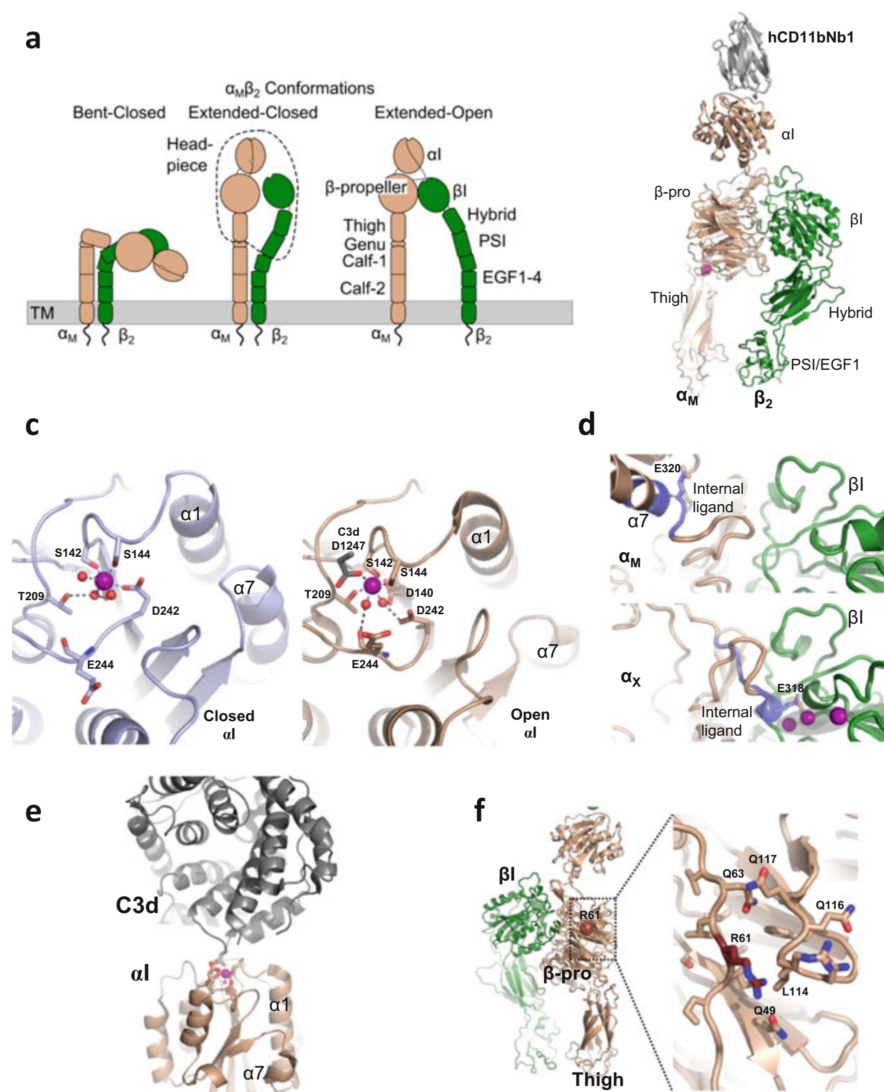

Fig. 1 The conformational states of αMβ2 and the C3d ligand. (**a**) Diagram of the three major conformations of αMβ2. In the extended open conformation the ligand affinity is high. (**b**) Cartoon representation of the crystal structure of the complex between the αMβ2 HP and the hCD11Nb1 nanobody (pdb entry 7P2D). Notice the characteristic proximity of β2 domains to the αM Thigh domains in the closed conformation of the headpiece. (**c**) Comparison of the closed conformation in the absence of a ligand (pdb entry 1JLM) and open conformation of the αI domain associated with ligand binding, here represented by the C3d aspartate (pdb entry 4 M76). Notice how the metal ion changes coordination sphere in response to the ligand and becomes directly coordinated by Thr209 and released from the coordination by Asp242. This propagates to conformational changes that allow the α7 helix to move out of the plane. (**d**) Comparison of the internal ligand region (blue backbone) in the structure of the αMβ2 HP in the closed conformation and an internally liganded structure of αXβ2 (pdb entry 4NEH). In the lower panel, the binding site for three metal ions is outlined by the gray background; the central ion is located in the MIDAS coordinating the glutamate side chain from the internal ligand. (**e**) Structure of the αI:C3d complex (pdb entry 4 M76). A recent

cell activation leading to the recruitment of both platelets and neutrophils can induce and augment neutrophil activation and intravascular release of neutrophil extracellular traps (NETs). Activated neutrophils and NETs in turn recruit more platelets, creating a cycle that drives a process termed immunothrombosis leading to thrombi rich in nucleic acids. Mice deficient in αMβ2 show delayed thrombosis, but largely unimpaired hemostasis (Wang et al. 2017). A plethora of additional αMβ2 ligands have been reported, but for most of these, the in vivo relevance is uncertain (Lamers et al. 2021).

2 The Structure of the αMβ2 Ectodomain

Structures of a β2-integrin in the open extended conformation of high ligand affinity are still lacking although low-resolution negative stain EM micrographs confirm the presence of the open conformation (Xie et al. 2010; Jensen et al. 2021). Until very recently, models of the αMβ2 ectodomain were based on structures of the homologous integrin receptors αXβ2 and αLβ2 in the overall closed bent conformation (Xie et al. 2010; Sen and Springer 2016; Sen et al. 2013). We have recently obtained the structure of the αMβ2 headpiece (αMβ2 HP) in the closed conformation by co-crystallization with the αI binding nanobody hCD11bNb1 (Jensen et al. 2022). Except for the αI domain, the conformation of the αMβ2 HP in this crystal structure (Fig. 1b) is rather similar to closed conformations known from structures of αXβ2 and αLβ2 (Xie et al. 2010; Sen and Springer 2016).

Within the αM subunit, the αI domain is the major ligand binding site. The function of the αI domain depends on Mg^{2+} binding to the metal-ion dependent adhesion site (MIDAS) (Michishita et al. 1993; Lee et al. 1995). A glutamate or an aspartate of the ligand directly coordinates the Mg^{2+} ion bound in the αI MIDAS (Lee et al. 1995; Bajic et al. 2013). The side chain of Thr209 directly coordinates the Mg^{2+} ion only in the 'open' conformation of the αM I-domain, and crystallographic studies have defined a large allosteric conformational shift between 'open' and 'closed' states which links the switching of the coordination of the metal ion from Asp242 to Thr209 to a shift in the position of helix α7 (Fig. 1c).

Prior structures of αXβ2 and αLβ2 demonstrated how the α-subunit β-propeller and the β2-subunit βI domain form a platform above which the αI domain has considerable rotational freedom. The precise position of the αI domain in αXβ2 and αLβ2 appears to be influenced by crystal packing and whether the so-called

Fig. 1 (continued) structure of the αI:iC3b complex (Fernandez et al. 2022) confirms that the thioester domain within iC3b (that corresponds to the C3d fragment) interacts with the αI domain in the same manner. (**f**) Left, the Arg61 residue (Arg77 in prepro numbering, see text) associated with SLE is located 40 Å from the αI domain. Right, a magnified view illustrating that the Arg61 is located next to the 111–118 loop in the β-propeller. With a histidine at position 61, the neighboring loop may adopt a different conformation and have altered dynamic properties

internal ligand (see below) coordinates the βI MIDAS (Xie et al. 2010; Sen and Springer 2016; Sen et al. 2013). In line with this idea, the orientation of the αI domain in our structure of the αMβ2 HP in the closed conformation (Fig. 1b) is unique. Compared to known structures of αXβ2 and αLβ2, the αI domain is rotated by 42–180°. This may not be of functional relevance since the αI-hCD11bNb1 part of the subcomplex is heavily involved in crystal packing interactions (Jensen et al. 2022).

3 The β2 I-Domain Relays outside-in and inside-out Signaling

The αI domains are only found in the integrin subunits αM, αX, αL, and αD associated with the β2 subunit and α1, α2, α10, α11, and αE that pair with the β1 or β7 subunits. For αI-less integrin receptors not having an αI domain, ligand binding occurs directly to the βI domain that is structurally homologous to the αI domain and likewise contains a MIDAS. Comprehensive structural studies of αI-less integrins have revealed the conformational rearrangements occurring upon ligand binding to the βI domain (Schumacher et al. 2021; Kotecha et al. 2017; Xiao et al. 2004; Arimori et al. 2021). In crystal structures of the α(IIb)β3 integrin it was observed that upon ligand binding, internal conformational changes in the βI domain, translates into 60° swing out of the hybrid domain, and in turn moves the PSI domain 70 Å (Xiao et al. 2004). This swing out of the β-subunit is supported by negative stain EM, and Förster resonance energy transfer studies (Nishida et al. 2006; Chigaev et al. 2003). The large conformational change separates the legs of the two integrin subunits, and in turn separates the transmembrane and cytoplasmic domains from each other. It thereby provides a mechanism to relay a signal upon ligand binding through the βI and hybrid domain, all the way into the intracellular environment, or reversely from the intracellular environment to the βI domain. The cytoplasmic domains are only loosely associated and the opening of the headpiece makes the cytoplasmic domains dissociate and allows them to interact with intracellular adaptor proteins (Luo et al. 2007).

The αMβ2 receptor is expected to respond in a similar manner to αM ligand binding and binding of a glutamate side chain (Glu320) from the αM subunit to the βI domain. This has led to the following model for intersubunit allosteric regulation of αI domain ligand affinity. During inside-out signaling, the βI domain MIDAS becomes able to bind Glu320 from the αM subunit. The Glu320 residue is located in the C-terminal end of the αI α7 helix, and its interaction with the βI domain exerts a pull on the α7 helix forcing the αI domain into the open conformation with high ligand affinity. Conversely, in ligand-induced outside-in signaling, ligand-induced α7 helix movement in the αI domain allows αM Glu320 to interact with the βI MIDAS site, and induces the transition of the βI domain into the open conformation that propagates to the remaining parts of the β2 subunit and cause swing out of the β2

subunit. A small highly conserved region encompassing Glu320 in αM is therefore known as the internal ligand (Fig. 1d) since it corresponds to the βI binding ligand of an αI-less integrin.

4 The αMβ2 Complexes with C3d and iC3b

The opsonin C3b is deposited on the surface of the complement activator through a covalent bond between the C3b thioester domain and the activator. Host cells present glycans that attract the fluid phase regulator factor H and host cells likewise express on their cell membrane complement regulators membrane cofactor protein (MCP/CD46) and CR1/CD35. These regulators interact with C3b and enable its cleavage by the protease factor I (FI) to iC3b (Zipfel and Skerka 2009). The iC3b may be further degraded to the C3dg and C3d fragments that contain little more than the thioester domain but iC3b is normally considered to be the in vivo relevant ligand for αMβ2 although C3dg may act as a ligand in vivo also (Lin et al. 2015).

The iC3b is a ligand for both αMβ2 and αXβ2. The primary binding site recognized by the αMβ2 αI domain is located in the thioester domain of iC3b whereas the αI domain in αXβ2 recognizes a distinct epitope quite far from the thioester domain (Bajic et al. 2013; Lin et al. 2015; Xu et al. 2017). In addition, regions outside the αI-domain contribute to iC3b binding, since its deletion leaves residual iC3b affinity in αMβ2 (Yalamanchili et al. 2000). The monovalent interactions between iC3b and the αMβ2 HP fragment are the strongest known monovalent interaction between a recombinant fragment of αMβ2 and a ligand. Using surface plasmon resonance, we measured a dissociation constant of 30 nm for the interaction between αMβ2 HP and iC3b. In the same study, we observed an apparent dissociation constant of 50 nM for the complex between monomeric fluid phase iC3b and intact αMβ2 presented by K562 cells and activated by Mn^{2+} or antibody treatment (Jensen et al. 2021). Likewise, we found that the αMβ2 HP interacted with the minimal ligand C3d through the αI domain with a dissociation constant of 520 nM (Jensen et al. 2021). Earlier, we demonstrated that the interaction of the isolated recombinant αI domain with iC3b occurs with a dissociation constant of 600 nM (Bajic et al. 2013). The interaction between the αI and iC3b/C3dg depends on Mg^{2+} bound in the MIDAS (Fig. 1c) that may be substituted in vitro by Ni^{2+} and Mn^{2+}.

These data illustrate that the core of the iC3b-αMβ2 interaction is formed by the iC3b thioester domain and the αMβ2 αI domain. This core interaction was captured in our structure of a Ni^{2+} stabilized complex formed by the αI domain and C3d (Bajic et al. 2013). The Ni^{2+} ion allowed us to crystallize the complex by increasing the stability of the αI-C3d complex. Ligand binding was further favored by the I316G mutation in the recombinant αI domain that favors the open conformation of the MIDAS and the shift of the α7 helix toward its C-terminal end (Fig. 1c, d). In this structure, the side chain of the C3d residue Asp1247 coordinates the Ni^{2+} ion bound to the αI MIDAS (Fig. 1c, e). Mutation of the aspartate to alanine abolishes the interaction and replacing the aspartate with a glutamate also impaired the interaction

demonstrating that the geometry of the metal ion coordinating ligand is important. The C3d-αI interface is of modest size and rather polar, and in addition to the Asp1247-metal ion interaction, the interface is stabilized by intermolecular hydrogen bonds and ionic interactions (Bajic et al. 2013). Residues in both αM and the iC3b thioester (TE) domain engaged in this core interaction are highly conserved and not present in αX and complement C4b which is homologous to C3b in both structure and function. In particular, C4b also becomes degraded to iC4b by factor I (Mortensen et al. 2015). Very recently, a crystal structure of iC3b in complex with the αI domain was reported which confirmed the core interaction between αI and the iC3b TE domain (Fernandez et al. 2022). Two different crystal packing interactions between the αI domain and regions in iC3b far from the TE domain were suggested to mirror cell bound αMβ2 interaction with iC3b on an opsonized surface. Further experimental evidence is needed to confirm the suggested in vivo relevance of these αI interactions.

To obtain structural insight into additional αMβ2-iC3b interactions outside of this core interface has proven challenging, presumably because the iC3b TE domain is flexibly attached to the rest of the molecule (Nishida et al. 2006). Interestingly, negative stain electron microscopy (nsEM) of the αMβ2 HP-iC3b complex confirmed the αI interaction with the iC3b-TE domain and suggested an additional interaction between a C-terminal domain of iC3b and the αMβ2 platform in a non-compact complex. However, only 2D classes were presented in this study and multiple complexes with significantly different orientations of iC3b relative to αMβ2 were observed (Xu et al. 2017).

Negative stain EM may result in perturbation of the sample due to interaction with the support and the staining procedure. Electron microscopy under cryogenic conditions (cryo-EM) offers an alternative, but despite considerable efforts, we have not managed to obtain suitable grids for structure determination of the αMβ2-iC3b complex. One possible reason is that the ~100 disordered residues present in iC3b cause iC3b and its αMβ2 complex to behave poorly at the air–water interface during cryo-EM grid preparation. Likewise, we have not been able to crystallize the iC3b-αMβ2 HP complex. In contrast, we recently showed using small angle X-ray scattering (SAXS) that the αMβ2-iC3b complex is rather compact and distinct from that captured by negative stain EM. In SAXS, the sample can be studied under native conditions with physiologically relevant concentrations of Ca^{2+} and Mg^{2+} in the buffer. Even though the resolution of the SAXS data was limited to around 25 Å, we could obtain qualitative atomic models of the complex by fitting the data with a homology model of αMβ2 HP in the open conformation and iC3b represented by the thioester domain connected with flexible linkers to the remaining ordered fragment of iC3b (Jensen et al. 2021). By comparison with the solution structure of iC3b, we concluded that interaction with the αMβ2 HP induces a large conformational change in iC3b, but we were unable to identify a unique conformation of the αMβ2 complex (Jensen et al. 2021). Perhaps this mirrors that the complex is dynamic even when cell-bound αMβ2 interacts with an iC3b-opsonized complement activator in vivo.

5 Structural Basis for αMβ2 Recognition of Platelet GPIbα

Activated platelets and leukocytes interact with each other through an αMβ2 interaction with the GPIbα chain of the platelet GPIb-IX-V complex (Wang et al. 2017; Simon et al. 2000). The platelet GPIbα chain is the largest component of the GPIb-IX-V complex and disulfide bonds to the adjacent GPIbβ subunit. Additional non-covalent interactions are formed with the GPIX and GPV subunits (Li and Emsley 2013). The 45 kDa GPIbα N-terminal domain (GPIbαN) is the major ligand binding domain of the complex and is situated at the end of a highly O-glycosylated macroglycopeptide (Uff et al. 2002). The GPIbαN domain adopts an arc-shaped solenoid structure, characteristic of its leucine-rich repeats (LRR). GPIbαN contains disulphide bonds between Cys20-Cys33 and Cys211-Cys264, Cys209-Cys248 within what are termed the N- and C-terminal LRR capping or flanking regions. The GPIbαN domain contains binding sites for not only αMβ2 but also the von Willebrand factor (VWF) A1-domain and thrombin (Morgan et al. 2019). Function-blocking monoclonal antibody studies conducted with the anti-GPIbα antibodies AP1 and VM16d, which map to within the C-terminal flank of the GPIbα, demonstrated inhibition of the αMβ2 mediated platelet-leukocyte interaction (Ehlers et al. 2003). A series of cell-based and biochemical studies confirmed residues 218–228 from GPIbαN as the principal determinants of the αMβ2 interaction. Importantly, the related integrin αLβ2 does not bind GPIbα and αL/αM chimeras were utilized to localize the binding site for GPIbαN within the αI-domain of αM to specific residues in the region of the MIDAS site (Ehlers et al. 2003).

A series of biophysical studies subsequently determined which GPIbαN acidic residue was utilized for integrin binding and how the GPIbα LRR flanking region was positioned relative to the integrin αM MIDAS binding face (Morgan et al. 2019). To define the key residues at the binding interface, NMR assignment was made of the spectra of the mouse αI-domain and mapped the residues contacting the mouse GPIbα N-terminal domain (GPIbαN) to the locality of the αI MIDAS surface. As the crystal structures were not available for the mouse proteins, these were determined to 2 Å (GPIbαN) and 2.5 Å (αI-domain) resolution respectively (Morgan et al. 2019). The mouse αI-domain crystal structure revealed an active conformation which is stabilized by a crystal contact from the α7-helix with a glutamate side chain completing the octahedral coordination sphere of the MIDAS Mg^{2+} ion. Remarkably, the amino acid sequence of the α7-helix and disposition of the glutamate matches the C-terminal capping region α-helix of GPIbα effectively acting as a ligand mimetic. There is a piece of integrin structural biology history associated with this observation as the first structure of an integrin I domain was determined for human αI domain from αM by the group of Robert Liddington in 1995 with a glutamate sidechain observed coordinating to the MIDAS metal ion (Lee et al. 1995). At the time it was hypothesized by the authors this was the method by which other integrins could utilize the MIDAS-bound metal ion to coordinate a critical acidic residue in the ligand. This is now an almost universal principle observed for integrin-ligand interaction. The 1995 Cell paper shows the coordination

geometry of the glutamate from the C-terminal α-helix (Lee et al. 1995) and a more recent paper illustrates that the helix α7 sits comfortably across the face of the α_M MIDAS site in the same way as GPIbαN (Morgan et al. 2019).

Utilizing the GPIbαN and αI crystal structures in combination with NMR measurements and docking analysis we developed a model whereby the acidic Glu222 from the GPIbα LRR C-terminal capping α-helix coordinates directly to the αI MIDAS Mg^{2+} ion. The αI:GPIbαN complex involves additional interactions consolidated by an elongated pocket flanking the GPIbαN leucine-rich repeat (LRR) capping α-helix. The GPIbαN α-helix has a HxxxE motif, which is equivalent by homology to RxxxD from the human GPIbαN. Subsequent mutagenesis of residues at this interface, coupled with surface plasmon resonance analysis, confirmed the importance of GPIbαN residues His218, Glu222 and the α_M MIDAS residue Thr209 to the formation of the complex (Morgan et al. 2019). Molecular docking of the αI: GPIbαN complex was performed using NMR distance restraints together with GPIbαN surface exposed residues Arg218, Asp222, and Asn223. In the resulting model, the αI:GPIbαN complex buries a surface area of 593 $Å^2$ and two key features that define the unique orientation are that (i) GPIbα Asp222 coordinates to the αM MIDAS Mg^{2+} ion and (ii) GPIbα Arg218 forms bidentate salt bridges with α_M residues Asp242 and Glu244 (Fig. 2a). As viewed down the central projection of the GPIbα capping α-helix, colored orange in Fig. 2b, residues Gly143 and Ser144 of αI hydrogen bond to the side chains of Asn221 and Arg217 of GPIbα, respectively. Flanking interactions also occur on the right of Fig. 2b as Arg208 from αI forms hydrogen bonds to the side chains of Asn223 of GPIbα, and its own H195 main chain carbonyl. The αI Phe246 side chain inserts into an elongated hydrophobic pocket in the GPIbαN LRR capping region defined by GPIbα residues Phe192, Ser194, Tyr215, Trp219 (Fig. 2b).

This study highlighted some of the commonly occurring technical issues associated with determining integrin I domain-ligand complexes (Morgan et al. 2019). The weak micromolar dissociation constant for the GPIbαN:αI complex renders co-crystallization with the recombinant proteins unsuccessful. NMR spectroscopy was thus required to characterize the interaction. Unfortunately, the human αI domain was unstable at the high protein concentrations required for NMR and did not result in high-quality NMR spectra. The mouse αI domain, by comparison, yielded high-quality NMR spectra and performed well in SPR binding studies showing clear metal ion dependance and high-quality binding curves (Morgan et al. 2019). This species-specific difference in stability is likely due to the propensity of the C-terminal helix to disengage from the body of the I domain in solution which may occur more easily in human than mouse αI domain.

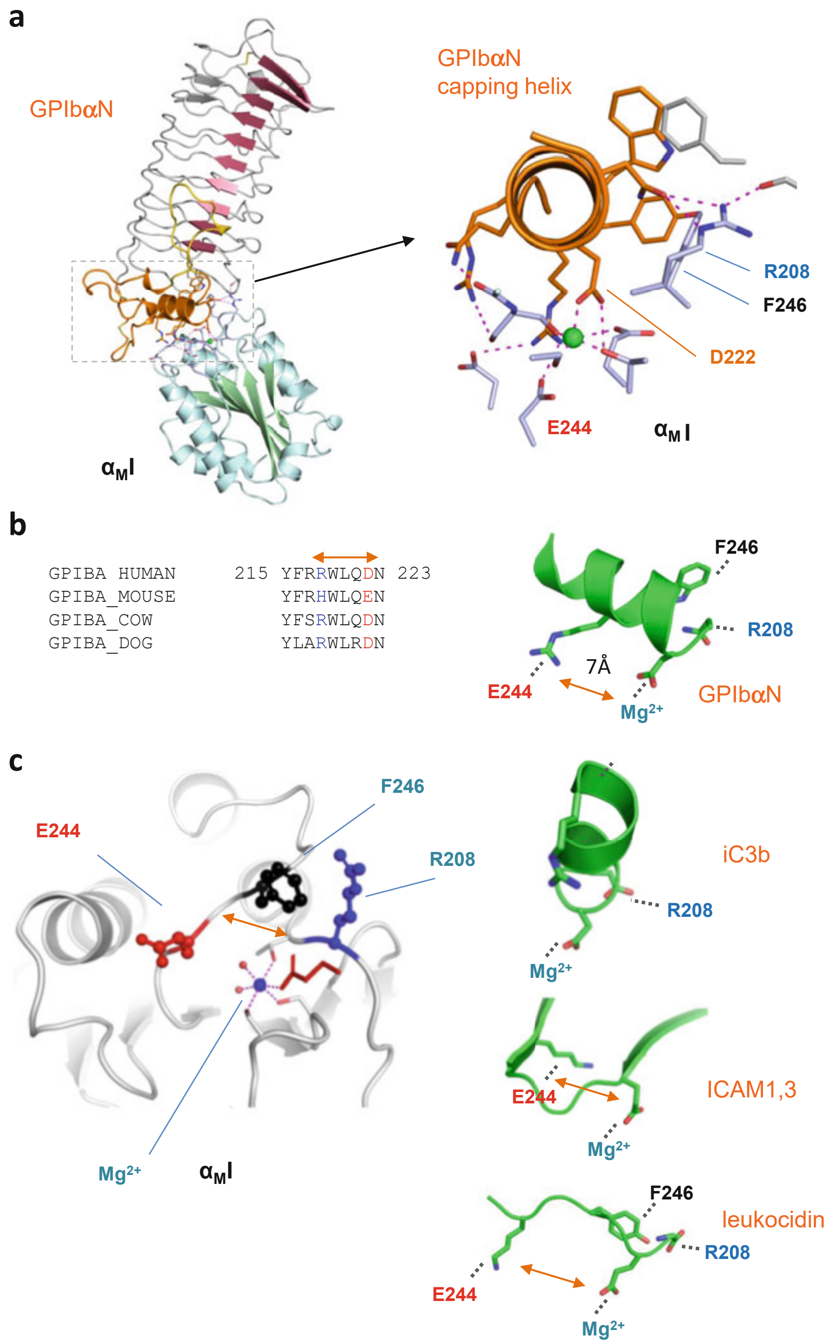

Fig. 2 The αMβ2 complex with platelet Glycoprotein Ibα. (**a**) Cartoon diagram of the docked complex of the crystal structures from the human α_MI-domain with platelet receptor N-terminal domain (GPIbαN) is shown. The αM I domain (αI) secondary structures are colored blue/green and GPIbαN colored red/orange. Left is a close-up view of the interface where the Mg^{2+} ion bound to

6 Recognition of FcgRIIA Sialic Acid by the αI Domain

The three-state model depicted in Fig. 1a possibly represents a too-simplified view of the possible conformational states that αMβ2 can adopt. Two crystal structures of αXβ2 in metastable states featured the overall closed bent conformation of the ectodomain like that in Fig. 1a but with the αI MIDAS in the open conformation and the αI α7 helix shifted toward the C-terminal end (Sen et al. 2013). The C-terminal end of the α7 helix is unfolded, and despite the overall closed bent conformation, the internal ligand region is located at the βI MIDAS in the β2 subunit (Fig. 1d). The αX glutamate 318 (equivalent to αM Glu320) coordinates the Mg^{2+} ion in the βI MIDAS, and in one of the two crystal structures, the βI domain is in a "cocked" state that shares features with a βI domain in the open conformation. But, the βI α7 helix maintains the conformation of the closed state in agreement with the overall bent-closed conformation (Sen et al. 2013).

An intermediate bent-closed state of αMβ2 akin to that observed for αXβ2 with an open αI domain capable of binding a ligand was suggested to underlie the cis-interaction between αMβ2 and FcγRIIa on neutrophils (Saggu et al. 2018). This interaction downregulates the IgG affinity of FcγRIIa and thereby inhibits FcγRIIA-mediated recruitment of neutrophils under flow. In this cis-interaction, terminal sialic acid residues on one specific Asn-linked glycan were suggested to coordinate the Mg^{2+} ion in the αI MIDAS of an αMβ2 receptor on the same cell (Saggu et al. 2018). Accordingly, treatment with neuraminidase markedly decreased the αI interaction with FcγRIIA demonstrating that sialic acid is required and likewise the presence of EDTA significantly reduced the interaction (Saggu et al. 2018). From a structural point of view, it remains to be seen how the rigid carboxylic

Fig. 2 (continued) the αM MIDAS site is shown as a sphere and electrostatic interactions are shown as dashed purple lines. The GPIbαN C-terminal LRR capping region is colored orange and key interacting residues are shown as sticks. (**b**) Amino acid sequence alignment of GPIbα residues 215–223 (human sequence numbering without the signal sequence) from the LRR capping α-helix for human, mouse, dog, and cow sequences. Key charged residues 218 and 222 are colored blue and red respectively. The orange double-headed arrow indicates an approximate 7 Å distance between the negative and positive charge side chain functional groups of carboxylate and guanidinium for Asp and Arg respectively. The 7 Å distance occurs in the context of an α-helix and corresponds to the distance between the MIDAS Mg^{2+} ion and Glu244 in the αM I-domain. (**c**) αM I domain principles of mosaic ligand binding to the MIDAS face from multiple ligands with distinct 3D structures. On the left the αM I domain structure is shown and has a relatively flat surface with features of positive charge (Mg^{2+}, Arg208), negative charge (Glu244), and hydrophobic character (Phe246). The position of the Glu/Asp from the coordinating ligand occupies a similar position coordinating the Mg^{2+} ion in all structures described to date. Shown on the right in green at the top is a cartoon diagram of the GPIbα C-terminal LRR capping α-helix. Charged residues involved in the interaction with αM I domain are shown as sticks with text for the αM residues they coordinate shown adjacent in bold. The spacing between a basic and acidic residue for ICAM1,3, GPIbα, and leukocidin (orange double-headed arrow) is the same as the spacing between the Glu244 and Mg^{2+} ion on the MIDAS face. Leukocidin has a similar aromatic hydrophobic amino acid packing against αM Phe246 as GPIbα

group linked directly to the endocyclic C2 atom of sialic acid can coordinate the Mg^{2+} ion in the same manner as the terminal and more accessible carboxylic group of an aspartate or glutamate side chain.

7 Structural Aspects of a Lupus Associated αM Variant

ITGAM (the gene encoding the αM subunit) coding region single nucleotide polymorphisms (SNPs) (rs1143678, rs1143679, and rs1143683) result in missense mutations that are strong risk factors for developing Systemic Lupus Erythematosus (SLE) (Fagerholm et al. 2013; Nath et al. 2008). Given that αM acts as a negative regulator of TLR signaling and B cell autoreactivity (Cui et al. 2018; Flick et al. 2004), it is not unexpected that these SNPs found in SLE patients may support disease via a reduction in the normal anti-inflammatory signaling of αMβ2 in leukocytes (Rosetti and Mayadas 2016). The R77H mutation occurs within the headpiece but does not change the surface expression of αMβ2 on neutrophils and monocytes and inside-out signaling appears not to be affected (Rhodes et al. 2012; MacPherson et al. 2011). In contrast, the mutation does interfere with ligand-induced outside-in signaling since it significantly decreases phagocytosis of iC3b opsonized red blood cells (Rhodes et al. 2012; MacPherson et al. 2011). In addition, monocytes carrying the mutation adhere less efficiently to surfaces coated with iC3b, fibrinogen, ICAM-1, and other ligands. The molecular mechanism underlying the functional defects of αM R77H was recently suggested to be due to interference with the formation of catch-bonds that increases the ligand affinity under flow conditions. Based on the rescue of the defect induced by a β2 specific antibody, it was proposed that in the R77H variant of αMβ2, the β2 subunit is less likely to adopt the fully extended conformation with maximum interaction between the βI domain and the αM internal ligand that transmits into higher affinity for the ligand like iC3 and ICAM-1 (Rosetti et al. 2015).

In our structure of the αMβ2 HP, Arg77 (Arg61 in mature numbering) is exposed on the edge of the αM β-propeller (Fig. 1f). The arginine side chain only appears to interact with the nearby loop Gly127-Pro134 by non-specific van der Waals interactions and does not engage in specific hydrogen bonds or electrostatic interactions that could directly explain the compromised allosteric coupling in αMβ2 containing the R77H variant. Arg77 is located ~40 Å from the ligand-binding αI domain and the β2 subunit. Hence, a direct interaction of Arg77 with residues in the αM-β2 domain interface involved in the allosteric coupling between ligand binding and transition to the extended open conformation is unlikely to underlie the functional defects. Comparison with the structure of the closed-bent conformation of the homologue αXβ2 (Xie et al. 2010; Sen et al. 2013) also suggests that Arg77 does not interact with other domains in either of the two subunits in the bent-closed or extended-closed conformations of αMβ2. However, a possible consequence of a histidine at position 77 could be that the conformation and dynamic properties of the neighboring loop (Fig. 1f) change. This could propagate to α_M residues located at the

interface to the β2 subunit. Transmission of force upon ligand binding resulting in outside-in signaling is crucially dependent on a stable αM-β2 interface, even a small perturbation may give rise to the observed abnormal outside-in signaling in the R77H αM variant.

8 Function Modulating Molecules Targeting αMβ2

In vivo studies demonstrate the importance of αMβ2 as protective against infection (Kadioglu et al. 2011) and as an aggravating factor in diseases with a poorly regulated inflammatory response, as observed in animal models of multiple sclerosis and Alzheimer's disease (Hong et al. 2016). For multiple sclerosis, there is evidence from the pharmacological mode of action of drugs and animal models that αMβ2 may play also in this case an aggravating role, at least in the relapsing-remitting form of the disease (Stapulionis et al. 2008; Jalilian et al. 2012). When it comes to stroke, blocking of αMβ2 using the hook worm-derived neutrophil inhibitory factor improved the outcome in animal models (Krams et al. 2003).

The examples of αMβ2 linked diseases and the coupling with SLE discussed above demonstrate that pharmacological regulation of αMβ2 activity could be of major interest. The complications with respect to therapeutic modulation of the receptor and the repertoire of natural and man-made molecules targeting $\alpha_M\beta_2$ has recently been extensively reviewed (Lamers et al. 2021). Inhibition of αMβ2 function is expected to lead to an increased susceptibility to infections due to decreased phagocytosis. However, interfering with αMβ2 function also enhances the risk of autoimmune disease due to the immunoregulatory roles of the receptor (Rosetti and Mayadas 2016). As one prominent example, αMβ2 plays a key role in clearance of iC3b opsonized apoptotic cells (Rosetti and Mayadas 2016).

The hCD11bNb1 nanobody used to determine the structure of the αMβ2HP binds to the ligand binding αI domain of the αM subunit (Fig. 1b) with a low nanomolar dissociation constant, and in biophysical experiments, the nanobody acts as a competitive inhibitor (Jensen et al. 2022). Comparison with the structure of the αI-C3d complex (Fig. 1e) and biophysical experiments suggested that the nanobody competes through steric hindrance exerted on the thioester domain of iC3b attempting to bind the αM subunit. However, in cellular assays, the nanobody stimulated the interaction of cell-bound αMβ2 with both free and surface-immobilized iC3b suggesting that it represents a novel proteinaceous αMβ2 specific agonist. To explain this discrepancy, we proposed that the hCD11bNb1 favors the extended conformation of αMβ2 due to predicted proximity of the nanobody to the cell membrane in the closed bent conformation (Jensen et al. 2022). In addition, the opposing effects of the hCD11bNb1 nanobody in biophysical and cell-based assays mirrors that the iC3b-αMβ2 complex is more dynamic than what is predicted from the crystal structure of αI-C3d complex (Bajic et al. 2013). This example nicely illustrates the complexity in the development αMβ2-modulating molecules. This is further complicated if modulating molecule binds close to the

αI MIDAS since in that case it may interfere to varying degrees with a large number of structurally very different αMβ2 ligands (Lamers et al. 2021).

9 The Small Molecule αMβ2 Agonist Leukadherin

The most advanced candidate for modulation of αMβ2 activity is the agonist Leukadherin-1 (LA1), small molecule that stimulates leukocyte αMβ2 interaction with ICAM-1- and iC3b- presenting cells (Maiguel et al. 2011). Mechanistically, LA1 suppresses leukocyte infiltration into tissues by increasing αMβ2-dependent cell adhesion to ICAM-1 on the endothelium, preventing subsequent extravasation (Panni et al. 2019; Schmid et al. 2018). Modeling suggests that LA1 and similar compounds bind at the interface between the αM and the β2 subunit (Faridi et al. 2009; Bjorklund et al. 2006), but no detailed structural information is available, and we are not aware of a biophysical characterization that reports on affinity and stoichiometry of the LA1-αMβ2 interaction. It should also be noted that LA1 and earlier similar compounds (Faridi et al. 2009; Bjorklund et al. 2006) contain a rhodamine group as many other pan-assay interference compounds (PAINS) that do not depend on specific interaction with the target. The rhodamine group can undergo light-induced reactions that lead to irreversible modification of the target protein (Baell and Walters 2014; Baell and Holloway 2010). Such compounds often elicit false positive signals in functional assays involving different target proteins. For this reason, experimental data reported on the effects of LA1 or similar compounds on the function of cell-bound αMβ2 must be evaluated carefully.

Despite these concerns, LA1 stands out as a small molecule with the potential for treatment of human SLE but has also shown very promising results in animal models of solid tumors (Panni et al. 2019; Schmid et al. 2018). The LA1 agonist was in clinical phase 1/2 trials for cancer treatment and preliminary data did not suggest adverse effects (DeNardo et al. 2021). In relation to SLE, ex vivo experiments with cells and animal studies suggested that partial activation of αMβ2 with LA1 offers a novel therapeutic strategy for the treatment of SLE (Faridi et al. 2017). LA1 reduced IFN-γ signaling and protected animals in a murine SLE model from end-organ injury and vascular dysfunction independent of the presence of the R77H mutation in agreement with another study using ex-vivo studies with NK cells presenting αMβ2 from R77H homozygous donors (Roberts et al. 2016).

10 Discussion and Conclusions

10.1 Fundamental Principles αM Ligand Recognition

The αI domain of αM binds diverse ligands to a common binding surface in line with the previously proposed mosaic model of αI domain MIDAS site ligand binding

(Ustinov and Plow 2005). Common principles for the αI domain interaction with ligands characterized structurally so far is that the critical Mg^{2+} ion coordinating acidic residue is placed at the end of a secondary structural element for endogenous ligands GPIbα, ICAM1,3 (Shimaoka et al. 2003), iC3b and C3d (Bajic et al. 2013; Fernandez et al. 2022) and exogenous bacterial ligand leukocidin (Trstenjak et al. 2020) (Fig. 2c). This can be either an α-helix (GPIbα, iC3b/C3d) or a β-strand (ICAMs) and being placed at the end of the secondary structural element is likely important for the acidic sidechain carboxylate to reach down and coordinate to the Mg^{2+} ion. The principle of the presence of the integrin-binding acidic residue at the end of an α-helix in GPIbαN is reminiscent of the crystal structure of the αI:C3d complex, whereby the integrin MIDAS metal ion binding residue Asp1247 of iC3b/C3d is also placed at the end of an α-helix (Bajic et al. 2013). A comparison of the αI:C3d and αI:GPIbαN complexes reveals the MIDAS-co-ordinating α-helix is oriented differently (90-degree rotation) relative to the αI surface. In addition, unlike GP1bαN, iC3b/C3d does not utilize the negatively charged patch (E244) on the surface of the αI for binding, but in common with GPIbαN it does utilize αI residue Arg208 (Bajic et al. 2013). In all cases, a folded domain is required to generate the necessary 3D shape to provide the correct spacial placement of the acidic residue with other coordinating side chains in order to achieve binding.

The αI:GPIbαN complex is the first detailed description of the interface formed between an integrin receptor and an LRR protein revealing an elongated pocket in the GPIbαN LRR C-terminal capping region is key to the interaction. The αM integrin is not the only integrin to engage multiple ligands and interestingly the more distantly related integrin α2β1 binds to collagen (Emsley et al. 2000) as the principal ligand and also has a well characterized additional interaction with the LRR C-terminal capping region of the matrix protein chondroadherin (Haglund et al. 2011). The binding site for the α2β1 integrin is localized to the chondroadherin sequence of residues LRRWLEAK that resembles the FRRWLQDN sequence from GPIbα (Haglund et al. 2011). Chondroadherin crystal structures reveal the LRRWLEAK sequence has an acidic residue which is surface exposed at the end of the α-helix of the LRR capping region in a similar manner to GPIbαN and it has been speculated that the groove adjacent to the α-helix could be utilized by the integrin (Paracuellos et al. 2017).

The αI MIDAS face interaction with an acidic residue from an α-helix of the ligand contrasts to the closely related integrin αLβ2 (leukocyte function-associated antigen-1) which is more selective and does not bind GPIbα or iC3b (Shimaoka et al. 2003). Thus, the flatter surface of the αI from αM may be able to accept more heterogenous ligand shapes compared to the αI domains from the integrin subunits αL and α2. A recently determined structure for αI from αM is the complex with the bacterial protein leukocidin which reveals an extensive interface formed by a β-sheet and a C-terminal peptide (Trstenjak et al. 2020). Nevertheless, leukocidin still has elements of molecular recognition around the αI MIDAS face that are similar to the other αM ligands. The distance of 7 Å between the acidic residues coordinating the Mg^{2+} ion and a basic residue coordinating Glu244 is present in leukocidin and ICAM1,3, GPIbα (orange arrow in Fig. 2c). In addition, similar to the modeled αI:

GPIbαN complex the leukocidin C-terminal peptide also forms a hydrophobic contact with Phe246 and the carboxy terminus contacts Arg208 which is also seen observed for GPIbα.

10.2 Structures of the αMβ2 Heterodimer

Our recent structure of the closed conformation of αMβ2 is the first step toward establishing the mechanism of outside-in signaling in this receptor. But detailed structures of multiple αMβ2-ligand complexes with the ectodomain or the headpiece fragment are required to establish the relationship between high-affinity ligand binding, the conformational freedom of the αI domain and the structural events underlying outside-in signaling in αMβ2. To avoid crystal-packing effects on the αI location and the conformation of αMβ2, single particle analysis by cryo-EM is likely to be the best approach for establishing the detailed molecular mechanism of outside-in and inside-out signaling of αMβ2.

In our structure of the αMβ2, the MIDAS sites and α7 helixes of both the αI and the βI domains are those expected for a closed bent conformation of the αMβ2 HP. However, two crystal structures of bent αXβ2 in which the internal ligand interacts with the βI MIDAS site exhibit variation in the orientation of the αI domain adopting the open conformation (Sen et al. 2013). The internal ligand region in these two αXβ2 structures is highly extended and overall these structures indicate that the distance between the MIDAS sites in the αI and βI domains as well as the orientation of the αI domain relative to the βI domain and the αM β-propeller is not necessarily fixed (Sen et al. 2013). One striking example supporting this notion is the complex between αXβ2 and its iC3b ligand, where negative-stain 2D classes revealed two opposite orientations of the ligand compared to the platform. This implies that two ligand-bound orientations of the αI domain differing by up to 180° were present in the sample (Chen et al. 2012). However, studies of αMβ2 by negative stain EM featured a more defined orientation of the αI domain relative to the platform (Adair et al. 2013). Our own 3D reconstructions of the unbound αMβ2 headpiece also offered evidence that the αI domain is at least somewhat restricted with respect to rotation relative to the platform (Jensen et al. 2021).

A particular challenge that needs to be addressed to advance our understanding of the αMβ2 structure-function relationships and the interplay with ligands is a detailed structural characterization of the large number of αMβ2 complexes where the affinity is weaker than the for the αMβ2-iC3b and αMβ2-C3d complexes. The model of the low-affinity complex between the αI domain and GPIbαN benefitted from an integrative approach where multiple sources of structural information were combined to provide a detailed model of the core interaction (Morgan et al. 2019). Such an integrative strategy may be applied for other low-affinity ligand-αMβ2 complexes unless the ligand affinity can be increased by tethering or affinity maturation.

In particular, a structural model of the complex between αMβ2 and the ligand ICAM-1 is required to understand the function of αMβ2 with respect to leukocyte

recruitment and to comprehend in detail the effects of αMβ2 modulating molecules like LA1 on the αMβ2 interaction with ICAM-1. Two acidic residues in the third Ig domain of ICAM-1 have been suggested to act as the MIDAS Mg^{2+} coordinating residue and interestingly, deletion of an Asn-linked glycan was observed to enhance the αMβ2 interaction with ICAM-1 significantly (Diamond et al. 1991). The affinity between the recombinant αI domain and soluble ICAM-1 was found to be low with a dissociation constant of 6.5 μM although it could be improved to 0.6 μM through stabilization of the open αI conformation with a disulfide bridge (McCleverty and Liddington 2003). This low affinity apparently represents a significant obstacle for structure determination of the complex whereas multiple structures are known of the equivalent complexes formed by αI domain from αL with ICAM-1/3/5. Other enduring mysteries of αM ligand binding are how it recognizes fibrinogen and the best-characterized inhibitor NIF from the canine hookworm *Ancylostoma caninum* (Ustinov and Plow 2002).

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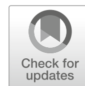
Part IV
Integrins in Hemostasis and Immune Control

Platelet Integrins: Critical Mediators of Haemostasis and Pathological Thrombus Formation



Timo Vögtle and Bernhard Nieswandt

Abstract Platelets are small anucleated blood cells that survey the integrity of the blood vasculature, ready to rapidly seal injuries in order to limit blood loss. Integrins on the platelet surface are essential mediators of this haemostatic function: their ability to rapidly shift from a low- to a high-affinity conformation to bind their ligands allows the platelet to quickly adapt an adhesive state, facilitating stable adhesion to the extracellular matrix and platelet aggregation under highly dynamic blood flow conditions. Furthermore, integrins act as bidirectional signalling machines that regulate cytoskeletal rearrangements and cellular effector functions. While integrin function is critical for haemostasis, it is also a prerequisite for pathological thrombus formation, resulting in vessel occlusion, ischaemia, and infarction of vital organs. Over the last decades, many investigators have provided fundamental insights into the cellular and molecular basis of platelet integrin function and regulation, particularly of the major integrin, αIIbβ3, advancing not only our understanding of platelet function but also of integrins in general. In this chapter, we will give an overview on the role of platelet integrins in normal platelet physiology and disease. We will further describe the inside-out signalling pathways that control integrin activity—with a special focus on talin and kindlin-3—as well as the outside-in signalling pathways initiated by the ligand-occupied integrins themselves.

1 Introduction

Platelets are small anucleated blood cells which originate from the cytoplasm of giant precursor cells, the *megakaryocytes* (MKs) in the bone marrow. The platelet count in the blood of healthy humans ranges from 150,000 to 450,000 per μL and averages at about 250,000 platelets/μL, while mice—representing the favoured

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animal for platelet research—exhibit a platelet count of approximately 1,000,000 per μL. The lifespan of platelets is about 10 days in humans and about 5 days in mice. Due to the lack of a nucleus, the de novo protein synthesis of platelets is limited. They harbour two major types of granules—α-granules and dense granules—which are loaded with various bioactive compounds and cell adhesion molecules that allow them to quickly react to cues in the environment.

Platelets are essential for haemostasis, as they survey the vascular integrity while circulating in the blood stream. In an intact vasculature, most platelets never undergo firm adhesion to the vessel wall and are cleared by the reticuloendothelial system in the spleen and liver at the end of their life time. However, upon disruption of the endothelial cell layer, platelets rapidly adhere to the exposed components of the subendothelial *extracellular matrix* (ECM). This triggers cellular activation and the release of secondary platelet agonists such as *adenosine diphosphate* (ADP) and *thromboxane A2* (TxA_2) and thereby initiates a self-amplifying loop that results in the recruitment of additional platelets that interact with each other to form a plug that seals the wound and limits blood loss. Under pathological conditions, e.g. upon rupture of an atherosclerotic plaque, thrombus formation may result in occlusion of the vessel and obstruction of blood flow and loss of oxygen supply, leading to irreversible ischaemic damage of vital organs (Jackson 2011). Among these complications, *myocardial infarction* (MI), triggered by occlusion of coronary arteries, and stroke, elicited by thrombus embolization to the brain, are two of the leading causes of disability and mortality worldwide (Lopez et al. 2006). Furthermore, platelets have emerged as critical regulators in processes beyond thrombosis and haemostasis, such as wound healing (Etulain 2018), maintenance of vascular barrier function (Gupta et al. 2020; Ho-Tin-Noe et al. 2018), tumour metastasis (Lavergne et al. 2017), and infection (Guo and Rondina 2019). Importantly, platelets also act as critical mediators that orchestrate haemostatic, inflammatory, and immune responses, in a process referred to as thrombo-inflammation (Stoll and Nieswandt 2019).

Integrins are essential for the platelet's haemostatic function, since their ability to rapidly shift from a low- to a high-affinity conformation for their ligands allows the cells to quickly acquire an adhesive state, thereby facilitating stable adhesion to the ECM and the formation of a three-dimensional thrombus under highly dynamic blood flow conditions. Platelets express three β1 integrins, α2β1, α5β1, and α6β1, which mediate binding to collagen, fibronectin, and laminin, respectively, and two β3 integrins, αvβ3 (binds to vitronectin) and αIIbβ3 (also referred to as *glycoprotein* (GP)IIb/IIIa), which binds to multiple ligands, most notably fibrinogen, fibrin, and vWF (Varga-Szabo et al. 2008).

Platelets, and especially integrin αIIbβ3, have been proved as an excellent model system to study integrin function in general, since the integrin-mediated increase in platelet adhesiveness and aggregation is a rapid process and can easily be measured and quantified, e.g., by light transmission aggregometry (Fig. 1a). Furthermore, conformation specific-antibodies have been developed for human and mouse αIIbβ3 (Bergmeier et al. 2002; Shattil et al. 1985), which allow direct and quick

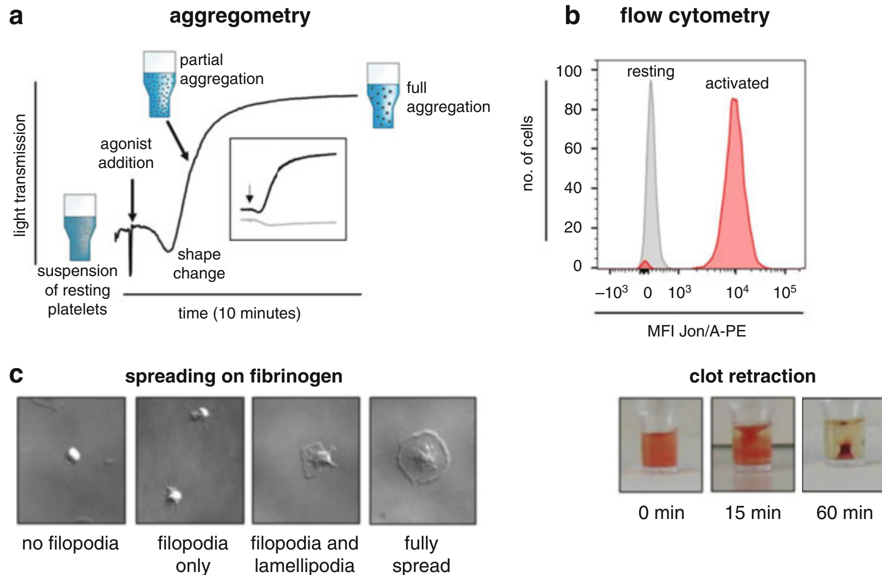

Fig. 1 Assays for assessing integrin function in platelets. (**a**, **b**) Inside-out activation of integrin αIIbβ3. (**a**) In aggregometry measurements, light transmission through a platelet suspension is recorded over time. While a suspension of single resting platelets scatters light, integrin αIIbβ3-dependent platelet aggregation upon agonist stimulation results in formation of large aggregates and increased light transmission. The initial decrease in light transmission is a consequence of preceding cytoskeletal rearrangements, resulting in a change from biconcave discs to spiculated spheres. The two boxed traces show the aggregation response of untreated (black) and abciximab-treated (αIIbβ3 inhibitor—grey) platelets towards the GPVI agonist collagen related peptide. (**b**) Detection of the active integrin αIIbβ3 with a conformation-specific antibody (JON/A-PE) in resting and activated mouse platelets by flow cytometry. (**c**, **d**) Assessment of integrin αIIbβ3 outside-in signalling. (**c**) Platelets are seeded on a fibrinogen-coated surface. Upon binding, integrin αIIbβ3 triggers actin cytoskeleton arrangements, mediating filopodia and lamellipodia formation, finally resulting in full spreading (Published with permission from Stefano Navarro). (**d**) Clot formation is induced by adding high doses of thrombin to platelet rich plasma (supplemented with erythrocytes for better visualization) and retraction of the clot is recorded over time

assessment of the integrin activation status by flow cytometry (Fig. 1b). Hence, several breakthrough findings in integrin biology were actually made in platelets.

In this book chapter, we will introduce all five different platelet integrins and discuss their role in platelet physiology and disease. Furthermore, we will describe the molecular mechanisms that control integrin function (inside-out signalling) as well as the signalling pathways initiated by the integrin themselves (outside-in signalling). Finally, we will discuss therapeutic targeting of platelet integrins and their role in disease.

2 Platelets in Thrombosis and Haemostasis

Platelet adhesion and aggregation on the exposed ECM at sites of vascular injury requires the coordinated interaction of platelet adhesion receptors, including integrins, with adhesive macromolecules and the amplification of activation by soluble mediators. This process is termed primary haemostasis and is classically divided into three major steps (Fig. 2): (i) tethering and adhesion of platelets, (ii) platelet activation, and finally (iii) platelet aggregation and thrombus growth (Stegner and Nieswandt 2011).

In the first step, platelets are tethered to ECM components, like collagen, laminin, or fibronectin, and 'roll' along the site of injury in a stop-and-go manner. Under high shear conditions, such as those prevailing in arterioles or stenosed arteries, this initial adhesion is mediated by the interaction of platelet receptor complex GPIb/V/IX with *von Willebrand factor* (vWF), which becomes immobilized on collagen (Savage et al. 1996). This interaction induces the rapid deceleration of the platelets, thereby enabling the interaction of collagen I with the immunoglobulin-like receptor GPVI, the major activating platelet collagen receptor (Nieswandt et al. 2001; Nieswandt and Watson 2003). Through an *immunoreceptor tyrosine-based activation motif* (ITAM) in the associated *Fc receptor* (FcR) γ-chain, GPVI induces intracellular signalling cascades. These lead to cellular activation and the release of second wave mediators, most notably ADP, released from dense granules, and TxA_2, synthesized by cyclooxygenase-1. Additionally, exposed *tissue factor* (TF) triggers local thrombin generation, which is further promoted by exposure of negatively charged phosphatidylserine on the surface of highly activated platelets, providing a platform for coagulation factor complexes (Heemskerk et al. 2013). ADP, thrombin, and TxA_2 further reinforce and sustain cellular activation by initiating different pathways via *G protein-coupled receptors* (GPCRs) (Offermanns 2006) and recruit additional platelets from the blood stream into the growing thrombus.

All these signalling pathways finally converge in the 'final common pathway' of platelet activation: the functional upregulation of the integrin adhesion receptors, which shift from a low- to a high-affinity state, allowing firm ligand binding. The most important integrin in this process is the highly abundant integrin αIIbβ3, which binds to multiple ligands, most notably fibrinogen, fibrin, and collagen-bound vWF, thereby mediating shear-resistant adhesion to the ECM (Varga-Szabo et al. 2008). Firm adhesion is supported by other integrins by binding to collagen (α2β1), fibronectin (α5β1), and laminin (α6β1). Importantly, binding of αIIbβ3 to fibrinogen also facilitates the bridging of neighbouring platelets that are not in direct contact with the ECM (Fig. 2). This *aggregation* of platelets is a prerequisite for the formation of a stable three-dimensional thrombi and hence for sealing the injury site and cessation of bleeding. On the downside, this mechanism is also a critical pathomechanism driving the formation of vessel occluding thrombi in ischaemic disease settings. Ligand-occupied integrins in turn transduce 'outside-in' signals, which promote platelet cytoskeletal rearrangements, thrombus stabilization, and clot

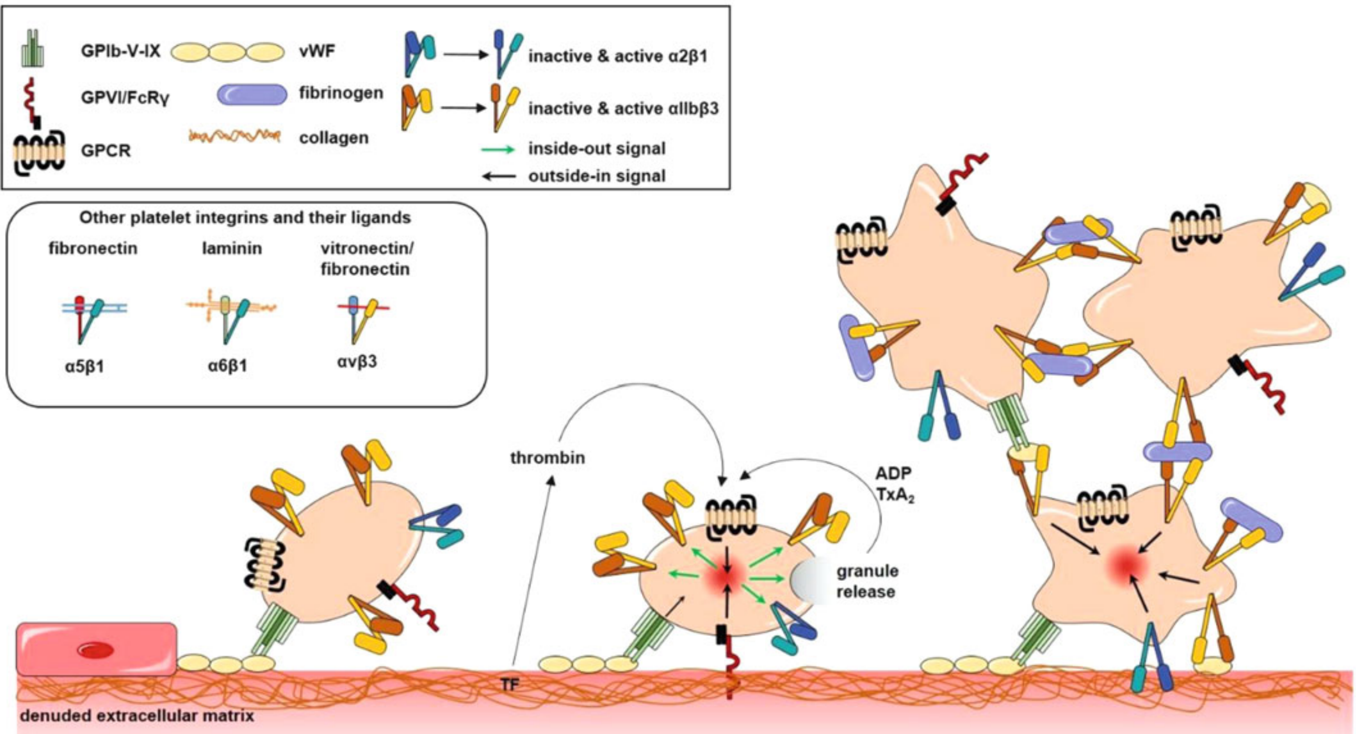

Fig. 2 Platelet adhesion and aggregation on the exposed extracellular matrix after disruption of the endothelial cell layer. The GPIbα-vWF interaction mediates platelet tethering and deceleration thereby enabling GPVI interaction with collagen. This triggers 'inside-out' signalling and the shift of integrins to a high-affinity state and the release of secondary mediators, notably ADP and TXA_2. In parallel, subendothelial *tissue factor* (TF) triggers local thrombin formation which also contributes to platelet activation. Interaction of integrins with extracellular ligands mediates thrombus formation and triggers 'outside-in' signalling events

retraction (shrinking and consolidation of a blood clot) (see below) (Durrant et al. 2017; Huang et al. 2019).

Platelet aggregation and their ability to form a thrombus can be readily investigated in vivo and ex vivo, e.g., by using aggregometers (Fig. 1a) or flow chamber assays, where blood is perfused over a thrombogenic surface (e.g., collagen) and thrombus formation can be observed under different rheological conditions. Because of the highly dynamic nature of the blood flow and the complex interactions between platelets, plasma factors, and endothelial cells, a detailed analysis of haemostasis and thrombosis also requires in vivo experiments in animals. In platelet research, the mouse has been established as the model of choice, since major signalling and effector functions are comparable to humans and because of its suitability for genetic modification. A typical assay for measuring haemostasis is the tail bleeding assay, where a small portion (typically 2–3 mm) of the tail tip is cut off and the time to cessation of bleeding is measured. Depending on the type of injury, bleeding in healthy mice typically stops after a few minutes, while bleeding for more than 20 minutes is usually regarded as a severe haemostatic defect and experiments are stopped for animal welfare reasons. Multiple in vivo thrombosis models have been developed: usually a vessel is surgically exposed and then injured, e.g., by mechanical force (forceps compression, needle injury), chemicals (e.g., $FeCl_3$), or by a laser and thrombus build-up is followed microscopically or with an ultrasonic flow probe. Noteworthy, these assays are pretty insensitive to variations in platelet numbers, tolerating up to 80% decrease (Morowski et al. 2013), and therefore well suited to reveal alterations in platelet (integrin) function.

3 The Physiological Function of Platelet Integrins

There are three β1-integrins present on the platelet membrane, α2β1, α5β1, and α6β1, as well as two β3 integrins, αIIbβ3 and αvβ3, the latter only expressed at very low numbers. Among those integrins, integrin αIIbβ3 stands out due to its high expression and its essential function for platelet aggregation, which also makes it a valuable therapeutic target. In resting platelets, all integrins are expressed in an inactive, low-affinity state and, following platelet activation, shift into a high-affinity state that allows binding of their respective ligand. This process is referred to as 'outside-in signalling' and will be discussed below. In this section, we will first introduce each of the platelet integrins and describe its specific role in platelet physiology.

3.1 Integrin αIIbβ3: The Central Integrin for Stable Thrombus Formation

αIIbβ3 is the most abundant receptor on the platelet surface, with about 60,000–80,000 copies per cell (Wagner et al. 1996; Burkhart et al. 2012) and an additional intracellular pool located in α granules, which becomes exposed on the surface upon activation (Woods et al. 1986). Integrin αIIbβ3 is specifically expressed on platelets, but was found also on some tumour cells (Lavergne et al. 2017). It binds to several adhesive proteins, each containing an *arginine–glycine–aspartic acid* (RGD) sequence, such as fibrinogen, fibrin, vWF, fibronectin, and thrombospondin (Gartner and Bennett 1985; Plow et al. 1985; Pytela et al. 1986). Of those interactions, the binding of activated αIIbβ3 to fibrinogen is of pivotal importance, since fibrinogen's dimeric nature enables the bridging of adjacent platelets, thereby allowing the formation of a stable thrombus—a critical step for haemostasis, but also a major pathomechanism underlying arterial thrombosis (Jackson 2011; Varga-Szabo et al. 2008). In addition, integrin αIIbβ3 is of major importance for platelet adhesion on the ECM, which is mediated by binding of collagen-immobilized vWF, fibrinogen as well as fibronectin and vitronectin (Fig. 2) (Varga-Szabo et al. 2008). αIIbβ3 also mediates the endocytosis of fibrinogen for storage in MK and platelet α-granules (Handagama et al. 1993).

The structure of αIIbβ3 has been extensively studied, and crystal structures—in the presence or absence of bound ligands—as well as cryo-electron microscopy images have been reported. A detailed summary of these findings is beyond of the scope of this chapter. Hence, readers are referred to Chaps. 'Roles for Integrin α3β1 in Development and Disease' and 'Integrins: Key Targets in Tissue Fibrosis and Tumor Stroma', which give a general overview of integrin structural features and to reviews on αIIbβ3 structure and its conformational changes upon activation (Shattil et al. 2010; Coller 2015; Bledzka et al. 2019). Thus, we will only give a brief overview on the key structural elements that determine αIIbβ3 function.

Like all integrins, αIIbβ3 is a heterodimer, composed of the αIIb and the β3 subunits that are non-covalently associated and comprise 1008 and 762 *amino acids* (aa), respectively. Each subunit contains a large extracellular domain, a transmembrane domain, and a short intracellular tail at the C-terminus (Fig. 3). The N-terminus of αIIb contains the seven-bladed β-propeller domain, which is followed by the thigh, calf-1 and calf-2 domain that constitute the remaining part of the α subunit's extracellular domain. The N-terminal portion of the β subunit is the βA-domain and contains three metal binding sites that play an important role in ligand binding: the *metal ion-dependent binding adhesion site* (MIDAS, binding Mg^{2+}) as well as the *synergistic metal ion-binding site* (SyMBS) and *adjacent to MIDAS* (ADMIDAS) that both bind Ca^{2+} (Coller 2015). The βA-domain is followed by a hybrid and PSI domain, which connect to four EGF domains that form a rod-shaped structure, and a membrane-proximal β tail-domain (Fig. 3) (Bledzka et al. 2019; Lau et al. 2009; Xiong et al. 2001).

The main contact area between the α and β subunits involves the β-propeller within the α subunit and the βA-domain of the β subunit, which form the globular 'head' of the integrin and provide the ligand binding site (Fig. 3) (Xiao et al. 2004). The residual extracellular domains form 'stalks'. Crystal structures suggest that the main binding site of the RGD sequences of the ligands lies between the seven-bladed propeller of the αIIb subunit and the βA-domain of the β3 subunit (Xiao et al. 2004). In resting platelets, however, the integrin is in a low-affinity or inactive state, which is characterized by a bent conformation that keeps the binding site for the RGD sequence hidden. Upon agonist-induced platelet activation, however, 'inside-out' signals (see below) trigger a conformational shift of the integrin towards a high affinity or active conformation, where the RGD binding site becomes unmasked (Shattil et al. 2010; Lau et al. 2009). This process is called switchblade-like opening and can be experimentally provoked by manganese ions (Smith et al. 1994), however, within the cells this process is triggered by the binding of intracellular adaptor molecules—most prominently kindlin-3 and talin-1—to cytoplasmic tails of the subunits (Shattil et al. 2010).

The cytoplasmic tails of the αIIb and β3 subunits have no enzymatic or actin-binding activity themselves, but provide a hub for protein complex assembly. A plethora of molecules that interact with the β3 subunit has been described and mediates integrin bidirectional signalling, (see below) (Durrant et al. 2017; Huang et al. 2019). The tail of the α subunit is composed of 20 aa containing an α-helix and interacts with filamin, which stabilizes the inactive conformation of the integrin. The β3 tail consists of 47 aa and contains two NxxY (where x denotes any aa) motifs—a membrane-proximal NPLY (residues 744–747) and a membrane-distal NITY (residues 756–759), which bind to talin-1 and kindlin-3, respectively (Bledzka et al. 2012), and become phosphorylated by *Src family kinases* (SFK) to induce outside-in signalling (Fig. 4b) (Law et al. 1996; Jenkins et al. 1998). The cytoplasmic tails of the two subunits interact with each other through their membrane-proximal regions. A widely accepted model suggests that these hydrophobic and electrostatic interactions keep the integrin in its inactive conformation. Indeed, mutagenesis of the residues predicted to build an inhibitory salt bridge (R995 in αIIb and D723in β3) results in permanent activation of αIIbβ3 (Hughes et al. 1996). Furthermore, there is evidence that during inside-out signalling this inhibitory hinge is disrupted by talin-1 (Anthis et al. 2009a), thereby 'unclasping' the cytoplasmic tails to allow integrin activation (Bledzka et al. 2019). The transmembrane domains have a key role in enabling the conformational 'inside-out' switch (Shattil et al. 2010).

Lack or dysfunction of αIIbβ3 gives rise to *Glanzmann-thrombasthenia* (GT), a bleeding disorder associated with impaired adhesion and abolished aggregation of platelets. Additional platelet defects are impaired clot retraction and reduced or absent fibrinogen uptake. Platelet count and size are normal. Disease causing mutations are found across both *ITGA2B* (encoding αIIb) and *ITGB3* (encoding β3) and include missense mutations, stop codons, small deletions, inserts or duplications and splice defects, often with frame-shifts. A clinical hallmark of GT are mucocutaneous haemorrhages which manifest to different extent, with some patients having only minimal bruising while others have frequent, severe, and potentially

fatal haemorrhages (Glanzmann 1918; Botero et al. 2020; Nurden et al. 2011). Mice deficient in β3 integrin show all the cardinal features of GT—abolished platelet aggregation, impaired clot retraction, greatly reduced fibrinogen uptake, and cutaneous and gastro-intestinal bleeding. These mice also display infinite tail bleeding times and spontaneous haemorrhage in all developmental stages (Hodivala-Dilke et al. 1999; Li et al. 2021). Of note, a recent publication observed additional alterations in β3-deficient mice, which are not observed in GT, such as decreased platelet count, splenomegaly, and extramedullary haematopoiesis, hence putting a note of caution on transferring observations from this mouse model directly to the human situation (Li et al. 2021).

Upon ligand binding, the cytoplasmic tails of αIIb and β3 establish further functional connections with a number of signalling molecules to trigger 'outside-in' signalling events that induce cytoskeletal rearrangements, which are discussed below. Due to the central role of αIIbβ3 for platelet aggregation, it has become a pharmacological target for the prevention of platelet-dependent thrombosis, e.g., by inhibiting its function with a Fab-fragment of an inhibitory antibody (see below). On the other hand, αIIbβ3 is also a major target of autoreactive antibodies in *immune thrombocytopenic purpura* (ITP), an autoimmune disease in which auto-antibodies are directed against the individual's own platelets, resulting in increased platelet destruction and frequently severe bleeding (Singh et al. 2021). In a mouse model, bolus injections of antibodies directed against αIIbβ3 induced Fc-dependent platelet clearance, which was unexpectedly associated with acute systemic reactions, hypothermia and frequently also massive subcutaneous and internal haemorrhages. In contrast, antibodies directed against the other most prominent ITP target—GPIb—induced Fc-independent platelet depletion and this was not associated with major bleeding episodes or acute systemic reactions, indicating that antibodies against αIIbβ3 are more pathogenic than auto-antibodies against other platelet receptors (Nieswandt et al. 2000).

3.2 Integrin αvβ3: The Vitronectin Receptor

Integrin αvβ3 is highly expressed in osteoclasts, and migrating smooth muscle cells (Wilder 2002); the expression in platelets, however is very low, with about 50–100 copies per cell (Coller et al. 1991). It was the first integrin to be crystallized (Xiong et al. 2001), but despite structural similarities with αIIbβ3, vitronectin but not fibrinogen is the preferred ligand for αvβ3, which also binds several other RGD-containing ligands, including osteopontin, fibronectin, thrombospondin, proteolysed collagen, vWF, and others (Wilder 2002). Studies with inhibitors suggested that αvβ3 to a minor extent supports platelet adhesion on fibronectin, vitronectin, and osteopontin (McCarty et al. 2004; Paul et al. 2003). A role in thrombosis and haemostasis has not been reported.

3.3 Integrin α2β1: A Collagen Receptor

Integrin α2β1 is a collagen receptor and expressed in a wide range of tissues—its function in different cell types and structure is extensively discussed in chapter "Integrins α1β1 and α2β1: The Generalist 1 Collagen Receptors", so here we will only focus on its function in platelets.

For a long time α2β1was considered as the central collagen receptor in platelets mediating shear-resistant adhesion to collagen and platelet activation at the exposed ECM upon vascular injury. This assumption was largely based on studies on two patients whose platelets exhibited reduced levels of α2β1 and showed severely impaired aggregation responses towards collagen resulting in mild bleeding disorders to more severe post-traumatic bleeding and excessive menorrhagia (Kehrel et al. 1988; Nieuwenhuis et al. 1985). However, at least in one case additional genetic defects were observed, hence questioning whether the bleeding complications were indeed a consequence of the loss of α2β1 (Kehrel et al. 1988). Clarification came from studies in mice with constitutive knockout of α2 ($Itga2^{-/-}$) or conditional knockout of β1 in blood cells ($Itgb1^{fl/fl\ Mx\text{-}Cre}$). Both resulted in abolished α2β1 expression in platelets but only moderately impaired activation responses of the cells to collagen, resulting in a minor effect on haemostasis of the animals (Nieswandt et al. 2001; Chen et al. 2002; Holtkotter et al. 2002). In experimental thrombosis models, platelet adhesion and initial thrombus growth were found to be unaltered in α2-deficient mice, however, the formed thrombi were smaller with more embolization compared to wild-type mice, suggesting a role for integrin α2β1 in thrombus stabilization (Gruner et al. 2003; He et al. 2003; Kuijpers et al. 2003). Overall, it is now firmly established that the central collagen receptor in platelets is GPVI, and that α2β1 has a supportive rather than an essential role in the adhesion process (Nieswandt et al. 2001; Holtkotter et al. 2002), as other receptors, most notably αIIbβ3, also mediate shear-resistant adhesion to the ECM (Varga-Szabo et al. 2008; Nieswandt and Watson 2003; Gruner et al. 2003).

Like αIIbβ3, also integrin α2β1 requires inside-out activation to undergo a conformational change to bind its ligand with high affinity (Jung and Moroi 2000), which can, e.g., be induced through ligation of GPVI (see below) (Nieswandt et al. 2001). On the other hand, ligand binding of α2β1 contributes to cellular activation of GPVI and reinforces GPVI-collagen interactions by 'outside-in' signalling (Chen and Kahn 2003; Inoue et al. 2003). Therefore, the two major collagen receptors act in a cooperative manner to reinforce each other's activity.

3.4 Integrins α5β1: The Fibronectin Receptor

α5β1 is broadly expressed in various cell types and referred to as the platelet's fibronectin receptor, even though also αvβ3 and particularly αIIbβ3 integrins also bind to fibronectin. Experiments using blockers of β3 integrins or genetic deletion of

αIIbβ3 demonstrated that α5β1 supports the adhesion of platelets to immobilized fibronectin under static and flow conditions (McCarty et al. 2004; Beumer et al. 1994). Interestingly, and in contrast to αIIbβ3, α5β1-mediated platelet adhesion to fibronectin does not facilitate lamellipodia formation and cell spreading (Fig. 1c). This is likely due to the integrin's inability to induce outside-in signals (see below), as evidenced by absent tyrosine phosphorylation or Ca^{2+} signals upon platelet spreading on fibronectin in the presence of β3 integrin blockers (McCarty et al. 2004). Of note, α5β1-mediated platelet adhesion under flow becomes more significant when platelets are perfused over cellular fibronectin in its fibrillar form, as found in the vessel wall, in comparison to soluble fibronectin derived from plasma (Maurer et al. 2015).

A recent study thoroughly analysed mice with a MK- and platelet-specific deletion of α5β1 ($Itga5^{fl/fl;Pf4\text{-}Cre+}$). Platelets deficient in α5β1 showed unaltered aggregation responses in response to all tested agonists, as well as unimpaired adhesion on fibrinogen, laminin, and vWF. In contrast, these platelets displayed a marked decrease in adhesion, activation, and aggregation on fibrillar cellular fibronectin and collagen. The latter might be a species-specific effect, as it was not observed when human blood was perfused over collagen in the presence of a blocking anti-α5 antibody. Despite these ex vivo defects, no alterations in a tail bleeding time assay, nor in three different experimental models of arterial thrombosis were observed, demonstrating that α5β1 is dispensable in haemostasis and arterial thrombosis, at least in mice (Janus-Bell et al. 2021).

3.5 *Integrin α6β1: The Laminin Receptor*

Integrin α6β1 is a ubiquitous receptor for laminins, a large family of conserved multidomain trimeric basement membrane proteins that are critical structural components of the ECM (Domogatskaya et al. 2012). In platelets, several studies have demonstrated that α6β1 supports the adhesion to laminin, under both static and flow conditions (Inoue et al. 2006; Nigatu et al. 2006; Schaff et al. 2013; Sonnenberg et al. 1988). Upon binding to its ligand, α6β1 generates outside-in signals and thereby stimulates platelet spreading in conjunction with GPVI (Inoue et al. 2006), but does not induce P-selectin exposure or platelet aggregation (Nigatu et al. 2006).

First studies were mostly performed with laminin isoform-α1β1γ1 (laminin 111) or laminin preparations from human placenta, hence the physiological relevance of these findings for thrombosis and haemostasis remained unclear. Using recombinant laminin isoforms, it was demonstrated that mouse and human platelets also adhere to laminins 411, 421, and 511—the predominant laminin isoforms in the vessel wall—under static and flow conditions (Nigatu et al. 2006; Schaff et al. 2013). This adhesion was completely abolished in mice with a platelet- and MK-specific deletion of α6 ($Itga6^{fl/fl;Pf4\text{-}Cre+}$), establishing α6β1 as the dominant laminin receptor in mouse platelets. Importantly, $Itga6^{fl/fl;Pf4\text{-}Cre+}$ mice presented markedly decreased thrombus formation in three different models of arterial thrombosis, while bleeding

time and blood loss in a tail bleeding assay remained unaltered, indicating normal haemostasis. Hence, the authors proposed α6β1 as a potential anti-thrombotic target (Schaff et al. 2013).

Notably, *Itga6*$^{fl/fl;Pf4\text{-}Cre+}$ mice showed markedly decreased spreading of tumour cells in mouse models of experimental and spontaneous lung metastasis. Subsequent studies revealed that α6β1 directly binds to ADAM9 on tumour cells, thereby inducing platelet activation and favouring the extravasation process of tumour cells. Hence, α6β1 might also represent a target for anti-metastatic therapies (Mammadova-Bach et al. 2016).

4 Inside-Out Activation of Integrins

Like many other integrins, platelet integrins are not constitutively active. In resting platelets, they are expressed in an inactive, low-affinity state, in which they do not bind their ligands and do not signal. This allows platelets to circulate in the blood stream as non-adhesive cells, despite the presence of high levels of the αIIbβ3 ligand fibrinogen in the plasma. It is only after platelet activation that integrins shift to a high-affinity state, allowing ligand binding and thus firm platelet adhesion and aggregation. This transmission of an intracellular activation signal from the small cytoplasmic tail to the large extracellular domain is referred to as 'inside-out' activation or signalling. This regulatory mechanism of platelet adhesiveness must be tightly controlled, in order to prevent complications such as bleeding or thrombosis.

4.1 Upstream Signalling Pathways

As already mentioned in the introduction, there are two major signalling pathways that trigger integrin inside-out activation in platelets (Stegner and Nieswandt 2011).

One pathway is initiated by engagement of the GPVI-FcR γ-chain complex or CLEC-2 and requires the phosphorylation of critical tyrosine residues in the ITAM or hemITAM, respectively, and is similar to signalling cascades used by multiple immunoreceptors. Signal propagation is mediated by tyrosine kinases of the *spleen tyrosine kinase* (Syk) and Src families, as well as downstream adapter and effector proteins and finally culminates in activation of PLCγ2 (Fig. 3) (Stegner and Nieswandt 2011). While mouse platelets harbour only these two (hem) ITAM receptors, human platelets also express *Fc gamma receptor IIA* (FcγRIIA). This receptor triggers the ITAM-tyrosine kinase signalling cascade upon binding of immune complexes and participates in host defence but also autoimmune diseases, such as heparin-induced thrombocytopenia (Patel et al. 2021). The other major pathway is triggered by GPCRs, which are stimulated by soluble platelet agonists, such as thrombin, ADP, and TxA_2. Three major types of heterotrimeric G proteins

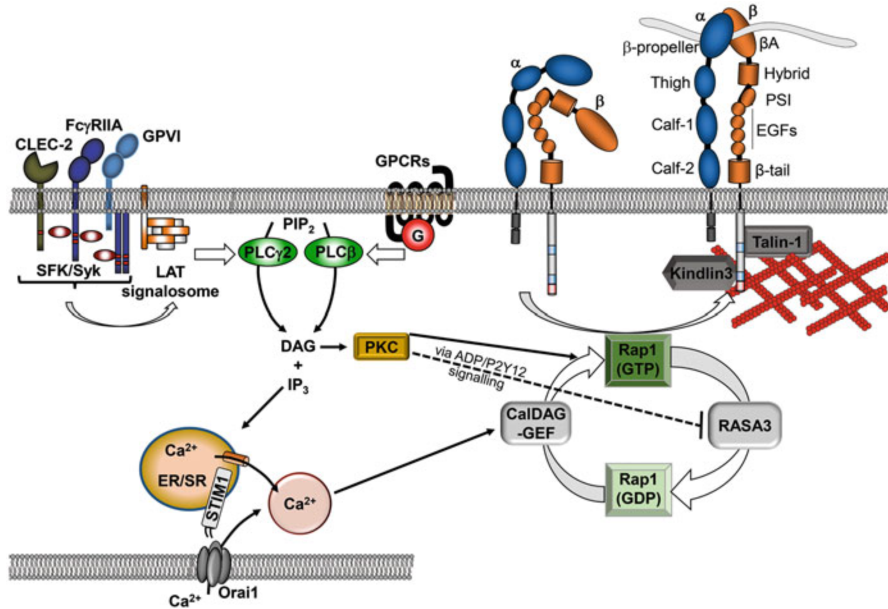

Fig. 3 Integrin inside-out activation. Agonist stimulation triggers PLC activation and the formation of IP_3 and DAG. IP_3 induces Ca^{2+} store release through IP_3 receptors in the endoplasmic/sarcoplasmic reticulum (ER/SR) membrane and subsequent STIM1/Orai1-mediated Ca^{2+} entry. DAG and Ca^{2+} activate PKC and CalDAG-GEFI, respectively, leading to activation and translocation of Rap1 to the plasma membrane. This process is counteracted by the GAP RASA3, which converts Rap1 to its inactive, GDP-bound form. Rap1 recruits talin-1, which binds to the integrin β3 cytoplasmic tail, triggering a conformational change in the extracellular domains and ligand binding. This final step requires also binding of kindlin-3 to the membrane-distal NxxY motif of the integrin β tail. Domain names refer to integrin αIIbβ3. For details see text

are found downstream of GPCRs in platelets: G_q, $G_{12/13}$, and G_i. The G proteins induce multiple signalling events like stimulation of Rho-GTPases (via $G_{12/13}$), *phosphoinositide-3-kinase* (PI3K)/Akt (via G_i) signalling, and activation of PLCβ (via G_q) (Fig. 3) (Offermanns 2006).

In GPCR as well as in (hem)ITAM signalling, the activated PLC isoforms hydrolyse the membrane phospholipid *phosphatidylinositol-4,5-biphosphate* (PIP_2) to *inositol-1,4,5-trisphosphate* (IP_3) and *diacylglycerol* (DAG). DAG activates *protein kinase C* (PKC) while IP_3 binds to IP_3-sensitive Ca^{2+} channels in the membrane of intracellular Ca^{2+} stores, thereby inducing store depletion and subsequent massive Ca^{2+} influx, termed *store-operated* Ca^{2+} *entry* (SOCE) (Fig. 3) (Braun et al. 2011; Putney 2009).

Furthermore, integrin activation in response to GPIbα engagement has been reported (Delaney et al. 2012; Du 2007). The underlying signalling mechanisms as well as its significance for haemostasis and pathological thrombus formation have not been fully elucidated.

4.2 Rap1: The Master Switch in Platelet Integrin Activation

The rise of DAG and Ca^{2+} promotes the activation of the *Ras-related GTPases* (Rap) 1A and B, two members of the larger family of Rap GTPases, that are highly abundant in platelets (Burkhart et al. 2012; Zeiler et al. 2014). They cycle between a GDP-bound 'off' state and GTP-bound 'on' state and thereby act as critical molecular switches of integrin activation and platelet function (Fig. 3) (Stefanini and Bergmeier 2019). Ca^{2+} activates *Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor I* (CalDAG-GEFI), which acts as GEF to promote the conversion of Rap1b from its inactive GDP-bound state to its active GTP-bound state (Crittenden et al. 2004; Kawasaki et al. 1998). DAG activates *protein kinase C* (PKC), which also promotes the formation of Rap1b-GTP, but more importantly, also triggers signalling processes to inhibit the activity of the *GTPase activating protein* (GAP) RASA3, which counteracts the activity of CalDAG-GEFI (Fig. 3) (Stefanini et al. 2015).

Studies in human platelets have already demonstrated that the levels of Rap1activation correlate with platelet integrin activation and platelet adhesiveness (Franke et al. 1997, 2000). The pivotal importance of the Rap1 activation circuit has been demonstrated by studies with knockout mice (Stefanini and Bergmeier 2019). Platelets deficient in the most prominently expressed Rap family member Rap1b (approx. 200,000 copies in mouse, 154,000 in human platelets (Burkhart et al. 2012; Zeiler et al. 2014)) exhibited impaired integrin activation and defective aggregation responses to all major agonists in vitro, which were apparent at low but hardly at high agonist concentrations. The slower shape change of *Rap1b*$^{-/-}$ platelets observed in these experiments point towards altered actin microfilament dynamics, indicating that Rap1b may act at the interface of integrins and cytoskeleton (see below). On the physiological level, the abnormal platelet function resulted in increased tail bleeding times and protection from arterial thrombosis following $FeCl_3$-induced vessel injury (Chrzanowska-Wodnicka et al. 2005). Despite these defects, there was still moderate integrin activation detectable, indicating that other small GTPases might partially compensate for the loss of Rap1b. An obvious candidate was Rap1a, which is also robustly expressed in platelets (approx. 20,000 and 125,000 copies in mouse and human platelets, respectively (Burkhart et al. 2012; Zeiler et al. 2014)). Indeed, this was confirmed in a study using genetically modified mice with platelet- and MK-specific deletions of Rap1a, Rap1b, or both (Stefanini et al. 2018). Already the lack of one isoform resulted in activation defects of integrin αIIbβ3 and β1 integrins, which were more pronounced in the absence of Rap1b, confirming the dominant role of this Rap family member. The combined loss of both isoforms led to an almost complete (80–90%) inhibition of integrin activation and platelet aggregation, even at high agonist concentrations. As a consequence, double-deficient animals showed defective haemostasis in response to a small laser-induced injury to endothelial lining, whereas single-deficient animals revealed no difference as compared to wild-type. To date, it is unclear which molecules or mechanisms mediate the

small residual integrin activation in the absence of Rap1a and Rap1b and whether this is of physiological relevance (Stefanini et al. 2018).

The predominant Rap-GEF in platelets is CalDAG-GEFI (or Rasgrp2), which is comprised of an N-terminal catalytic GEF domain and a C-terminal regulatory domain with a pair of highly Ca^{2+}-sensitive EF hand motifs and an atypical C1 domain. The EF hand motifs have a high affinity for Ca^{2+} and provide a remarkable sensitivity towards minor fluctuation of Ca^{2+}, while the atypical C1 domain has only weak affinity for DAG (Stefanini and Bergmeier 2016). In accordance with a regulatory role of CalDAG-GEFI on Rap1b activity, platelets from CalDAG-GEFI-deficient mice (Crittenden et al. 2004; Bergmeier et al. 2007) and from patients with inactivating CalDAG-GEFI mutations (Canault et al. 2014; Lozano et al. 2016) exhibited severe defects in integrin activation that resulted in an impaired aggregation response in vitro. CalDAG-GEFI deficiency provided resistance against experimental lethal collagen/epinephrine-induced pulmonary thromboembolism (Crittenden et al. 2004) and abolished thrombus formation in an arterial thrombosis model in mice (Bergmeier et al. 2007). However, in both, mouse and human, mutations in CalDAG-GEFI are associated with a moderate to severe haemostatic defect (Canault et al. 2014; Lozano et al. 2016; Piatt et al. 2016), suggesting that only partial inhibition of CalDAG-GEFI might be a promising therapeutic strategy (Piatt et al. 2016).

Further studies demonstrated that CalDAG-GEFI mediates rapid but reversible activation of Rap1b in platelets, whereas delayed and sustained Rap1b activation—required for full integrin activation—is initiated by DAG/PKC-dependent ADP release and downstream signalling of the G_i-coupled ADP receptor P2Y12 (Cifuni et al. 2008; Stefanini et al. 2009). Subsequent studies identifiedRASA3 (also known as GAP1IP4BP) as the missing link in the ADP/P2Y12 signalling pathway. Acting as a GAP, RASA3 catalyses GTP hydrolysis, and thereby counteracts CalDAG-GEFI in the regulation of Rap1a/b activation (Fig. 3). Thereby, RASA3 is supposed to be constitutively active in circulating platelets, in order to antagonize unwanted Rap1 activation and thus to keep platelets in an inactive state. In line with this, mice with reduced RASA3 expression (*Rasa3*$^{hlb/hlb}$) exhibited pre-activated circulating platelets with increased levels of Rap1-GTP and high-affinity integrin αIIbβ3. These hyperactive platelets undergo more rapid clearance in the circulation, as evidenced by a markedly reduced life span and increased removal in spleen and liver (Stefanini et al. 2015; Lee et al. 2022). This increased turnover and the resulting thrombocytopenia could be normalized by concomitant deletion of CalDAG-GEFI, and double-deficient platelets were hyperresponsive to agonists, as compared to CalDAG-GEFI-deficient platelets, both in vitro and vivo. Interestingly, a more recent study demonstrated a partial recovery of platelet counts by blocking integrin αIIbβ3 with a Fab-Fragment, and proposed a novel mechanism of integrin-dependent platelet clearance (Lee et al. 2022).

RASA3-deficient platelets were insensitive to the clinically used P2Y12 inhibitor clopidogrel, suggesting that one of its modes of action is to prevent the inactivation of RASA3, thereby prohibiting sustained Rap1 signalling (Stefanini et al. 2015; Stefanini and Bergmeier 2016). Therefore, the authors proposed a model that in

resting, circulating platelets, in the absence of vascular injury, CalDAG-GEFI activity is low and RASA3 is active in the plasma membrane to restrain uncontrolled Rap1 activation and maintain a quiescent, non-adhesive state (no integrin activation). Activation of platelets, however, results in an increase in cytoplasmic Ca^{2+} levels and DAG/PKC-mediated ADP release and subsequent activation of CALDAG-GEFI and inactivation of RASA3, respectively, resulting in robust and sustained activation of RAP1b and consequently integrin activation (Fig. 3) (Stefanini et al. 2015; Stefanini and Bergmeier 2016).

4.3 Talin-1 and kindlin-3: Essential Adaptor Proteins for Platelet Integrin Activation

While Rap1 proteins have multiple downstream actions where the identity of effector molecules remains elusive, the players in integrin activation are well established: In the current model, Rap1forms an integrin-activation complex with talin-1, kindlin-3, and additional adaptor proteins at the cytoplasmic tail of the integrin β subunit (Shattil et al. 2010; Nieswandt et al. 2009). Membrane association of Rap1b to the plasma membrane is mediated by post-translational prenylation as well as by a stretch of basic residues within its carboxyl terminus (Heo et al. 2006). On the other hand, studies in HEK cells have demonstrated that PKA-dependent phosphorylation of an adjacent serine residue can promote Rap1 translocation from the plasma membrane into the cytosol, thereby terminating integrin activation (Takahashi et al. 2013). Whether this process takes place or is relevant for platelets remains to be established.

Since platelets lack a nucleus and are short-lived ex vivo, they are not accessible to transfection or cell culture studies. Hence, before the broad availability of knockout mouse models, many studies on platelet-integrin activation pathways were performed in cell lines stably expressing human αIIbβ3 (most prominently A5 *Chinese hamster ovary* (CHO) cells (Frojmovic et al. 1991)). While experiments with these cells provided valuable insights into the basic mechanisms and proteins involved in integrin activation, some caution is warranted with regard to transferring the results to platelet physiology, as not all findings could be confirmed in genetically modified mice (e.g., in case of RIAM, see below).

The established master regulator of integrin function is talin. It forms the core of integrin adhesion complexes by linking the integrins to the actin cytoskeleton and recruiting numerous proteins—it is therefore essential for integrin activation (Calderwood et al. 2013). There are two mammalian talin isoforms encoded by distinct genes: talin-1 is expressed ubiquitously and expressed at very high levels in platelets, while talin-2 is highly expressed in brain and striated muscle and hardly in haematopoietic cells and whose function remains obscure (Monkley et al. 2001). The following chapter will therefore focus on talin-1, which is often simply referred to as talin (Calderwood et al. 2013).

Talin-1 is a large protein with approx. 2500 aa and a molecular weight of ~270 kDa, comprised of a head and a flexible rod region, connected by a small linker region. The C-terminal talin head (approx. 400 aa, ~50 kDa) is an atypical *protein 4.1R, ezrin, radixin, moesin* (FERM) domain and constitutes four subdomains (F0, F1, F2, F3) (Calderwood et al. 2013). The F3 has a *phosphotyrosine-binding* (PTB) domain fold that comprises the *integrin-binding site 1* (IBS-1) mediating binding to the conserved membrane-proximal NPxY motif in the integrin β tails (Calderwood et al. 2002; Garcia-Alvarez et al. 2003). It is proposed that the ionic interaction between the talin F3 domain and the membrane–proximal helix of the β tail disrupts an inhibitory salt bridge between the β3 and αIIb tail, thereby separating their cytoplasmic tails in order to allow integrin activation (Shattil et al. 2010; Hughes et al. 1996; Anthis et al. 2009a). In addition, the talin head interacts with multiple other proteins, e.g. Rap1 (Plak et al. 2016; Zhu et al. 2017a) via F0, *focal adhesion kinase* (FAK), *Rap1-guanosine triphosphate-interacting adaptor molecule* (RIAM) and with actin (*actin-binding site 1*, ABS1) with the F2-F3 subdomains (reviewed in Calderwood et al. 2013). The talin-rod (approx. 2000 aa, ~220 kDa) comprises 62 α-helices that fold up into 13 bundles (R1–R13) of four to five α-helices and a C-terminal dimerization helix and also has multiple interaction sites. There are at least two additional actin-binding sites, multiple binding sites for vinculin and a second integrin-binding site, whose significance has remained controversial (Calderwood et al. 2013). A current concept (Calderwood et al. 2013) suggests that talin is present as an auto-inhibited homo-dimer in the cytosol. In response to activation by Rap1 GTPases it gets translocated to the plasma membrane (Lee et al. 2009; Watanabe et al. 2008), where interaction with the negatively charged phospholipid PIP_2 releases the auto-inhibitory F3-R9 head-to-tail interaction, and therefore activates talin.

The first mechanistic insights into talin-1 function came from the key discovery that the talin-1 head directly binds to the cytoplasmic tails of β integrins and increased the ligand binding affinity of integrin αIIbβ3 in CHO cells (Calderwood et al. 1999). In accordance, knockdown of talin-1 in mouse embryonic stem cell-derived MKs led to inhibition of integrin αIIbβ3 activation towards ADP or thrombin stimulation, demonstrating that talin-1 performs the key final step in integrin activation (Tadokoro et al. 2003).

In mice, global deletion of talin-1 results in embryonic lethality between embryonic days 8.5 and 9.5 due to severe defects in cell migration before and during gastrulation (Monkley et al. 2000). Therefore, mice with platelet- and MK-specific talin-1 deficiency ($Tln1^{fl/fl;\text{Pf4-Cre+}}$) were used to investigate the role of talin-1 in platelet function and physiology (Nieswandt et al. 2007; Petrich et al. 2007a). $Tln1^{fl/fl;\text{Pf4-Cre+}}$ platelets were unable to activate αIIbβ3 in response to any tested agonist and therefore neither aggregated nor bound fibrinogen in vitro and also did not form aggregates in in vivo thrombosis assays or ex vivo under flow conditions (Nieswandt et al. 2007; Petrich et al. 2007a). Importantly, flow cytometry showed abolished binding of the 9EG7 antibody that specifically recognizes an activation-dependent epitope in the β1 subunit. This finding demonstrated that talin-1 is required for conformational changes in all platelet β1 integrins and provided direct evidence that

the regulation of β3 and β1 integrins occurs through similar pathways (Petrich et al. 2007a).Talin-1-deficient platelets also exhibited defects in spreading on immobilized fibrinogen (Nieswandt et al. 2007; Shen et al. 2013), however, this could be overcome by addition of Mn^{2+}, suggesting that this is due to an defect in integrin activation, rather than a defect in outside-in signalling (see below) (Shen et al. 2013). This complete abolishment of integrin function resulted in resistance to experimentally induced arterial thrombosis, which was, however, accompanied by severe defects in primary haemostasis, as indicated by infinite bleeding times (Nieswandt et al. 2007; Petrich et al. 2007a; Stefanini et al. 2014) and lethal spontaneous as well as gastro-intestinal bleeding (Petrich et al. 2007a; Stefanini et al. 2014). In contrast, these gastro-intestinal bleeding complications were virtually absent in a mouse model expressing the L746A mutation of the β3 integrin, which resulted in abolished talin-1 binding and defective integrin αIIbβ3 activation. Since mice were still protected in models of experimental thrombosis, the authors speculated that blocking integrin αIIbβ3-talin-1 interaction might have a potent anti-thrombotic effect without the extensive pathological bleeding associated with complete lack of αIIbβ3 function. Nevertheless, mice with the L746A mutation still exhibited severely defective haemostasis in tail bleeding time assays (Petrich et al. 2007b). More favourable observations in support of this hypothesis came from a mouse model bearing a talin-1 W359A mutation that selectively disrupts talin binding to the NxxY motif of the integrin β3 subunit, resulting in slower αIIbβ3 activation and delayed platelet aggregation (Stefanini et al. 2014). W359A mice were likewise protected from experimental thrombosis, however, showed no gastro-intestinal bleeding and only mildly prolonged tail bleeding times.

Studies in heterologous cell lines stably expressing integrin αIIbβ3 suggested that, like in other cells, Rap1 facilitates integrin activation by targeting talin-1 to the plasma membrane through the formation of a complex with the adapter molecule RIAM (Lee et al. 2009; Han et al. 2006). Indeed, a minimized Rap-RIAM fusion protein, containing the talin-1-binding site of RIAM fused to the membrane-targeting sequence of Rap1a, was sufficient to target talin-1 to the plasma membrane and to activate integrin αIIbβ3 (Lee et al. 2009). Furthermore, sh-RNA-mediated RIAM knockdown blocked thrombin-mediated talin-dependent αIIbβ3 activation in A5 CHO cells and in vitro differentiated MKs (Watanabe et al. 2008). Surprisingly, studies in two independently generated RIAM knockout mice revealed that RIAM-deficient mice—in contrast to talin-1-deficient mice—are viable, healthy, and fertile and strikingly showed no defects in platelet integrin activation. Consequently, adhesion and aggregation responses under static and flow conditions as well as haemostasis and arterial thrombus formation were indistinguishable between wild-type and RIAM-null mice (Stritt et al. 2015; Su et al. 2015). In contrast, absence of RIAM produces severe defects in β2 integrin-mediated leukocyte adhesion and trafficking, however, the major leukocyte β1 integrin family member, α4β1, was only partially affected by RIAM deficiency (Su et al. 2015; Klapproth et al. 2015). Thus, there is considerable integrin- and cell-type specificity in the requirement of RIAM for integrin activation, with β2 integrins being more dependent on RIAM than β1 and β3 integrins, even when expressed in the same cell (Calderwood 2015).

Overall, this data suggests that in many adherent cell types (because of the lack of developmental defects) and in platelets, RIAM-independent talin1-recruitment occurs. To date, it is not clear what protein substitutes for RIAM in platelets—an alternative potential mechanism is the direct interaction of membrane-anchored Rap1b with the F0 domain of talin-1. Despite having little sequence homology to canonical Rap1 effectors, talin-1-F0 binds to human Rap1b and disruption of the interaction strongly impaired integrin activation, cell adhesion, and spreading in αIIbβ3-expressing CHO A5 cells (Zhu et al. 2017a). While it was recently shown that the direct interaction of Rap1b and talin is of relevance in *Dictyostelium discoideum* (Plak et al. 2016), studies in a new mouse model (*Tln1*$^{R35E/R35E}$) found only a minimal contribution of Rap1 binding to talin1-F0 to αIIbβ3 activation murine platelets (Lagarrigue et al. 2018). Hence, further studies are required to investigate the mechanism of RIAM-independent talin recruitment in integrin activation and platelet function.

Although talin-1 binding to integrins is essential for integrin activation, it is by itself not sufficient for this process to occur. There are multiple other integrin-associated proteins that cooperate with talin-1 to activate integrins (Calderwood et al. 2013). Solid evidence has demonstrated that talin needs to cooperate with proteins of the kindlin family for integrin activation (Calderwood et al. 2013; Rognoni et al. 2016). In mammals, the kindlin family comprises three members of evolutionarily conserved focal adhesion molecules: kindlin-1, kindlin-2, and kindlin-3. Kindlin-1 is mainly expressed in epithelial cells, kindlin-2 is broadly expressed, but absent in blood cells. Kindlin-3 is found in haematopoietic cells (Rognoni et al. 2016) and is particularly highly expressed in platelets (Burkhart et al. 2012; Zeiler et al. 2014; Kim et al. 2014). Kindlins consist of ~680 aa and have a size of approximately 75 kDa and share considerable sequence and structural similarities. They have a similar domain organization to the atypical talin FERM domain, comprising F0, F1, F2, and F3 subdomains. Recent results have shown that the kindlins FERM domain adopts a compact cloverleaf-shaped conformation that is distinct from the linear talin FERM domain structure and important for activation of integrin αIIbβ3 (Sun et al. 2020). The F3 subdomain contains a PTB domain fold that can directly bind the NxxY motif in β integrins. In contrast to talin, the kindlin FERM domain contains a *pleckstrin homology* (PH) domain in the F2 subdomain, interacting with multiple phosphoinositides, which supports targeting to focal adhesions. Also, in contrast to talin, kindlins do not contain actin-binding sites; therefore, linkage to the cytoskeleton is mediated by their binding to the *ILK-PINCH-parvin* (IPP) complex and/or migfilin (Rognoni et al. 2016). Extensive biochemical studies in genetically modified mice and in αIIbβ3-expressing CHO cells as well as biophysical studies revealed that kindlins bind the tails of several β integrins and, in concert with talin, trigger integrin activation (Bledzka et al. 2012; Harburger et al. 2009; Montanez et al. 2008; Moser et al. 2008). Notably, Kindlin-3 binds the membrane-distal NxxY motif, while talin binds the membrane proximal one (Fig. 4b) (Bledzka et al. 2012), however it is still unclear whether they bind consecutively or jointly to control integrin activation (Calderwood et al. 2013; Moser et al. 2009a).

The importance of kindlin function in platelet integrin activation was shown in mice lacking kindlin-3 (*Fermt3*$^{-/-}$ mice) (Moser et al. 2008). *Fermt3*$^{-/-}$ mice died within one week after birth and suffered severe haemorrhages in different regions of the body, anaemia, leukocytosis, osteopetrosis, and defective haematopoiesis (Rognoni et al. 2016; Moser et al. 2008). Platelets from foetal liver cell chimeric *Fermt3*$^{-/-}$ mice displayed severe defects in β1 and β3 integrin activation, similar to talin-deficient platelets. This complete loss of integrin function resulted in defective adhesion, aggregation, and thrombus formation in vitro and in vivo and in infinite tail bleeding times. *Fermt3*$^{-/-}$ platelets did not spread on immobilized fibrinogen, implying that this protein might also be involved in outside-in signalling (Moser et al. 2008). Notably, kindlin-3-deficiency also abolished firm adhesion and arrest of neutrophils on activated endothelial cells (Moser et al. 2009b). Thus, the phenotype of *Fermt3*$^{-/-}$ closely resembles leukocyte adhesion deficiency type III (LAD III) in humans, a syndrome characterized by severe bleeding and impaired adhesion of leukocytes to inflamed endothelia, due to impaired activation of integrins. Indeed, in all investigated cases so far, LADIII was caused by loss of KINDLIN-3 expression (Kuijpers et al. 2009; Malinin et al. 2009; Svensson et al. 2009).

While there is compelling evidence that kindlins enhance talin-1 mediated integrin activation, the exact mechanism remains unknown. In vitro, kindlin, the talin head, and β3 integrin tails form a ternary complex (Bledzka et al. 2012; Moser et al. 2008), albeit sequential binding or binding to different integrin molecules (Moser et al. 2009a) cannot be excluded in pull-down experiments. Kindlins do not promote initial talin recruitment or talin binding to αIIbβ3, and no direct interaction of kindlin and the talin-1 head was observed in surface plasmon resonance measurements (Bledzka et al. 2012), suggesting that kindlins co-activate integrins through an independent mechanism occurring after talin-1 recruitment (Kahner et al. 2012). This could occur via direct potentiation via a yet unknown mechanism, the induction of clustering of talin-activated integrins, the displacement of an inhibitor from, or the recruitment of an additional adaptor to the integrin β tail (Calderwood et al. 2013). An example for such an additional adaptor might be *adhesion and degranulation promoting adapter protein* (ADAP) a blood-cell specific adaptor and a critical component of platelet integrin αIIbβ3 inside-out signalling (Kasirer-Friede et al. 2007). Recent studies revealed that ADAP interacts with kindlin-3 and talin-1 in platelets, and that ADAP-deficient platelets exhibited reduced co-localization of talin with αIIbβ3 and reduced fibrinogen binding in response to platelet stimulation (Kasirer-Friede et al. 2014).

Furthermore, there are additional regulators of integrin activation in platelets, however, this is beyond the scope of this review and this topic been discussed recently (Huang et al. 2019).

5 Integrin Outside-In Signalling

Like in other cells, platelet integrins do not only serve as adhesion molecules, but are also functional receptors that, upon ligand binding, initiate intracellular signalling cascades (Fig. 4a) (Durrant et al. 2017; Hynes 2002). These outside-in signalling events induce cytoskeletal reorganization, required for platelet spreading, stable thrombus formation, retraction of the formed clot (Fig. 1) and further enhance activation of αIIbβ3.

Investigation of the platelet outside-in signalling pathway is challenging, since under physiological conditions there is a strong spatio-temporal overlap with inside-out signalling pathways (Durrant et al. 2017). Outside-in signalling has been demonstrated for α2β1 (Inoue et al. 2003) and α6β1 (Inoue et al. 2006), but the vast majority of the research on integrin outside-in signalling in platelets has been conducted on αIIbβ3 (Durrant et al. 2017)—therefore our knowledge on this process is almost entirely based on research of this receptor. Thus, the findings in this section refer to studies on integrin αIIbβ3, if not stated otherwise. The reason for this bias is not only the outstanding importance of this integrin for thrombosis and haemostasis,

Fig. 4 Integrin outside-in signalling. (**a**) Simplified overview of key signalling molecules in integrin outside-in signalling. For details see text. Please note that only key mediators and not every component of the signalling pathways are indicated. The upstream mechanisms of Rac1 and Cdc42 during outside-in signalling are poorly defined. (**b**) Binding sites on the β3 C-terminal tail for selected interaction partners involved in bidirectional signalling. The two NxxY motifs are highlighted in blue, the C-terminal RGT sequence in red. Arrows indicate calpain cleavage sites. The shown amino acid sequence (aa716-762) is identical in mouse and human β3. Figure is adapted from Durrant et al. (2017), Huang et al. (2019)

but also owes to the fact that it is well accessible by inhibitors and functional assays. Most common assays analyse the retraction of a formed clot over time (Fig. 1d) or the morphological changes of platelets upon adhesion on the αIIbβ3 ligand fibrinogen, which triggers lamelli- and filopodia formation and finally full platelet spreading (Fig. 1c). However, since several proteins are involved in both, inside-out and outside-in signalling pathways (Watson et al. 2005), it is not always clear, whether an observed defect—particularly in the spreading assay—is really due to impaired outside-in signalling, or a consequence of impaired integrin binding to its ligand, or both. It is, at least partially, possible to overcome the limitation, e.g. by the addition of manganese in spreading experiments, which forces integrins into their active conformation (Smith et al. 1994), but these experiments are not performed routinely. It is also important to mention a recent study which found that, besides αIIbβ3, also GPVI signals upon platelet adhesion to fibrinogen and contributes to platelet spreading on this surface. This effect, which was observed in human but not mouse platelets, highlights the need for careful analysis of in vitro observations and also explains previously observed differences between mouse and human platelets, such as the enhanced spreading capability on fibrinogen of human compared to mouse platelets (Mangin et al. 2018).

In addition to changes in the affinity of individual integrin heterodimers described above, also the clustering of integrins influences the binding of ligands by avidity modulation of the adhesive complexes and thus supports outside-in signalling (Shattil et al. 2010; Hato et al. 1998). Clustering may be promoted by inside-out signals that drive the recruitment of multivalent protein complexes to cytoplasmic domains, the binding of multivalent extracellular ligands, the release of integrins from cytoskeletal constraints that leads to their free diffusion in the membrane, or as a result of transmembrane domain separation in the absence of a ligand (Shattil et al. 2010; Buensuceso et al. 2003; Fong et al. 2016). The investigation of integrin clustering is demanding, since most integrin ligands are multivalent and their binding may be influenced by the cellular regulation of integrin clustering and also multimeric ligand binding itself may modify the nature of the interaction. Furthermore, the high density of integrin αIIbβ3 on the platelet surface exacerbates microscopy studies. Therefore, the understanding of the mechanisms of integrin clustering is incomplete—however, advances in super-resolution microscopy techniques and expansion microscopy hold the promise to improve our understanding of integrin clustering and its impact on outside-in signalling at the nano scale (Heil et al. 2022).

Outside-in signalling cascades are highly ordered, both in space and time, in order to regulate the complex cytoskeletal rearrangements required for the two opposite processes of cell spreading (filopodia and lamellipodia formation, outward moving of cell membrane) and clot retraction (inward movement of cell membrane and cytoskeletal complexes) (Fig. 1c, d) (Durrant et al. 2017). In a widely accepted model, different mechanisms, such as the competition of proteins for binding sites as well as the conformation, phosphorylation, and proteolytic cleavage of the β integrin C-terminus, switch the functional outcome of integrin signalling from cell spreading in the initial phase to retraction in the later stage (Durrant et al. 2017; Shen et al. 2013; Flevaris et al. 2007). In consequence, as it has been shown for RhoA (Arthur

et al. 2000), this means that the activity of some signalling components is initially suppressed and then later enabled in the signalling process.

To date, more than 100 proteins have been implicated in integrin outside-in signalling—only the key mechanisms of this process are discussed here and readers are referred to an excellent recent review on this topic (Durrant et al. 2017).

5.1 *Events at the β Integrin's C-Terminus*

Integrin outside-in signalling requires the assembly of a multi-protein complex at the integrin's C-terminus, which induces downstream signalling events and acts as an adapter that connects the integrin to the cytoskeleton. An early key event is the phosphorylation of specific downstream substrates and the β3 C-terminus itself by SFKs, particularly c-Src, one of the most abundantly expressed SFK in platelets (Fig. 4a) (Law et al. 1996; Obergfell et al. 2002; Arias-Salgado et al. 2003). Originally, inactive c-Src was proposed to constitutively associate to RGT residues in the β3 C-terminal end under resting conditions (Obergfell et al. 2002; Arias-Salgado et al. 2005), but a more recent study suggests that it binds predominantly after platelet activation (Fig. 4b) (Wu et al. 2015). First evidence for the importance for SFK came from KO and inhibitor studies: Human platelets treated with the SFK inhibitor PP2 and mouse platelets multiple-deficent in c-Src, Hck, Fgr, and Lyn failed to spread on fibrinogen and showed defective phosphorylation of the downstream target Syk (Obergfell et al. 2002). Interestingly, platelets treated with RGT-containing peptides that abrogate the interaction of c-Src with β3, and platelets from knockin mouse with a deleted RGT sequence within β3 (β3Δ760-762) showed selective inhibition of integrin αIIbβ3 outside-in signalling and recapitulated the observed adhesion and spreading defects on fibrinogen in the absence of significant inside-out activation defects (Ablooglu et al. 2009; Su et al. 2008). The β3Δ760-762 mice also showed strongly impaired thrombus formation in the $FeCl_3$ model of arterial thrombosis, but no spontaneous bleeding, excess bleeding after surgery, faecal blood loss, or anaemia, thus contrasting with the severe haemorrhagic phenotype associated with marked deficiency or loss of αIIbβ3 (Ablooglu et al. 2009). Notably, integrin α2β1 has also been reported to induce an SFK-mediated signalling pathway, although c-Src does not bind to the β1 cytoplasmic tail (Inoue et al. 2003; Arias-Salgado et al. 2003, 2005). Hence, signalling might be activated by an alternative mechanism or other SFK members, like Lyn and c-yes, which are able to directly bind to both β3 and β1 integrins (Arias-Salgado et al. 2003).

One target of the SFKs are the two conserved tyrosine residues within the β3 NxxY motifs (Fig. 4b). These phosphorylation sites facilitate the binding of signalling molecules (Law et al. 1996; Jenkins et al. 1998), but can also negatively regulate the binding of other molecules, such as talin (Anthis et al. 2009b). In a mouse model expressing an αIIbβ3 variant, in which these tyrosine residues are mutated to phenylalanine (β3diYF mice), platelets showed selective defects in integrin outside-in signalling but normal inside-out activation (Law et al. 1999).

Phenotypically, this translated into defective clot-retraction responses in vitro, and an in vivo bleeding defect, which was characterized by a pronounced tendency to rebleed, thus providing evidence for a role of outside-in signalling in platelet physiology. Noteworthy, not all signalling events are blocked by these mutations, since SFKs have multiple other targets including Syk (Obergfell et al. 2002) or RhoGaps that mediate the transient inhibition of RhoA in the early phase of outside-in signalling (see below) (Arthur et al. 2000; Gong et al. 2010).

The activation of SFKs is regulated by altering their phosphorylation status (dephosphorylation of the inhibitory Y529, phosphorylation of Y416/18) (Obergfell et al. 2002; Senis et al. 2021). In the outside-in signalling of αIIbβ3 this is proposed to be initiated by $G\alpha_{13}$ that interacts with an ExE motif in the β3 cytoplasmic tail (Fig. 4b) (Shen et al. 2013; Gong et al. 2010). $G\alpha_{13}$ knockdown platelets showed impaired spreading on fibrinogen and accelerated clot retraction, which was attributed to impaired SFK activation and concomitant enhanced RhoA activity (see below) (Gong et al. 2010). This defect in platelet spreading could also be verified with an ExE-motif-based blocking peptide (myr-FEEERA) that specifically interferes with $G\alpha_{13}$ binding to the β integrin cytoplasmic tail, but not with integrin inside-out activation and ligand binding function (Shen et al. 2013). In mice, this inhibitor suppressed occlusive laser-induced arterial thrombosis but did not affect tail bleeding times—therefore targeting the $G\alpha_{13}$-integrin interaction might represent a new anti-thrombotic strategy (Shen et al. 2013). In a later study, it was shown that $G\alpha_{13}$ also interacts with β1 integrins and controls cell migration in nucleated cells—whether this mechanism also plays a role for β1 integrins in platelets, however, remained elusive (Shen et al. 2015). However, it is noteworthy that the spreading defects of $G\alpha_{13}$ knockdown platelets that formed the basis of these studies could not be confirmed in platelets of $G\alpha_{13}$ knockout mice (Pleines et al. 2012).

Apart from inducing outside-in signals that finally result in actin polymerization and reorganization, also the coupling of integrins to the actin cytoskeleton is critical. Global approaches defining core components of the integrin adhesome have established the actin-binding proteins such as α-actinin, filamin, tensin, and vinculin as linkers between integrins and the cytoskeleton (Horton et al. 2015; Legate et al. 2009). In addition to its key role in integrin activation, talin-1 also facilitates direct coupling between β integrin cytoplasmic tails and the actin cytoskeleton (Calderwood et al. 2013). While there have been conflicting reports on the role of talin-1 in outside-in signalling (Nieswandt et al. 2007; Petrich et al. 2007a; Shen et al. 2013), more recent work suggested a model where talin-1 is initially displaced by $G\alpha_{13}$ from the integrin β3 terminus, and thus dispensable in the early phase of outside-in signalling (responsible for platelet spreading)—however, it later re-associates to the β3 C-terminus in order to mediate clot retraction (Shen et al. 2013; Haling et al. 2011).

In addition to multiple protein binding sites, the β3 cytoplasmic tail also contains cleavage sites for the intracellular calcium-dependent protease calpain and undergoes limited proteolysis during platelet aggregation induced by physiological agonists (Fig. 4b) (Du et al. 1995). Based on experiments in CHO cells expressing wild-type, calpain cleavage-resistant or -mimicking mutants, it is hypothesized that

calpain-mediated cleavage of the β3 cytoplasmic tail removes the c-Src binding site (RGT) during spreading, thereby dampening c-Src's inhibitory effects on the RhoA-signalling pathway, subsequently allowing local RhoA-dependent cell retractile signals. Hence, β3 cleavage by calpain might serve as a molecular switch that changes the functional outcome of integrin signalling from cell spreading to retraction (Flevaris et al. 2007; Gong et al. 2010). This is in line with the spreading defects of platelets from mouse models with a calpain cleavage-mimicking mutation in the β3 C-terminus (Ablooglu et al. 2009).

5.2 Downstream Signalling Cascades

Many of the molecules implicated in outside-in signalling are also involved in signalling of the platelet ITAM receptor GPVI and include tyrosine kinases, adapter molecules, and phospholipase C (Fig. 4a) (Watson et al. 2005). The tyrosine kinase Syk is a direct downstream target of SFKs and has been shown to be critical for GPVI, but also B cell receptor signalling (Stegner and Nieswandt 2011; Watson et al. 2005; van Eeuwijk et al. 2016). A first study reported that Syk-deficient platelets exhibited normal c-Src activation upon adhesion on fibrinogen, but showed no spreading and phosphorylation of Vav1, Vav3, and SLP-76 (Obergfell et al. 2002). The spreading defect of Syk-deficient platelets, however, could not be confirmed in a later study and also spreading of mouse platelets treated with a Syk-inhibitor was unaltered, suggesting that Syk is not essential for SFK-mediated outside-in signalling (van Eeuwijk et al. 2016). Irrespective of this controversial role of Syk, mouse models deficient in the downstream targets SLP-76 (Judd et al. 2000), Vav1/3 (Pearce et al. 2007), and PLCγ2 (Wonerow et al. 2003) all exhibited defective spreading on fibrinogen. In GPVI-ITAM signalling SLP-76 co-operates with multiple other adapter proteins—*linker for activation of T cells* (LAT), *GRB2-related adaptor downstream of Shc* (GADS), *and growth factor receptor-bound protein 2*(Grb2)—all of which help to recruit SLP-76 to the plasma membrane and thus are all required for proper signalling (Dutting et al. 2014; Hughes et al. 2008). It is therefore remarkable that SLP-76 is the only of these four adaptor molecules required for platelet spreading on fibrinogen (Dutting et al. 2014; Hughes et al. 2008), and clearly points towards different modes of SLP-76 recruitment in these two signalling pathways.

A previous study had reported an important role for the ITAM-bearing FcγRIIA receptor—expressed in human but not mouse platelets—in Syk activation and subsequent spreading on fibrinogen (Boylan et al. 2008). This surprising finding could however not be reproduced in a very recent study (Ahmed et al. 2021), which also suggested that the originally observed defects in platelets from patients with low FcγRIIA expression might be caused by an accompanying deficiency in GPVI, which had been meanwhile identified as an additional receptor for fibrinogen (Mangin et al. 2018).

Enzymatic activity of PLCγ2 results in the production of IP_3 and DAG, the latter activating PKC. Of the various PKC isoforms expressed platelets, PKCβ and PKCθ both associate with the β3 integrin cytoplasmic tail. Furthermore, platelets deficient for either isoform spread poorly on fibrinogen, but exhibited normal agonist-induced fibrinogen binding, demonstrating a specific role in αIIbβ3 outside-in signalling (Buensuceso et al. 2005; Soriani et al. 2006).

FAK serves as a tyrosine kinase and as a scaffold protein in integrin signalling. Its initial autophosphorylation is induced by actin polymerization and coordinated signalling through αIIbβ3 and agonist receptors (Shattil et al. 1994), and is further amplified by SFKs. This promotes the recruitment of binding partners and the activation of its kinase activity, enabling phosphorylation of its substrates that include the actin-binding protein α-actinin (Durrant et al. 2017). FAK-null platelets exhibit diminished spreading on immobilized fibrinogen (Hitchcock et al. 2008).

Although all four class I PI3Ks are expressed in platelets, studies have revealed PI3Kβ to be particularly important for αIIbβ3 outside-in signalling. Interference with its function by genetic or pharmacological approaches results in defective adhesion to and spreading on fibrinogen, as well as delayed clot retraction and unstable thrombi (Durrant et al. 2017). The PI3K activation pathway downstream of αIIbβ3 involves Src, Syk, Pyk2, and the E3 ubiquitin ligase c-Cbl. PI3Ks regulate cell function primarily via downstream of PIP_3-binding proteins, of which the kinase Akt is the best characterized, with isoforms Akt1 and Akt3 appearing important for αIIbβ3-mediated platelet spreading (Durrant et al. 2017).

The roles and signalling mechanisms of the molecules mentioned above have been studied downstream of αIIbβ3. Of note, phosphorylation of Src, Syk, SLP-76, and FAK as well as a role of PLCγ2 has also been observed upon engagement of α2β1 (Inoue et al. 2003). Phosphorylation of Syk and PLCγ2 and activation of PI3K were observed downstream of α6β1 (Inoue et al. 2006; Chang et al. 2005). This raises the possibility that some mechanisms described above might occur similarly downstream of β1 integrins.

The RhoGTPase family members RhoA, *cell division control protein 42* (Cdc42), and *Ras-related C3 botulinum toxin substrate 1* (Rac1) are key regulators of platelet function and of particular importance for cytoskeletal rearrangements induced by αIIbβ3 outside-in signalling (reviewed in Aslan and McCarty 2013). Like Rap1, they act as signalling switches that are active in their GTP-bound state and inactive in a GDP-bound state and are cyclically regulated by specific GEFs and GAPs (Aslan and McCarty 2013). Rac1 regulates the formation of lamellipodia protrusions and membrane ruffles, and Cdc42 triggers filopodial extensions in various cell types. In platelets, Rac1-deficiency resulted in abrogated lamellipodia formation upon spreading on fibrinogen and impaired thrombus stability at sites of vascular injury in vivo (McCarty et al. 2005; Pleines et al. 2009). Furthermore, a role for Rac1 in clot retraction was reported (Flevaris et al. 2009). For Cdc42, a major role in platelet filopodia formation was proposed, based on biochemical analysis of the signalling pathways downstream of integrin α6β1 (Chang et al. 2005). However, two independent studies analysing Cdc42-deficient platelets produced conflicting results: one study reported an essential role for Cdc42 for filopodia formation upon spreading on

immobilized fibrinogen (Akbar et al. 2011), while another found that Cdc42 is dispensable in this process (Pleines et al. 2010). The reason for this discrepancy is not clear to date, but may be due to differences in the genetic KO mouse models used. Downstream effectors of Cdc42 and Rac1 are *Wiskott–Aldrich syndrome protein* (WASP) family members, which relay signals to the *actin-related protein 2/3 complex* (ARP2/3), which in turn induces actin branching (Aslan and McCarty 2013).

RhoA is known to play a central role in organization of the actin cytoskeleton by its ability to form stress fibres and the regulation of platelet actomyosin contractility. These effects are mediated by downstream effector molecules such as *mammalian diaphanous homologue* (mDia) and *Rho-associated coiled-coil–containing protein kinase* (ROCK), with the latter triggering *myosin light chain* (MLC) phosphorylation (Aslan and McCarty 2013). As mentioned above, different studies support a model, where RhoA activity downstream of integrin αIIbβ3 is initially inhibited by c-Src-dependent activation of p190RhoGAP (p190) (Arthur et al. 2000; Gong et al. 2010). This transient RhoA inhibition alleviates contractile forces that would otherwise impede formation of protrusive structures, such as filopodia and lamellipodia. Over time, this inhibition is released by mechanisms that involve the displacement of the c-Src activator Gα13 by talin (Shen et al. 2013) and the proteolytic cleavage of the c-Src binding site in the β3 integrin terminus by calpain (Flevaris et al. 2007). The reinstated RhoA activity then mediates platelet retraction. Indeed, and in line with this model, studies with RhoA-deficient platelets showed that this GTPase is dispensable for filopodia and lamellipodia formation on fibrinogen but essential or integrin αIIbβ3-mediated clot retraction (Pleines et al. 2012). In vivo, platelet- and MK-specific RhoA-deficiency resulted in reduced thrombus stability in two thrombosis models as well as in prolonged tail bleeding times, however, it remained unclear to what extent this phenotype was influenced by the accompanying defects in GPCR signalling. In addition, RhoA might also regulate microtubule organization (Pleines et al. 2012).

5.3 Transmembrane Proteins

Integrins also crosstalk with other cell surface receptors and proteins during outside-in signalling. Among these are *immunoreceptor tyrosine-based inhibition motif* (ITIM)-containing receptors. Whilst these receptors are mostly regarded as negative regulators of GPVI/FcRγ-ITAM signalling (Coxon et al. 2017), studies have found defective αIIbβ3 function in mice lacking the ITIM-family members *platelet endothelial cell adhesion molecule-1* (PECAM-1) (Wee and Jackson 2005), *carcinoembryonic antigen-related cell adhesion molecule 1* (CEACAM1) (Yip et al. 2016), and phosphorylation of G6b-B downstream of αIIbβ3 (Senis et al. 2009). Tetraspanins are a superfamily of membrane proteins with four transmembrane regions that interact with specific partner proteins to distribute them into highly organized plasma membrane microdomains. In platelets, a supportive role

for the tetraspanins CD151 and *tumour-suppressing subchromosomal transferable fragment cDNA 6* (TSSC6) was reported in αIIbβ3 outside-in signalling (Huang et al. 2019). Due to space limitations, we refer to reference (Huang et al. 2019) for more detailed information on transmembrane proteins that regulate platelet integrin function.

6 Therapeutic Targeting of Integrins

Because of its pivotal importance for platelet function, αIIbβ3 has been and remains a target for the development of powerful anti-thrombotic agents for more than 30 years. Three structurally distinct αIIbβ3 inhibitors (also referred to as GPIIb/IIIa blockers) have been in clinical use: abciximab (Reopro), eptifibatide (Integrilin), and tirofiban (Aggrastat) (Bledzka et al. 2013). Abciximab is a Fab-fragment of the chimeric mouse/human monoclonal antibody 7E3. The mechanism of action is thought to involve steric hindrance and/or conformational effects to block access of large molecules to αIIbβ3, rather than direct interaction with the RGD binding site. Noteworthy, abciximab also binds the vitronectin receptor αvβ3, expressed on endothelial and smooth muscle cells, monocytes, polymorphonuclear leukocytes, T lymphocytes and platelets, and the leukocyte integrin αMβ2 (Mac-1), which might dampen inflammatory reactions, but the clinical implications are unclear (Bledzka et al. 2013; Ostrowska et al. 2014). In 2018, the production of abciximab was discontinued due to persisting problems in the production supply chain. Eptifibatide is a cyclic heptapeptide, containing a *lysine–glycine–aspartic acid* (KGD) sequence. The starting point for its design was barbourin, a snake venom disintegrin with potent anti-aggregating activity, which relies on a KGD sequence, as opposed to the more frequent RGD sequence. The potency was greatly enhanced by cyclizing the peptide via a disulphide bond. Eptifibatide is reported to inhibit fibrinogen binding to αIIbβ3 more specifically than abciximab (Bledzka et al. 2013). Tirofiban is a non-peptide αIIbβ3 inhibitor—its design is based on the RGD peptide, with the peptide-bonds being ultimately eliminated from the structure. Tirofiban does not interact with αvβ3 and αMβ2 (Bledzka et al. 2013). All three compounds are/were administered intravenously—attempts to develop orally available small molecules in this class failed because they acted as partial agonists and had prothrombotic effects. Thus, oral inhibition of αIIbβ3 is regarded as a failed strategy (Cox 2004; Armstrong and Peter 2012).

Although the benefits of αIIbβ3 antagonists have not always been consistent across all clinical trials, they have become an important pillar of anti-platelet therapy and have been applied millions of times and potentially have saved many lives. However, the clinical scenarios in which αIIbβ3 antagonists proved to be efficient are more limited than originally hoped: their use is restricted to the prevention of cardiac ischaemic events in patients undergoing *percutaneous coronary intervention* (PCI) and/or for patients with unstable angina pectoris who are expected to undergo PCI—particularly those who are not eligible for P2Y12 inhibitors (e.g. clopidogrel).

P2Y12 inhibitors are increasingly used, due to higher efficacy and much lower costs (Bledzka et al. 2013).

The very potent anti-platelet effect of αIIbβ3 inhibitors also comes at the cost of bleeding complications, which occur more frequently than with other platelet inhibitors (Hanna et al. 2010). After bleeding, thrombocytopenia and severe reactions to re-administration are the most serious side effects of αIIbβ3 antagonists (Bledzka et al. 2013). There is strong evidence that thrombocytopenia results, at least in part, from drug-induced conformational changes in the receptor, which leads to the exposure of neo-epitopes (ligand-induced binding sites, LIBS) that are targeted by pathogenic antibodies (Armstrong and Peter 2012; Bougie et al. 2012). Furthermore, conformational changes have been reported to prime fibrinogen binding and elicit outside-in signalling, thereby inducing paradoxical platelet activation (Bassler et al. 2007; Peter et al. 1998). Despite these downsides, integrin αIIbβ3 still remains an attractive anti-thrombotic target, and novel strategies to develop, e.g., allosteric or conformation-specific inhibitors hold the promise to reduce the unwanted side effects of current therapeutics (Armstrong and Peter 2012). One example are the small molecules RUC-2 and RUC-4, which disrupt binding of Mg^{2+} to the MIDAS, and do not induce conformational changes in either the αIIb or the β3 subunits in the αIIbβ3 headpiece and hence do not 'prime' αIIbβ3 to bind its ligand (Zhu et al. 2012; Li et al. 2014). Similarly, antibodies directed against the PSI domain of β3 were reported to interfere with fibrinogen binding and to confer protection from thrombosis with minimal effect on tail bleeding times in mice (Zhu et al. 2017b). Another promising strategy is the conformation-specific blockade of the active form of αIIbβ3 by single-chain antibodies, which are small recombinant antibody fragments consisting of the variable regions of the antibody heavy and light chain fused together to a single molecule. These anti-αIIbβ3 single-chain antibodies did not cause a conformational change of αIIbβ3 nor paradoxical platelet activation but exhibited strong of strong anti-thrombotic properties without bleeding time prolongation (Armstrong and Peter 2012; Schwarz et al. 2006). Furthermore, they can also be fused to effector molecules, allowing not only blocking of αIIbβ3 but also targeting and enrichment of, for example, fibrinolytics to the site of platelet activation (Wang et al. 2014).

As already mentioned above, inhibition of outside-in signalling is another promising strategy. Indeed, potent inhibition of aggregation was observed for a synthetic RGT-peptide (inducing dissociation of Src from β3 C-terminus) (Su et al. 2008; Huang et al. 2015) and the ExE-motif-based blocking peptide myr-FEEERA (interferes with Gα13 binding and Src activation) (Shen et al. 2013), which both interfere with outside-in but spare inside-out signalling. Interestingly, myr-FEEERA suppressed experimental occlusive thrombus formation in mice as potently as eptifibatide, but did not exhibit its detrimental effect on tail bleeding times, suggesting that inhibitors of outside-in signalling might not cause the bleeding complications observed for the current inhibitors. However, it has to be considered that the ExE motif is present in most β integrins, thus disruption of integrin signalling by myr-FEEERA is likely to affect also the function of other integrins in other cell types, with so far unknown consequences (Shen et al. 2013).

Apart from αIIbβ3, no other platelet integrin is currently used as a therapeutic target.

7 Integrins Beyond Thrombosis and Haemostasis

Recent studies revealed that platelets also play an important role in processes beyond thrombosis and haemostasis, such as in tumour metastasis and tumour growth (Lavergne et al. 2017), maintenance of vascular barrier function (Gupta et al. 2020; Ho-Tin-Noe et al. 2018), infection and inflammation (Guo and Rondina 2019), as well as thrombo-inflammation (Stoll and Nieswandt 2019). This raises the questions, whether integrins are also involved in mediating these novel functions and if so, whether they can be targeted therapeutically.

Thrombo-inflammation is the complex interplay between thrombosis and inflammation that can result in acute organ damage. Platelets act as critical mediators in this process, bridging haemostatic, inflammatory, and immune responses. The prototype and the so-far best-described thrombo-inflammatory disease is *ischaemia/reperfusion* (I/R) injury following ischaemic stroke (Stoll and Nieswandt 2019; Burkard et al. 2020). Ischaemic stroke is caused by a thrombo-embolic occlusion of a central brain artery, resulting in cessation of blood supply of a larger brain area. Primary goal of therapy is the recanalization of the occluded vessels, either by mechanical thrombectomy or by enzymatic thrombolysis. However, even despite successful recanalization, infarct sizes often continue to grow following reperfusion, which is caused by thrombo-inflammatory processes in the reperfused brain area.

Because of the beneficial effect of αIIbβ3 inhibition in acute coronary syndrome patients undergoing PCI, where I/R is a known pathological process, blocking of αIIbβ3 was suggested as a strategy to combat I/R injury also in ischaemic stroke. Early experimental studies showed a beneficial effect of αIIbβ3 blockade by the non-peptide inhibitor SDZ GPI 562 on neurological outcome and infarct sizes in a mouse model of ischaemic stroke. The protective effect was in part attributed to the prevention of microvascular thrombi in the infarcted brain area, which are considered to contribute to tissue damage (Choudhri et al. 1998). Contradictory to that, another study in mice found that inhibition of αIIbβ3 by F $(ab)_2$ fragments of the function-blocking antibody JON/A did not reduce infarct sizes as compared to control-treated animals. In addition, the treatment was associated with severe intracranial haemorrhage eventually leading to death of the treated mice (Kleinschnitz et al. 2007). The lack of a protective effect is also in line with a recent study showing that infarct growth precedes cerebral microthrombi following experimental stroke in mice, suggesting that these aggregates are not causative for secondary infarct growth (Gob et al. 2021). Similar consequences of αIIbβ3 inhibition were also observed in different clinical studies, where application of abciximab significantly increased the risk of symptomatic intracranial haemorrhage, but did not result in any reduction in death or disability in survivors (Ciccone et al. 2014). Tirofiban is discussed controversially regarding its use in acute ischaemic stroke patients, since some, but not all

studies indicated the safety and efficacy of this drug in distinct subgroups of ischaemic stroke patients (Burkard et al. 2020). Thus, despite the high potency of αIIbβ3 inhibition in patients with acute myocardial infarction this receptor has to be considered as unreliable, yet druggable target in the therapy of ischaemic stroke.

A different cardiovascular complication of the brain is cerebral venous (sinus) thrombosis (CVT), representing an unusual and rare manifestation of venous thrombosis that has gained increased attention as a complication in patients with COVID-19 and rarely in response to vector-based vaccines (Aamodt and Skattor 2022). A recent study revealed that CVT can be induced by a Fab-fragment of an antibody raised against the platelet hemITAM receptor CLEC-2. Interestingly, integrin αIIbβ3 acts as an important co-receptor in this process and is not only required for aggregate formation, but is also essential for the initiation of platelet activation in this pathological setting. As a consequence, blocking of αIIbβ3 prevented disease development in mice and increased survival on application even after disease onset and was clearly more effective than heparin, the first-line treatment for CVT in humans. Thus, inhibition of αIIbβ3 might be a considered as a therapeutic option for CVT not controlled by heparin (Stegner et al. 2022).

In a seminal study with thrombocytopenic mice it was shown that platelets are essential to maintain vascular integrity and to prevent bleeding during inflammatory challenge in skin, lungs, and brain (Goerge et al. 2008). Interestingly, integrin αIIbβ3 is not required for prevention of bleeding in the inflamed skin, instead, single-platelets seal neutrophil-induced vascular breaches in a GPVI-dependent manner (Goerge et al. 2008; Gros et al. 2015). This integrin αIIbβ3-independent haemostasis has also been observed in other cellular contexts and seems more widespread than previously recognized (Rayes et al. 2019). This is in contrast to the role of αIIbβ3 in the thrombo-inflammatory setting of ischaemic stroke and highlights that different mechanisms ensure haemostasis in different inflammatory settings and vascular beds. Thus, the presence of inflammation does *per se* not exclude the option of αIIbβ3 inhibition, but more research is needed to investigate the involvement of αIIbβ3 in distinct inflammatory diseases.

Platelets play a significant role in the coordinated immune response to infection and hence are also involved in the misdirected host response that contributes to organ damage in the course of sepsis, one of the leading causes of infection-related hospitalizations and mortality (Shannon 2021). Given that thrombosis and vascular complications are common in sepsis, integrin αIIbβ3 has been tested as a therapeutic target in sepsis in animal studies, with some of them showing improved outcomes. Whether these results can be extrapolated to humans remains unclear, since there have been no clinical trials with αIIbβ3 inhibitors (Akinosoglou and Alexopoulos 2014). A recent study investigating the transcriptional and translational landscape of platelets in sepsis found elevated levels of αIIb in septic patients and mice, which correlated with increased mortality, however the functional consequences remained unidentified (Middleton et al. 2019). It is well established that human platelets can directly bind to and become activated by bacteria, thereby forming platelet-bacteria aggregates. This activation process depends on FcγRIIA, which recognizes IgG-opsonized bacteria, but also relies on synergistic signalling of integrin αIIbβ3,

since its blockade abolished secretion and FcγRIIA tyrosine phosphorylation. This collaboration of the two receptors seems to be a common mechanism for activation triggered by gram-positive (Arman et al. 2014) as well as gram-negative bacteria (Watson et al. 2016), however, the consequences for the platelet phenotype has not been investigated extensively.

Platelets can also physically and functionally interact with tumour cells and can promote their haematogenous spread and subsequent metastasis formation (Lavergne et al. 2017). As already discussed above, a recent study found that integrin α6β1 directly binds to ADAM9 on tumour cells, thereby inducing platelet activation and favouring the extravasation process in experimental and spontaneous lung metastasis (Mammadova-Bach et al. 2016). Furthermore, it is recognized that integrin αIIbβ3 plays a critical role in tumour cell-induced platelet activation and tumour metastasis. The challenges in elucidating its exact role, important considerations on potential off-target effects of integrin blockage as well as limitations of current αIIbβ3 inhibitors in anti-metastatic therapy are discussed in an excellent recent review (Lavergne et al. 2017).

8 Conclusions and Future Perspectives

The pivotal importance of integrins in mediating essential platelet functions has quickly emerged just right after the discovery of this receptor class. Extensive investigation of the prominently expressed integrin αIIbβ3 has boosted our knowledge on integrin and platelet function and ultimately resulted in the approval of potent anti-thrombotic drugs. However, platelet integrin biology goes far beyond αIIbβ3-mediated platelet aggregation and the precise roles of the other platelet integrins remain comparatively ill-defined.

Open questions also remain for the signalling cascades and mechanisms leading to integrin inside-out activation as well as for the very complex outside-in signalling pathways. Technical advancement in super-resolution microscopy, proteomics, biophysics, and structural biology as well as more complex and physiological in vitro systems are likely to provide new and deeper insights, which will support the development of new targeting strategies for platelet integrins. Again, the gaps in our knowledge in this field are much bigger for the β1 integrins, as compared to αIIbβ3 and it will be interesting to see, whether the molecular mechanisms and signalling pathways discovered for αIIbβ3 are also in place for other platelet integrins or whether there are significant differences.

The biggest gain of knowledge on platelet integrin function we expect to see in the just emerging area of new platelet functions beyond thrombosis and haemostasis, such as tumour metastasis and thrombo-inflammation. The complexity of the disease processes, involving close interaction of platelets with immune cells and other cell types as well as the organ-specificity of the underlying mechanisms will require extensive and sophisticated animal studies as well as carefully planned clinical studies in order to validate the suitability of platelet integrins as novel therapeutic

targets in diseases with so far poor treatment options. Successful therapies will highly depend on the development of novel therapeutic agents, which will need to show that the limitations of current αIIbβ3 inhibitors are indeed a consequence of an inappropriate strategy and not of an inappropriate target.

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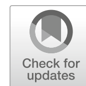
Integrins as Receptors for Bacterial and Viral Pathogens



Maria Mrakovcic, Samriti Sharma, and Petra Dersch

Abstract Integrins are important cell surface receptors named cell adhesion molecules that mediate cell–cell, cell–matrix, and cell–pathogen interactions. Integrins exist as obligate heterodimers of integral transmembrane α and β subunits linking the extracellular matrix to the intracellular cytoskeleton. In mammals, they are composed of eighteen α subunits and eight β subunits which form a range of distinct heterodimers with varying activities. Integrins transduce signals from the environment directly or via extracellular matrix molecules into the cell, to adjust cell processes and cell behavior. In addition, multiple microbial pathogens including bacteria, parasites, and viruses use integrin receptors to adhere to, enter, or subvert host cells to create a specialized niche which allows colonization, multiplication, and spreading of the microbe. This review highlights the fundamental role of integrins in bacterial and viral pathogenesis. We illustrate their role in the virulence process in more detail using well-studied examples of the molecular interplay of microbial factors with integrins.

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1 Introduction

1.1 Integrin-Mediated Cell Functions

The detailed analysis of integrin functions over the past years revealed that integrins not only mediate cell–cell and cell–extracellular matrix interactions, but they are also involved in a wide variety of biological processes, including the intersection of integrin-mediated signaling with signaling from other cell receptors implicated in proliferation, differentiation, and migration, and the manipulation of cell processes by pathogens (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012; Hoffmann et al. 2011; Stewart and Nemerow 2007).

Integrins exist as heterodimers of the noncovalently associated transmembrane glycoprotein subunits α and β. They are highly conserved in evolution of multicellular organisms from sponges to humans. Eighteen α subunits and eight β subunits were identified in mammals which can form a large set of distinct heterodimers which can have different cell type-dependent binding specificities (Hynes 2002). Furthermore, post-translational modifications of the subunits as well as alternative splicing of the mRNAs of these subunits increase the diversity of the integrin family. A subset of the integrin family binds to extracellular matrix (ECM) components such as fibronectin or vitronectin that contain the RGD (Arg-Gly-Asp) sequence; others recognize the overall conformation of these ligands (e.g., of laminin and collagen) (Hynes 2002). ECM proteins are widely distributed on host cells in basement membranes and cells of the connective tissue and form a dynamic interlinked 3D network supporting cell structures, cell barrier functions, and the regulation of cell signaling and physiology (Karamanos et al. 2021; Valdoz et al. 2021). Integrin interaction partners are not only limited to ECM but also include other factors, e.g. complement factors or plasma proteins such as fibrinogen as well as the hematopoietic cell receptors such as the intercellular adhesion molecules 1-5 (ICAM1-5) and vascular cell adhesion molecule 1 (VCAM-1) (van der Flier and Sonnenberg 2001).

Binding of extracellular ligands is achieved by the large ectodomain of integrins. It is transduced through the transmembrane domains and transmitted to the short cytoplasmic tails. This induces the formation of large intracellular protein complexes, which link ligand-bound integrins to the actin cytoskeleton. This integrin-mediated connection with external factors/stimuli and the internal side of the plasma membrane is highly dynamic and can be modulated in either direction—a process called outside-in and inside-out signaling (Gahmberg and Grönholm 2021). In this respect, integrin binding to ECM changes the shape and composition of the cytoskeleton beneath, and vice versa, modulation of the cytoskeletal interactions can control the affinity and avidity of the integrins with ECM (Bachmann et al. 2019).

This linkage and signaling is necessary for many integrin-controlled cell structuring processes, including ECM assembly and the formation of cell-substratum interactions (focal adhesions) (Gahmberg and Grönholm 2021). This is especially important for cell growth, migration and proliferation, formation and remodeling of

tissues and organs in multicellular organisms during development, angiogenesis, and hemostasis as well as in response to injury, inflammation, wound healing, and infection (Hynes 2002; Huttenlocher and Horwitz 2011; Brakebusch and Fässler 2005). In addition, integrin-promoted adhesion is crucial for the regulation of immune system during leukocyte trafficking, migration, and phagocytosis (Luo et al. 2007; Evans et al. 2009).

In the past few years, research has revealed that ligand-initiated integrin phosphorylation (in particular of the β chain enabled by α chain phosphorylation) regulates integrin activity by controlling the association of cytoplasmic factors and formation of signaling complexes on the cytoplasmic tail of the β chain (Gahmberg and Grönholm 2021). This triggers the activation of many other signaling events, e.g. the association and phosphorylation of other signaling proteins and cytoskeletal-adaptors (Gahmberg and Grönholm 2021). Induced signaling events by β_1, β_2, and β_3 integrins are best-known, of which β_1 integrins were in particular found to be crucial for pathogen–host cell interactions (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012; Hoffmann et al. 2011; Stewart and Nemerow 2007).

Cell adhesion via β_1 integrin-mediated signaling involves the replacement of bound filamin A by the recruitment of talin and kindlin-2. Subsequently, protein kinases such as the Src kinase, the focal adhesion kinase (FAK), and the spleen tyrosine kinase (Syk) are associated and activated. This process can be enhanced by transphosphorylation of the Src molecules through integrin clustering triggered by multivalent ligands (Gahmberg and Grönholm 2021). Additionally, downstream signaling molecules are recruited and activated, among them are protein kinases (PKCs, PKAs, cAMP-dependent kinases), phospholipases (PLCs), and α-actinin, as well as the small Rho GTPases RhoA, Rac1, and Cdc42 (Brakebusch and Fässler 2005; Mitra and Schlaepfer 2006; Meyer et al. 2000). Many interactions between integrins and the actin cytoskeleton occur via a direct contact of the actin-binding proteins such as filamin, talin, and α-actinin with the cytoplasmic tail of the β-integrin subunit or through other associated actin-binding proteins such as vinculin. Many of these factors are also implicated in pathogen-triggered signaling processes through integrins (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012; Hoffmann et al. 2011; Stewart and Nemerow 2007).

1.2 Fundamental Role of Integrins in Microbial Pathogenesis

During the long-lasting coexistence of microbes and mammals, microbial pathogens have evolved a variety of strategies to adhere to, invade, and subvert host cells in order to avoid or resist the innate immune responses, and to successfully proliferate and persist within host niches. The ability to bind to host cells is the most important step to initiate an infection and avoid immediate clearance by host defense mechanisms. Thus, it is not astonishing that pathogenic microorganisms such as bacteria, viruses, and even some parasites developed sophisticated strategies to interact with

cell adhesion molecules such as integrins and ECM proteins bound to integrins (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012; Hoffmann et al. 2011; Stewart and Nemerow 2007).

The first interaction of pathogens with ECM molecules was identified over 50 years ago (Kuusela 1978). Since then, an arsenal of highly sophisticated bacterial adhesion and invasion factors (known as adhesins/invasins) and protein secretion machineries have been revealed which promote the first contact to target cells through integrins (Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012). Moreover, multiple viruses have been identified that successfully infect and enter host cells by direct binding or indirect engagement of integrins (Hussein et al. 2015; Stewart and Nemerow 2007). The most common integrin-recognition motif is a short amino acid sequence harboring an RGD (Arg-Gly-Asp) sequence displayed on bacterial adhesins/invasins or viral envelope/capsid-associated proteins (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007). Currently known adhesive factors of pathogenic bacteria and viruses, as well as their targeted integrins or ECM molecules are listed in Table 1.

A comparative analysis of the bacterial and viral factors that promote binding of the pathogen to host cells via integrins is difficult due to the structural and functional diversity of these pathogenicity factors. They are highly diverse, including monomeric and multimeric proteins with varying domain structures and compositions such as the classical monomeric type Va β-barrel autotransporters, the trimeric autotransporter adhesins of the Vc type as well as fimbriae and pili (Kochut and Dersch 2013). The more insight we gained into the complex behavior of some pathogens, the more we realized that many microbes have multiple adhesive molecules that promote interactions to a specific but also overlapping set of host cell integrins (for details and references, see Table 1). From *in vitro* cell culture assays or *in vivo* animal model systems we learnt that pathogens use integrins to (i) adhere to and enter host cells, (2) to move inside and between cells, and (3) modulate cell functions such as vacuole formation, cell growth and migration, and avoidance of phagocytosis (Scibelli et al. 2007). For bacteria, three general strategies of host cell entry have been described as, the “zipper”-, the “trigger”-, and the “invasome”--mechanism (Scibelli et al. 2007). Examples of the role of integrins can be found for each of these entry mechanisms, and important information can be gained on how pathogens subvert host cell signaling via integrins to initiate the formation of a phagocytic cup, trigger engulfment of the bacteria, and promote their internalization into a membrane-bound vacuole known as the bacterial phagosome. The interaction with integrins in these processes can be generally achieved either by direct binding to integrin receptors or by indirect binding using components of the ECM as bridging proteins (Fig. 1).

Table 1 Role of pathogens targeting integrins and ECM proteins in disease development.

Pathogen	Associated disease, pathogenic process	Pathogenic process	Involved integrin(s) and/or ECM proteins	Involved microbial factor	References
Bacteria					
Acinetobacter baumannii	Pneumoniae, bacteremia, wound infections	Cell adhesion	Collagen (I, III, IV, V), laminin	**Ata** (trimeric autotransporter adhesin)	Vaca et al. (2020), Bentancor et al. (2012), Riess et al. (2004)
			Fibronectin	**OmpA** (Omp38) **Omp33** (porins)	
Bartonella henselae	Cat-scratch disease, bacillary angiomatosis and peliosis, vascular tumor formation, activation of hypoxia-inducible factor 1, secretion of vascular endothelial growth factor	Cell adhesion, cell invasion, prevents phagocytosis	β_1 integrins, collagen (III), laminin, fibronectin	**BadA** (trimeric autotransporter adhesin)	Vaca et al. (2020), Kaiser et al. (2008), Dabo et al. (2006)
			Fibronectin	**Pap31** **Omp43** **Omp89** (porins)	
Bartonella bacilliformis	Carrion's disease	Cell adhesion, cell invasion	$\alpha_5\beta_1$ fibronectin	**Brp/BbadA** (trimeric autotransporter adhesin)	Vaca et al. (2020), Henriquez-Camacho et al. (2015)
Bordetella parapertussis	Pertussis (mild)	Cell adhesion, cell invasion	β integrins	**P.70** (pertactin)	Li et al. (1991)
Bordetella pertussis	Pertussis or whooping cough	Cell adhesion, cell invasion, elicits immune responses	$\alpha_5\beta_1$	**FHA, P.69** (pertactin)	Relman et al. (1990), Saukkonen et al. (1991), Ishibashi et al. (2001), Everest et al. (1996), Osicka et al. (2015)
			Integrin complement receptor (CR3)	**CyaA** (adenylate cyclase toxin)	
Borrelia burgdorferi	Lyme disease, transmitted by tick bites	Cell adhesion, cell invasion, activation of	Fibronectin $\alpha_5\beta_1$	**BBK32**	Vaca et al. (2020), Coburn and Cugini (2003), Behera

(continued)

Table 1 (continued)

Pathogen	Associated disease, pathogenic process	Pathogenic process	Involved integrin(s) and/or ECM proteins	Involved microbial factor	References
		proinflammatory cytokines and matrix metalloproteases (MMPs)	Collagen (IV, VI)	**BBA33**	et al. (2006, 2008), Coburn et al. (1993), Probert and Johnson (1998), Zhi et al. (2015), Verma et al. (2009), Brissette et al. (2009, 2010), Wood et al. (2013)
			Laminin	**BmpA, B, C, D**	
			Fibronectin $\alpha_5\beta_1$	**RevA, B**	
			Collagen (I, III, IV), Fibronectin, laminin	**CspA, Z**	
			Laminin $\alpha_3\beta_1$	**ExpX** **BBB07** **BB0172**	
			β_3 integrins $\alpha_v\beta_3$ $a_{IIb}\beta_3$	**P66**	
Campylobacter jejuni	Gastroenteritis	Cell adhesion, cell invasion	$\alpha_5\beta_1$, fibronectin	**CadF** (Omp)	Vaca et al. (2020), Konkel et al. (2020)
			Fibronectin	**FlpA**	
Chlamydia trachomatis	Sexually transmitted diseases, reproductive dysfunction	Signaling, immune defense	β_1 integrins	**Ctad1**	Stallmann and Hegemann (2016)
			$\alpha_4\beta_7$	–	
Coxiella burnetii	Q fever	Cell invasion	$\alpha_v\beta_3$ $\alpha_M\beta_2$ (CR3)	–	Capo et al. (1999, 2003)

Escherichia coli spp.	Diarrhea, urogenital infections, hemolytic uremic syndrome (HUS)	Cell adhesion, activation of signaling in host cell	Fibronectin, laminin, collagen $\alpha_5\beta_1$	**Flagellin**	Vaca et al. (2020), Erdem et al. (2007), Moraes et al. (2015), Frankel et al. (1996), Allsopp et al. (2012)
			Fibronectin, laminin, collagen	**AAF/II** (fimbriae)	
			Collagen IV fibronectin	**Lfp1** (fimbriae)	
			Fibronectin, laminin	**UpaB** (autotransporter)	
			$\alpha_5\beta_1$ $\alpha_4\beta_1$	**Intimin**	
Haemophilus influenzae	Respiratory tract infections, meningitis	Cell attachment, cell invasion, induction of proinflammatory responses	Fibronectin, collagen (IV), laminin	**Hap** (autotransporter)	Vaca et al. (2020), Spahich et al. (2014), Su et al. (2016,2019), Singh et al. (2013)
			Laminin	**PE, PF** (surface proteins)	
			Laminin Fibronectin	**P4** (surface protein)	
Helicobacter pylori	Gastritis, peptic ulcer, gastric cancer	Injection of CagA virulence factor cell adhesion, cell invasion blocking T cell functions, exploits recycling of lymphocyte function associated antigen (LFA)-1	Laminin	**AlpA, B**	Barden and Niemann (2015), Buß et al. (2019), Skoog et al. (2018), Hennig et al. (2005), Kwok et al. (2007)
			$\alpha_5\beta_1$	**CagL**	
			β_1 integrins $\alpha_5\beta_6$	**CagY**	
			β_2, fibronectin	**VacA**	
Mycobacterium leprae	Leprosy	Cell adhesion, cell invasion	β_1 integrins, β_4 integrins, collagen, laminin, fibronectin	**ML2028**	Schorey et al. (1995), Byrd et al. (1993), Jin et al. (2019)

(continued)

Table 1 (continued)

Pathogen	Associated disease, pathogenic process	Pathogenic process	Involved integrin(s) and/or ECM proteins	Involved microbial factor	References
Mycobacterium avium complex *Mycobacterium avium* subsp. *paratuberculosis*	Pulmonary infections, fever, fatigue Johne's disease in cattle, Crohn's disease, paratuberculosis	Cell adhesion, cell invasion	β_1 integrins, fibronectin	**FAP**	Secott et al. (2004)
Neisseria gonorrhoeae and *N. meningitidis*	Gonorrhea, meningitis	Cell adhesion, cell invasion of cells, cytokine release	Fibronectin, vitronectin, $\alpha_5\beta_1$, $\alpha_v\beta_3$	**Opa, Opc,**	Dehio et al. (1998), Virji et al. (1995), van Putten et al. (1998), Nägele et al. (2011), Unkmeir et al. (2002), Jones et al. (2008)
			β_2-integrins (CR3, CR4)	**LPS** **NadA**	
Porphyromonas gingivalis	Periodontal disease	Cell adhesion, cell invasion	Fibronectin, vitronectin, $\alpha_5\beta_1$, $a_v\beta_3$ β_2-integrins (CR3)	**Fimbriae**	Nakagawa et al. (2005), Wang et al. (2007b), Hajishengallis et al. (2006)
Salmonella spp.	Gastroenteritis, systemic infections in humans, typhoid-like fever in mice	Cell adhesion, cell invasion	$a_1\beta_1$ β_1 integrins	**OmpV, PagN**	Vaca et al. (2020), Kaur and Mukhopadhaya (2020), Barilleau et al. (2021), Kingsley et al. (2004)
			Fibronectin, laminin	**Rck**	
			laminin	**PagC**	
			Fibronectin, collagen (I)	**ShdA** (autotransporter)	
			Fibronectin	**MisL** (autotransporter)	

Shigella flexneri	Diarrhea	Cell adhesion, cell invasion, decreases I, integrin affinity to ECMs	$\alpha_5\beta_1$	**IpaB,C** **IpaA**	Watarai et al. (1996), Demali et al. (2006)
Staphylococcus aureus	Sepsis, pneumonia, endocarditis, infections of soft tissues, arthritis-like symptoms	Cell adhesion, cell invasion	Fibronectin, $\alpha_5\beta_1$	**FnBPA, FnBPB**	Hoffmann et al. (2011), Kuusela (1978), Graham et al. (2005), Speziale and Pietrocola (2020), Niemann et al. (2021), Maurin et al. (2021), Meenan et al. (2007), Sinha et al. (1999), Zong et al. (2005), Chavakis et al. (2005), Ní Eidhin et al. (1998), McDevitt et al. (1997), Thomas et al. (2021), Schlesier et al. (2020), Pietrocola et al. (2020), Viela et al. (2019), Ponnuraj et al. (2003)
			$\alpha_v\beta_3$	**IsdB**	
			Fibronectin, $\alpha_5\beta_1$	**Atl**	
			Fibrinogen, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$	**ClfA,B Vhp**	
			Collagen	**CNA**	
			Collagen (I, IV, VI)	**Eap**	
			Fibronectin, collagen (I), vitronectin	**Emp**	
Streptococcus (Group A)	Toxic shock, necrotizing fasciitis	Cell adhesion, cell invasion, cytokine release	$\alpha_5\beta_1$, $\alpha_x\beta_2$ Fibrinogen	**M1**	Nobbs et al. (2009), Cue et al. (2000), Wang et al. (2007a), Herwald et al. (2004), Caswell et al. (2007), Humtsoe et al. (2005), Moschioni et al. (2010)
			Fibronectin, $\alpha_5\beta_1$ $\alpha_x\beta_2$	**Sfb1/F1** **F2/Pfbp/FbaB/PrtF2** **SfbX** **FnbA, FnbB** **FnB** **SfbII/SOF** **FbaA**	
			$\alpha_2\beta_1$	**Scl1/SclA** **Scl2**	

(continued)

Table 1 (continued)

Pathogen	Associated disease, pathogenic process	Pathogenic process	Involved integrin(s) and/or ECM proteins	Involved microbial factor	References
Streptococcus (Group B)	Neonatal meningitis, bacterial pneumoniae, sepsis	Immune activation, bacterial entry to CNS	Collagen, laminin, $\alpha_1\beta_1$	**ACP**	Graham et al. (2005), Nobbs et al. (2009), Bolduc and Madoff (2007), Love et al. (1997), Moschioni et al. (2010), Mu et al. (2014), De Gaetano et al. (2018)
			Collagen, $\alpha_5\beta_1$	**SspA, SspB (antigen i/II)**	
			Fibronectin, β_1 integrins	**Srr2, SfbA**	
			Collagen	**Cpa**	
			Collagen, vitronectin, laminin	**PilA, RrgA, PbsP Acb**	
Yersinia pseudotuberculosis, Y. enterocolitica, Y. pestis	Gastroenteritis, gastroenteritis, plague	Cell adhesion, cell invasion, cell adhesion, cell invasion, proinflammatory signaling	$\alpha_5\beta_1$	**InvA**	Isberg and Leong (1990), Tran Van Nhieu and Isberg (1993), Isberg and Barnes (2001), Schulze-Koops et al. (1993), Leong et al. (1990), Clark et al. (1998), Van Nhieu and Isberg (1991), Isberg and Van Nhieu (1995), Leong et al. (1995), Saltman et al. (1996), Leong et al. (1993), Takada et al. (1992), El Tahir and Skurnik (2001), Leo et al. (2008), Nummelin et al. (2004), Heise and Dersch (2006)
			Laminin, fibronectin, collagen	**YadA**	
			Laminin, fibronectin, collagen	**Ail**	

Viruses					
Adenovirus	Self-limiting diseases in the respiratory and gastrointestinal tract, and conjunctivitis	Cell attachment, cell entry, cell signaling, escape from the endosome	$\alpha_v\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_L\beta_2$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$,	**CAR** (Coxsackie- and adenovirus receptor)	Hussein et al. (2015), Hoffmann et al. (2011), Bieri et al. (2021), Nestić et al. (2019), Nemerow and Stewart (2016), Storm et al. (2017)
Coxsackievirus A and B (Enterovirus, Hantavirus)	Meningitis, herpangina, encephalitis, pericarditis, hepatitis, Hand-Foot-Mouth-Disease, devil's grip	Cell attachment and entry, virus uncoating	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$	Capsid protein **VP1**	Hussein et al. (2015), Hoffmann et al. (2011)
Ebolavirus	Ebola hemorrhagic fever	Cell entry	$\alpha_5\beta_1$		Schornberg et al. (2009)
Echovirus (Enterovirus B)	Influenza-like symptoms, meningoencephalitis, carditis	Cell attachment, cell entry	$\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_v\beta_1$	Capsid protein **VP1**	Hussein et al. (2015), Hoffmann et al. (2011), Jokinen et al. (2010), Merilahti et al. (2016)
Epstein-Barr virus (HHV-4)	Infectious mononucleosis, Burkitt lymphoma, hemophagocytic lymphohistiocytosis, Hodgkin's lymphoma	Cell attachment, cell entry, cell signaling	β_1 integrins, α_v integrins, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$	**LMP1**, **LMP2A**, **gH, gL**	Morris et al. (2016), Fotheringham et al. (2012), Chesnokova and Hutt-Fletcher (2011)
Foot-and-mouth disease virus (FMDV)	Foot-and-mouth disease	Cell attachment, cell entry	$\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_v\beta_1$,	**Coat protein**	Hussein et al. (2015), Hoffmann et al. (2011), Kotecha et al. (2017)
Hantavirus	Hemorrhagic fever with renal and pulmonary syndromes	Cell attachment, cell entry, cell signaling	$\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$		Hoffmann et al. (2011)

(continued)

Table 1 (continued)

Pathogen	Associated disease, pathogenic process	Pathogenic process	Involved integrin(s) and/or ECM proteins	Involved microbial factor	References
Herpes simplex virus (HSV, HHV-1)	Cold sores, genital herpes	Cell attachment, cell entry, activator of innate immunity	β_1 integrins	**gB**	Hussein et al. (2015), Hoffmann et al. (2011), Campadelli-Fiume et al. (2016)
			$\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_v\beta_3$	**gH, gL**	
Human herpesvirus 8 (HHV-8/Kaposi's sarcoma-associated herpesvirus—KSHV)	Kaposi's sarcoma, primary effusion lymphoma, HHV-8-associated multicentric Castleman's disease, KSHV inflammatory cytokine syndrome	Cell attachment Cell entry	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_9\beta_1$,	**gB**	Hussein et al. (2015), Hoffmann et al. (2011), Campadelli-Fiume et al. (2016)
Human cytomegalovirus (HCMV, HHV-5)	Fever, pharyngitis, symmetrical adenopathy	Cell attachment, cell entry	$\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$	**gB**	Hussein et al. (2015), Hoffmann et al. (2011), Campadelli-Fiume et al. (2016)
Human immunodeficiency virus type 1 (HIV)	Acquired immunodeficiency syndrome (AIDS)	Cell attachment cell entry, cell-to-cell spread	$\alpha_4\beta_7$	**gp41, gp120**	Nawaz et al. (2011), Cicala et al. (2011), Liu and Lusso (2020), Cafaro et al. (2021), Urbinati et al. (2021), Monini et al. (2012)
			$\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$	**Tat**	
Human papillomavirus	Cervical cancer, warts, oropharyngeal cancer, laryngeal papillomatosis	Cell attachment, cell entry	$\alpha_6\beta_4$, $\alpha_3\beta_1$, fibronectin, laminin		Hussein et al. (2015), Heuser et al. (2016), Aksoy et al. (2014)
Human and murine Polyomaviruses (JC virus, Merkel cell polyomavirus, Simian virus 40)	Progressive multifocal leukoencephalopathy, Merkel cell cancer, etc.	Cell attachment, cell entry	$\alpha_4\beta_1$, $\alpha_v\beta_3$ $\alpha_2\beta_1$	Capsid protein **VP1**	Langner et al. (2004)

Influenza virus	Flu	Cell attachment, cell entry	β_1 integrins (CD49a/ CD29) β_7 integrins (CD103)	**Hemagglutinin, neuraminidase**	Li et al. (2015), Reilly et al. (2020)
Reovirus	Diarrhea, intestinal distress	Cell attachment	β_1 integrins	Capsin protein **λ2**	Hoffmann et al. (2011), Koehler et al. (2021)
Rotavirus	Diarrhea	Cell attachment, cell entry	$\alpha_x\beta_2$, $\alpha_v\beta_3$	**VP7**	Hussein et al. (2015), Hoffmann et al. (2011), Fleming et al. (2014), Graham et al. (2005), 2006), Seo et al. (2008)
			$\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$	**VP4** (cleaved into VP5 and VP8 by trypsin)	
			$\alpha_1\beta_1$, $\alpha_2\beta_1$	Rotavirus **enterotoxin**	
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	COVID-19	Cell attachment cell entry Cell signaling	β_1 integrins, $\alpha_v\beta_3$	**Viral spike protein**	Park et al. (2021), Dakal (2021), Makowski and Olson-Sidford (2021), Carvacho and Piesche (2021)
Vaccinia virus	Rashes and fever	Cell attachment, cell entry	β_1 integrins		Hussein et al. (2015)
West Nile virus	West Nile fever	Cell attachment, cell entry	$\alpha_v\beta_1$, $\alpha_v\beta_3$	Envelope protein E	Hussein et al. (2015)

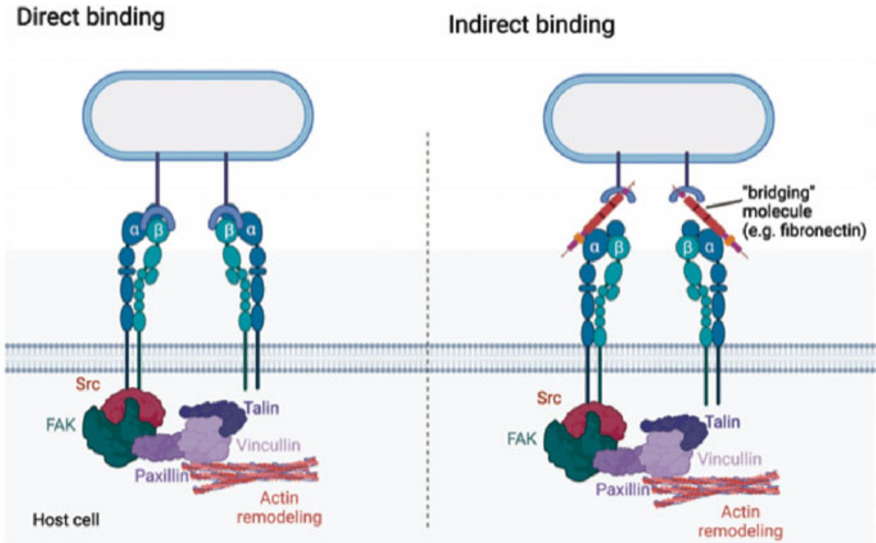

Fig. 1 Direct and indirect targeting of integrins by bacteria. Bacteria bind directly or indirectly via ECM molecules (such as fibronectin). This leads to phosphorylation of the cytoplasmic tail of the integrin chains, leading to subsequent recruitment and activation of various cell signaling molecules involved in cytoskeletal reorganization

2 Pathogen Interactions with Integrins

2.1 Direct Binding to Integrins

2.1.1 On Professional Phagocytic Cells

Macrophages and neutrophils are predominantly involved in pathogen recognition, binding, internalization, and elimination (phagocytosis). This phagocytic process is triggered by (i) F_c receptor-mediated uptake implicating the recognition of antibody-opsonized pathogens, and by (ii) receptor-mediated uptake of complement-opsonized pathogens (Sivaloganathan and Brynildsen 2021). During F_c receptor-mediated phagocytosis bacteria are attacked by bactericidal oxygenated compounds (reactive oxygen species/ROS; oxidative burst) and degradative enzymes and toxic peptides (defensins) are released upon lysosome fusion with the bacterial phagosome to kill the intruder. However, this defense strategy is circumvented by some intracellular pathogens which efficiently replicate in phagocytes by using complement receptors, such as CR3 ($\alpha_{MAC}\beta_2$ or CD11b/CD18) or CR4 ($\alpha_X\beta_2$ or CD11c/CD18) for their entry, which allow a bypass of the oxidative burst (Agramonte-Hevia et al. 2002). Prominent examples of pathogens that use this strategy are *Legionella pneumophila*, *Rickettsia typhi*, and *Bordetella pertussis*; however, pathogens like *Francisella tularensis*, *Escherichia coli*, *Neisseria meningitidis, Coxiella burnetii*, and *Neisseria gonorrhoeae* seem to use β_2-integrins

for their uptake into phagocytes (for details, see Table 1). The complement receptor-dependent phagocytic process appears to involve numerous integrin-triggered signaling events and implicates additional integrin receptors of the phagocytes. For example, uptake efficiency of *Coxiella* via $\alpha_{MAC}\beta_2$-mediated internalization was found to also depend on the activation of $\alpha_v\beta_3$ integrins (Capo et al. 1999).

2.1.2 On Non-Phagocytic Cells

A common theme in bacterial- or viral-host cell adhesion and internalization involves surface proteins with RGD- or non-RGD motifs that interact directly with integrins on epithelial and/or endothelial cells as well as lymphocytes, which normally are not phagocytic. In particular β_1 and β_3 integrin receptors have been shown to be very efficient in promoting pathogen entry. A very well-characterized and prominent example is the invasin-promoted cell entry via a set of β_1 integrins (Isberg and Leong 1990; Tran Van Nhieu and Isberg 1993; Isberg and Barnes 2001), the pertactin-triggered internalization of *Bordetella pertussis* (Leininger et al. 1991), the P66-induced uptake of *Borrelia* species (Coburn and Cugini 2003), and infection of various tissues by many viruses, e.g. the human echovirus 1 or Ebola virus (Jokinen et al. 2010; Schornberg et al. 2009). It is startling that the engagement of β_1 integrins, which are normally not implicated in endocytic processes triggers a phagocytosis-like uptake process of pathogens into non-phagocytic epithelial and endothelial cells. The driving mechanisms are still not fully understood, but it was speculated that pathogens are internalized by the force-generating machinery which is used to organize immobilized ECM proteins bound to β_1 integrins into a fibrillar network-like structure on the cell surfaces (Mao and Schwarzbauer 2005). Moreover, it was shown that the engagement and subsequent multimerization (clustering) of integrins trigger an endocytic entry mechanism (Ezratty et al. 2005). Observed clustering of the integrin receptors was found to trigger recruitment of signaling molecules, and the subsequent activation of signal transduction pathways which initiate internalization of the pathogen into target cells.

To initiate an integrin-mediated uptake process, a disengagement of the integrins from their natural ECM ligands is required. Thus, it is not surprising that individual pathogen ligands can often bind to integrins with a much higher affinity than the native host ligands. The primary invasion-triggering protein invasin (InvA) of enteropathogenic *Yersinia* species was shown to have a 1000-fold higher affinity to β_1-integrins than its natural ligand fibronectin (Tran Van Nhieu and Isberg 1993; Isberg and Barnes 2001). It is also well-known that ligands of some pathogens adopted similar structural determinants to bind with closely related domains of the α- and β-subunits of integrins. The most common binding motif includes the short tripeptide sequence RGD which is also found in many ECM proteins, and promotes the ability to interact with multiple integrin variants, which include $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha IIb\beta_3$ by bacteria and viruses (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Singh et al. 2012; Hoffmann et al. 2011). However, non-RGD-binding integrins are also targeted by bacteria and viruses through other peptide

motifs (Table 1), e.g. the rotavirus spike proteins VP4, VP5, and VP7 utilize a YGL (Tyr-Gly-Leu) sequence to bind to $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, $\alpha_2\beta_1$ and $\alpha_x\beta_2$, respectively, to enter host cells (Fleming et al. 2014; Graham et al. 2004, 2005, 2006). Moreover, rotavirus enterotoxin NSP4 recognizes $\alpha_1\beta_1$ and $\alpha_2\beta_1$ which initiates diarrhea (Seo et al. 2008). Simultaneous or cooperative engagement of several integrins by various ligands as well as the oligomeric nature of many adhesive factors (including the *Yersinia* InvA protein) promotes specific conformational changes and integrin receptor clustering, that enhance intracellular signaling events important for pathogen entry (Dersch and Isberg 1999).

In the early stage of infection, bacteria and viruses do not solely interact with integrins, but also establish contacts with other cell surface molecules to enhance (i) cell attachment, (ii) the induction of endocytic uptake of the pathogen, and/or (iii) the initiation of cytoplasmic signaling processes associated with the infection. In particular binding to host cell carbohydrate moieties (e.g., of glycoprotein, glycolipids) is critical for the entry of many viruses as this interaction triggers conformation changes in the virus structure that is crucial for the interaction with uptake-promoting receptors such as integrins (Hussein et al. 2015; Stewart and Nemerow 2007). For instance, the Human Immunodeficiency Virus (HIV-1) uses $\alpha_4\beta_7$, $\alpha_v\beta_5$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ as well as the galactosylceramide and glycosphingolipids to interact with host cells (Table 1).

2.2 Indirect Binding Via Extracellular Matrix Proteins

Various adhesins of numerous pathogens have been identified that bind integrins via ECM molecules, a strategy referred to as the "**bridging mechanism**"—often illustrated as sandwich model (Fig. 1) (Scibelli et al. 2007; Speziale and Pietrocola 2020; Arora et al. 2021). The exposure of ECM components in a dynamic, organized network on epithelial and endothelial cell surfaces makes them the preferred targets for initial microbial attachment and attacks (Vaca et al. 2020; Singh et al. 2012). To date, pathogenic microorganisms are shown to interact predominantly with fibronectin, collagen, and laminin, however interactions with elastin or vitronectin have also been identified (Table 1).

Fibronectin is a large heterodimeric, multidomain glycoprotein that occurs in a soluble (plasma) form in body fluids and in a fibrillar insoluble (cellular) form in the ECM. Both monomeric subunits are interconnected by a disulfide bridge and are composed of a linear series of repetitive five to six functional units, which contain various interaction sites for other ECM components or cell surface molecules such as heparin and fibrin (Henderson et al. 2011). Multiple fibronectin-binding proteins (FnBPs) of pathogens have been identified in the past. Strikingly, they were shown to possess distinct fibronectin-binding domains which interact with different sites of the fibronectin molecule. For instance, a tandem β-zipper region (GGXXXXV(E/D)(F/I)XX(D/E)T(Xx15)EDT has been revealed in FnBPs of *Staphylococcus aureus* and *Streptococcus pyogenes* which interacts with the N-terminal FI1-5 domain of

fibronectin (Hussein et al. 2015). Moreover, various canonical binding sites have also been identified for other bacterial adhesins in the FI2-5 and FI6/FII1-2, FI7-9 and FIII9-10, or FIII13 modules of fibronectin (BBK32 *Borrelia burgdorferi*, Omps *Bartonella henselae*, FlpA *Campylobacter jejuni*, UpaB, Lpf1 *Escherichia coli*, Ail *Yersinia pestis*, ShdA *Salmonella enterica*; Table 1). Exclusive binding of some microbial adhesion factors, such as the *Yersinia* YadA protein, and FnBPs of *S. aureus*, to the fibrillar form of fibronectin bound to integrins further enables the pathogen to adhere preferentially to host cells and prevents competition for circulating fibronectin in the serum (Schulze-Koops et al. 1993; Niemann et al. 2021).

Collagen, an important component of the ECM occurs as 28 different types of molecules which are made up of three α-helical polypeptide strands twisted together into a triple helix (Karamanos et al. 2021; Ricard-Blum 2011). The subunits are encoded by more than 45 different genes, but one distinctive feature (collagenous domain) is the regular arrangement of amino acids (Gly-Xaa-Yaa) in each of the three polypeptide chains. Notable for pathogenesis are fibrils that are found in connective tissues made by alloys of fibrillar collagens (mostly types I, II) and sheets constituting basement membranes (mostly type IV) (Karamanos et al. 2021; Ricard-Blum 2011). Thus, it is not surprising that collagen-binding proteins of pathogens predominantly target collagen types I and IV (Vaca et al. 2020; Singh et al. 2012) (Table 1).

Laminin consists of three subunits (α_1, β_1, and γ_1 with 15 isoforms) that form a heterotrimeric, multidomain protein with a cross-shaped structure. In particular the C-terminal five globular domains G1–G5 of the α-chain involved in integrin binding are also targeted by several bacterial factors (e.g., Ail *Y. pestis*, Paf *Pseudomonas aeruginosa*, P4 & Hap, PE & PF *Haemophilus influenzae*) (Vaca et al. 2020; Singh et al. 2012) (Table 1).

Use of ECM molecules as prime targets for host cell attachment and entry has multiple advantages: (i) ECM components are widely distributed on host tissues and this guarantees the pathogen an access at various locations throughout the infection, (ii) ECM molecules interact tightly with a broad range of integrins and other co-receptors, allowing the pathogen to gain an enormous adhesive and invasive potential, and (iii) ECM-mediated integrin interaction provides flexibility to the incoming pathogen to manipulate integrin-dependent cell signaling pathways for its successful internalization or even initiation of certain cell processes such as cytokine induction/inflammatory responses, cell migration or cell death pathways are initiated. Targeting of specific ECMs can also direct the pathogen to certain subtypes of integrins which may be specifically prone for the initiated pathogenic process. For instance, fibronectin-bound integrins in fibrillar adhesion formations seem to be associated with less/differently tyrosine-phosphorylated proteins compared to other adhesive structures, but they include multiple cytoskeletal components and signaling molecules, such as FAK, PI3 kinase, Src, Rho GTPases (RhoA, Cdc41, and Rac1), protein kinase C (PKC), MAPK (MEK, ERK1/2), NF_KB which play critical roles in the internalization of the pathogen into target cells at the site of attachment (Hussein et al. 2015; Vaca et al. 2020; Scibelli et al. 2007; Hoffmann et al. 2011).

2.3 Pathogen-Mediated Degradation of ECM Proteins

During infection, pathogens can also secrete proteases or manipulate host proteases in order to degrade ECM proteins and manipulate ECM-bound integrin functions. Among the secreted bacterial proteases are elastase and alkaline proteases of *P. aeruginosa* which cleave laminin and collagen type III and IV (Schmidtchen et al. 2003), the HtrA protease of *Helicobacter pylori* and *Listeria monocytogenes* degrading fibronectin, aggrecan and proteoglycans (Radhakrishnan et al. 2021; Backert et al. 2018), the *Clostridium* spp. collagenases and hyaluronidases (Shi et al. 2010; Pruteanu et al. 2011) as well as the *Y. pestis* Pla protein cleaving laminin and fibrin (Haiko et al. 2010). Moreover, several bacterial virulence factors are known to manipulate host proteases such as plasmin that degrade laminin, fibronectin, and collagen. For instance, the omptin family including Kop of *Klebsiella pneumoniae*, PgtE of *Salmonella*, and Pla of *Yersinia* degrades the plasminogen activator inhibitor (PAI) and α_2-antiplasmin controlling the synthesis of plasmin from plasminogen (Haiko et al. 2010). Several invasive pathogens also recruit plasminogen onto their surface and use special surface secreted enzymes to cleave it to plasmin, e.g. streptokinase from *Streptococcus* spp. and staphylokinase from *S. aureus* (Parry et al. 2000). A number of secreted bacterial proteases can also activate host metalloproteases which degrade various types of collagen and laminin (Singh et al. 2012).

To further illustrate how bacteria and viruses exploit integrin receptors to trigger pathogenic processes in more detail, we will focus on five of the well-characterized examples of integrin-mediated bacterial and viral pathogenesis in the following part of this chapter. We will further provide an overview of identified engagement of integrins by bacterial and viral pathogens in Table 1 to illustrate the diversified use and fundamental role of integrins in pathogenesis. The presented table lists the pathogens alongside their ligand/adhesive surface factors and the respective host cell integrin variants involved in the triggered pathogenic process. Information is also provided (if known) by which mechanism they utilize integrins to infect or manipulate host cells.

3 Selected Examples of Bacteria- and Virus-Triggered Integrin-Mediated Pathogenic Processes

3.1 Staphylococcus aureus

S. aureus, a non-spore forming gram-positive bacteria is a common human pathogen which is known to cause diseases ranging from infections of the soft tissues and skin to bacteremia, endocarditis, osteomyelitis, and hospital-acquired pneumonia. Although this bacterium occurs as a commensal in approximately 30% of the population worldwide, it produces a range of clinical manifestations in humans

resulting in two different epidemiology as health-care associated infections and community associated infections (Tong et al. 2015). To initiate the infection *S. aureus* interacts with the host tissues using its surface adhesins also known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that bind to the extracellular matrix proteins like fibronectin, fibrinogen, collagen, elastin, and keratin (Speziale and Pietrocola 2020; Arora et al. 2021; Foster et al. 2014). *S. aureus* adhesion to host cells is a prerequisite for its colonization in the tissue which is then followed by release of bacterial toxins resulting in tissue damage and dissemination to different organs (Josse et al. 2017; Maurin et al. 2021). *S. aureus* is predominantly an extracellular bacterium. However, it can invade host cells such as endothelial cells, epithelial cells, and osteoblasts that can result in the recruitment of monocytes and granulocytes, thereby generating an inflammatory response (Tong et al. 2015). Thus, it is not surprising that *S. aureus* expresses a broad range of adhesive/invasive surface molecules which promote interactions with a wide spectrum of ECM components such as fibronectin, laminin, collagen, and fibrinogen (Speziale and Pietrocola 2020; Arora et al. 2021) (Table 1). The best-characterized examples are the fibronectin-binding proteins.

3.1.1 Fibronectin–Integrin Interactions

The most common and highly investigated staphylococcal MSCRAMMs are fibronectin-binding protein A (FnBPA) and B (FnBPB) that bind to fibronectin (Josse et al. 2017). These staphylococcal surface proteins interact with two separate sites of fibronectin located within a 30 kDa N-terminal region, and 120–140 kDa C-terminal region (Kuusela et al. 1984). FnBPA and FnBPB are encoded by closely related genes *fnbA* and *fnbB* (located 682 bp downstream of *fnbA* stop codon) as identified in *S. aureus* strain 8325-4 (Speziale and Pietrocola 2020; Arora et al. 2021). FnBPA consists of (a) a signal peptide in the N-terminal followed by A-domain and fibronectin-binding repeats, (b) a proline-rich repeat, (c) and a C-terminal wall-membrane spanning region (Speziale and Pietrocola 2020; Jönsson et al. 1991). Its fibronectin-binding domain is a 38 amino acid sequence motif and consists of three complete repeats of D-units and a partial fourth unit (D1–D4), which are present close to the wall-spanning domain. FnBPB displays high amino acid sequence homology to FnBPA at several regions. The primary ligand binding D-domain in the two proteins is highly conserved (Jönsson et al. 1991). FnBPs D-domain binds to the N-terminal of fibronectin. LPXTG motif, which is crucial for attachment to the host cell, is located in the membrane spanning region. In 2007, Meenan *et. al.* identified a new classification of fibronectin-binding repeats (FBR) that consists of 11 fibronectin-binding sites in FnBPA (Meenan et al. 2007). These predicted FnBPA1-11 binding sites interact with varying affinity to fibronectin type-1 modules present at its N-terminal. When placed within two fibronectin-F1, FnB-peptide forms a β-strand that runs antiparallel to the individual F1 module. These F1-modules at the N-terminal of human fibronectin engage specific motifs in fibronectin-binding repeats and the number and sequence conservation of these

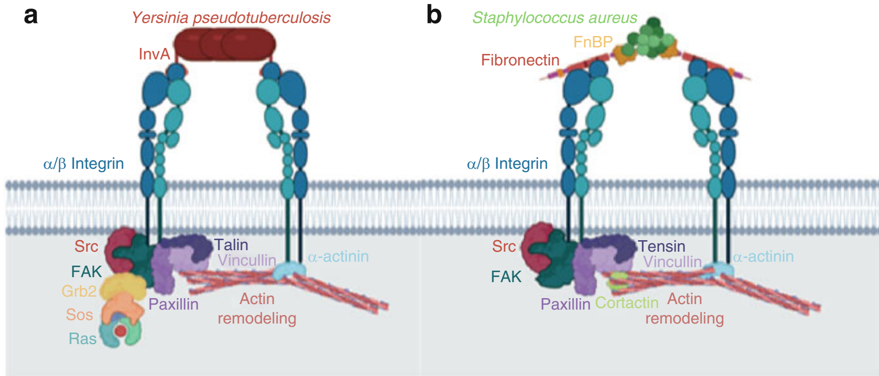

Fig. 2 Examples of integrin-mediated adhesion and invasion of bacteria. (**a**) Fibronectin-binding proteins (FnBPA and FnBPB) of *Staphylococcus aureus* and (**b**) the Invasin (InvA) protein of *Yersinia pseudotuberculosis* promote either an indirect or direct interaction to β_1 integrins leading to tight adhesion, phagocytic cup formation, and internalization of the bacteria in a membrane-bound vacuole

motifs are important for high affinity interactions between fibronectin and FnBRs (Meenan et al. 2007). Hence, according to this classification it can be said that high affinity binding sites for fibronectin are present outside the traditionally identified D-domain of FnBPs and that *S. aureus* can interact with fibronectin via a tandem β zipper mechanism. Due to the repetitive nature of the FnBPs, it is assumed that they associate with multiple fibronectin molecules. The interaction between staphylococcal FnBPs and fibronectin results in the formation of a bridge that gives access to the bacteria to contact and cluster fibronectin-bound $\alpha_5\beta_1$ integrins present on the host cell surface (Fig. 2a). This way the bacteria triggers endocytic cell signaling pathways and gains entry into the host cell via the zipper mechanism (Josse et al. 2017; Sinha et al. 1999). Notably, this type of bridging mechanism has been widely recognized in many pathogens including *Neisseria gonorrhoeae, Streptococcus pyogenes*, and *Yersinia* spp. for invading the host cell (Scibelli et al. 2007) (Table 1).

A recent study further demonstrated that not the crude amount but the supramolecular structure of fibronectin bound to integrins, i.e. the formation of fibrillar adhesion-like protein complexes plays an essential role in bacterial uptake (Niemann et al. 2021). This type of complex formation is followed by the recruitment of tensin, FAK, Src, zyxin, and vinculin (Agerer et al. 2005; Fowler et al. 2003). One factor that can activate FAK and Src kinases during the integrin-mediated internalization is cortactin which is an actin-binding protein that associates with the Arp 2/3 complex and promotes actin polymerization, but can also interact with dynamin-2, a regulator of membrane endocytosis (Selbach and Backert 2005). In fact, fibrillar adhesion-like contact sites induced by FnBPA expressing staphylococci were reported to promote bacterial movement on the surface of endothelial cells, where actin and Rab5 recruitment, and eventual invasion of the pathogens were observed (Schroder et al. 2006). Additionally, fibronectin/integrin-mediated cell adhesion and invasion

processes are mediated by other surface-bound (IsdB, Atl) or secreted ECM-binding (Emp) molecules of *S. aureus* (Table 1), although their interaction with the individual ECM components is less well investigated.

3.1.2 Collagen-Integrin Interactions

The collagen-binding adhesin (CNA) was the first adhesion factor isolated *from S. aureus* (Patti et al. 1992). This 135 kDa CNA protein is encoded by *cna* gene and contains a signal peptide in its N-terminal and LPXTG motif in its C-terminal for host cell attachment. The collagen-binding domain (CBD) is a 168 amino acid polypeptide located at its N-terminal and confers variable affinities to collagen (Patti et al. 1992). Crystallization of CNA with synthetic collagen revealed that CNA is composed of two separate subdomains (N1, N2) with antiparallel 9-11 β-strands and 1-2 α-helices separated by a flexible loop, which are wrapped around the collagen triple helix forming a hole/tunnel like structure (Arora et al. 2021; Zong et al. 2005). The collagen triple helix first interacts with the N2 domain which leads to a repositioning of domain N1 around the bound collagen. The subsequent locked position occurs through the insertion of an extended domain of N2 into the N1 domain, a principle described as the Collagen-Hug model which is a variation of the dock, lock-latch mechanism. Homologous proteins with related structures are the collagen-binding proteins SdrG of *S. epidermidis* and Ace of *Enterococcus faecalis* (Liu et al. 2007; Bowden et al. 2008).

In contrast to CNA, other staphylococcal collagen-binding proteins are not anchored to the bacterial cell wall, but secreted and therefore named “secreted expanded repertoire adhesive molecules” (SERAMs) (Chavakis et al. 2005). The extracellular adherence protein (Eap) which interacts with collagens I, IV, and V as well as the (Emp) binding to collagen I, fibronectin, and vitronectin has been identified (Table 1).

3.1.3 Fibrinogen-Staphylococcal MSCRAMM Interactions

Fibrinogen is an important plasma protein that is involved in the formation of blood clots and wound healing. This 340 kDa dimeric protein consists of six polypeptide chains, two α, two β, and two γ, and forms aggregates by adhering to the $\alpha_{IIb}\beta_3$ integrin receptors of platelets (Farrell et al. 1992). *S. aureus* can attach to fibrinogen present in the blood plasma or biomaterials coated with fibrinogen and form clumps. This is due to the presence of fibrinogen-binding MSCRAMM clumping factor A (ClfA) and B (ClfB) that can interact with the γ chain C-terminus in fibrinogen and help in the adhesion of *S. aureus* to plasma proteins (Ganesh et al. 2008*;* Ní Eidhin et al. 1998). The *clfA* gene encodes for a 92 kDa polypeptide that possesses the typical features of a gram-positive surface protein: an N-terminal region consisting of secretory signal and non-repetitive region A composed of N1, N2, and N3 subdomains responsible for ligand binding, an R-region consisting of

serine-aspartate repeats, a LPXTGX motif, and a cytoplasmic C-terminal for anchoring in the cell wall. ClfA is homologous to FnBPA and FnBPB of *S. aureus* and contains a binding site for $\alpha_{IIb}\beta_3$ integrin of the platelets (McDevitt et al. 1997). Subdomains N2 and N3 of the A region in ClfA binds to γ chain C-terminus in fibrinogen in a dock, lock-latch mechanism (Ganesh et al. 2008). Interestingly, FnBPA described above is also known to interact with fibrinogen and elastin via N2-N3 subdomains (Geoghegan et al. 2013). Quite recently, another *S. aureus* protein has been identified with significant sequence identity with the von Willebrand factor-binding protein (vWbp) and accordingly named Vhp. Up to date three distinct alleles, *vhpA*, *B*, and *C*, have been identified in the *S. aureus* core genome, and all isoforms bind fibrinogen with high affinity, targeting a site located in the D fragment of fibrinogen (Thomas et al. 2021).

3.2 Streptococcus *spp.*

Similar to staphylococci, *Streptococcus* species also evolved a number of surface proteins for binding to matrix components of their host. Various proteins from streptococci bind to ECM molecules, and different classes of integrins ($\alpha_5\beta_1$ $\alpha_2\beta_1$, and $\alpha_v\beta_3$) on host cells were found to bind to streptococci-immobilized ECM molecules, which results in internalization of the bacteria (Jiang et al. 2019; Nobbs et al. 2009). Group A streptococci (GAS) that can cause uncomplicated infections of the skin and throat, such as pharyngitis or life-threatening illnesses, toxic shock and necrotizing fasciitis express the surface protein M1, which promotes streptococcal survival in the blood and binds fibronectin to mediate invasion into epithelial cells (Cue et al. 2001; Ochel et al. 2014). M1 protein-mediated GAS entry into epithelial cells was shown to be abrogated by $\alpha_5\beta_1$-specific antibodies (Cue et al. 2000) and was found to require activation of the PI3 kinase, paxillin, and the integrin-linked kinase (ILK) (Purushothaman et al. 2003; Wang et al. 2007a). It has also become evident that M1-protein mediated ingestion also depends on the co-engagement of the membrane cofactor protein (MCP or CD46), a widely distributed complement regulatory human protein, indicating that cooperative action between integrins and co-receptor (MCP) might facilitate fibronectin-mediated uptake through integrin receptors, which is quite similar to the entry of $OpaA^+$ gonococci (Rezcallah et al. 2005). However, a more detailed analysis of the mechanism by which co-receptors and β_1-integrins cooperate is required to fully understand the relevance of receptor interaction for the internalization process. Notably, also $\alpha_v\beta_1$ integrins have been shown to play a role in M1 protein/fibrinogen interactions with neutrophils (Herwald et al. 2004).

70% of human GAS isolates produce another fimbrial adhesin Sfb1 (also termed F1 or PrtF1) which also mediates invasion into epithelial cells (Talay et al. 2000). Similar to the FnBPs of *S. aureus*, Sfb1 attaches the plasma content and the ECM protein fibronectin to the surface of the bacteria by a cooperative binding mechanism similar to tandem β-zipper mechanism observed for the staphylococcal FnBPs. The

fibronectin-binding region of Sfb1 is comprised of a 43-residue upstream domain and a repeat domain (RD2) comprised of five tandem 37-residue sequences. By using recombinant Sfb1 derivatives, the Sfb1 repeat region was found to interact first with the 30 kDa N-terminal fragment of fibronectin. This enabled tight adherence and was a prerequisite for subsequent activation of the adjacent Sfb1 spacer region to bind 45 kDa collagen-binding fragment of fibronectin, which efficiently triggered the invasion process (Talay et al. 2000). Sfb1-mediated uptake appears to activate similar signaling pathways like M1, including the activation of PI3 kinase, paxillin, and ILK, but distinct morphological changes at the port of entry suggest that different signals are also induced (Wang et al. 2007a). A requirement for the tyrosine kinases FAK and Src, as well as the small GTPases Rac and Cdc42 has also been reported. The important role of integrin-mediated signaling in GAS uptake was further supported by the fact that a GAS infection of primary human tonsil fibroblasts resulted in a strong stimulation of TGF-β1 production, which in turn induced $\alpha_5\beta_1$ integrin and fibronectin expression and increased intracellular invasion by GAS (Wang et al. 2007a). Multiple homologs, such as F2(Pfbp), FnbA, FnbB, and SfbX are found in different GAS (Table 1).

Recently, another streptococcal adhesin, the cell wall-associated collagen-like protein Scl1 (also known as SclA) of GAS has been shown to induce internalization of GAS via direct interactions with human $\alpha_2\beta_1$ integrin receptors (Caswell et al. 2007). The surface-exposed portion of Scl1 contains a distal variable region, forming the globular domain, that allows interaction with low-density lipoproteins (Han et al. 2006), and a central rod-shaped collagen-like domain, which is composed of repeating GXY sequences, that directly interacts with $\alpha_2\beta_1$ integrins with a relatively high affinity ($K_D = 17$ nm) (Humtsoe et al. 2005). Although the exact function and role of this protein in GAS pathogenesis remains unelucidated, it was shown that Scl1-$\alpha_2\beta_1$ integrin binding induces intracellular signaling, including phosphorylation of FAK, Cas, and paxillin, which enhances GAS internalization, and increases the overall capability of the pathogen to survive intracellularly and re-emerge (Humtsoe et al. 2005).

Group B Streptococcus (GBS) is the leading cause of bacterial pneumonia, sepsis, and meningitis among neonates and a cause of morbidity among pregnant women and immunocompromised adults (Arisoy et al. 2003). Little is known about the events that lead to GBS cell uptake, but expression of the alpha C protein (ACP) was shown to result in GBS internalization and translocation across human cervical epithelial cells (Bolduc and Madoff 2007). ACP is the prototype of a family of surface proteins known as alpha-like proteins found on 90% of the GBS clinical isolates. APC consists of two distinct N-terminal domains D1 and D2, followed by a number of tandem repeats and a C-terminal region containing the LPXTG peptidoglycan-anchor. D1 forms a β sandwich with strong homology to fibronectin's integrin-binding region (FnIII10) and this domain of ACP appears to strongly interact with collagen and laminin-binding $\alpha_1\beta_1$ integrin receptors (Bolduc and Madoff 2007). However, it is still unknown, what biological effect GBS may have on its hosts, when bound to $\alpha_1\beta_1$ integrins.

Besides GAS and GBS, other streptococci species have also been shown to produce surface proteins, which promote adherence and internalization into host cells via β_1 integrins, but considerably less information is available on their molecular function (Table 1). These adhesins include the SspA and SspB (antigen I/II family) proteins of oral *Streptococcus gordonii*, which interact with $\alpha_5\beta_1$ integrins through sequences within the N-terminal region in a fibronectin-independent manner (Love et al. 1997). Interestingly, this commensal bacterium triggers a low frequency internalization process, and a much lesser induction of proinflammatory cytokine release than pathogenic *S. pyogenes* (Nobbs et al. 2007).

3.3 Yersinia *spp.*

The genus *Yersinia* includes three pathogenic species, *Y. pestis, Y. enterocolitica*, and *Y. pseudotuberculosis* that cause diseases in humans and animals. *Y. pestis* is the causative agent of plague and is usually spread by flea bite or through aerosols. On the contrary, *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogens that are transmitted via contaminated food and water and result in infection of the gastrointestinal tract (Wren 2003). Despite different routes of infection, all three pathogenic *yersiniae* display tropism to lymphoid follicles, as *Y. pestis* find its way from dermis to nearby lymph node via the host lymphatic system whereas enteropathogenic *yersiniae* colonize the microfold cells or M-cells of the intestinal epithelium and translocate to the underlying lymphoid follicles in the Peyer's patches. Inside these follicles, *Yersinia* can replicate extracellularly and reside as microcolonies (Oellerich et al. 2007; Davis et al. 2015). However, this extracellular pathogen is also known to survive in macrophages and neutrophils in an early stage of infection, which implies that the bacteria can use these lymphoid follicles as a replicative niche, and an uncontrolled bacterial replication can thus result in its systemic spread to spleen, liver, and mesenteric lymph nodes (Moreau et al. 2010; Spinner et al. 2014; Pujol and Bliska 2003).

For a successful pathogenesis, *Yersinia* uses different virulence factors encoded on the chromosome and a 70 kb virulence plasmid (known as pYV in *Y. pseudotuberculosis* and *Y. enterocolitica,* and pCD1 in *Y. pestis*) type III secretion system (Ysc-Yop T3SS complex). They use these factors to (a) adhere and invade host cells and disseminate into deeper tissues, and (b) block and escape immune defense mechanisms of the host (Atkinson and Williams 2016). To aid in cell entry, the bacteria express surface proteins such as Ail, Pla, and Psa in *Y. pestis*, and Ail, InvA, and YadA in *Y. enterocolitica* and *Y. pseudotuberculosis* that develop initial contact with host cells (both phagocytic and non-phagocytic) either via direct interaction with integrins or by attaching to proteins of the ECM, and secretion of Yops into these cells (Mikula et al. 2012).

3.3.1 Direct Interaction of Invasin (InvA) with β_1-Integrins

Invasin is one of the best-characterized and most efficient bacterial adhesins/ invasins. This protein is expressed chromosomally by *invA* gene in *Y. enterocolitica* and *Y. pseudotuberculosis* but is absent in *Y. pestis* due to an insertion of 708 bp IS200-like element in the central region of the gene (Miller and Falkow 1988; Simonet et al. 1996; Isberg et al. 1987; Grassl et al. 2003). Presence of InvA imparts an invasive phenotype which is an essential factor for enteropathogenic *Yersiniae* in the early phase of infection (Miller and Falkow 1988). Invasin aids in the attachment and internalization of *Yersinia* into the M-cells of the small intestine followed by their translocation to the underlying Peyer's patches (Marra and Isberg 1996; Pepe et al. 1995).

The 986 amino acid invasin protein consists of an N-terminal domain (500 aa) that contains a signal peptide necessary for its insertion in the outer membrane, followed by a β-barrel transmembrane and an extracellular C-terminal domain (497 aa) to interact with β_1 integrins present on the host cell surface (Tran Van Nhieu and Isberg 1993; Isberg and Barnes 2001; Leong et al. 1990). The crystal structure of 503-986 residues of *Y. pseudotuberculosis* invasin describes an array of surface-exposed β-stranded domains D1-D4 also known as the bacterial immunoglobulin-like domains (BIG) followed by a C-type lectin-like domain D5 (consisting of α+β strands) at the end of the terminal (Hamburger et al. 1999). These domains are highly conserved between the two enteropathogenic *Yersinia* except the absence of self-associating domain D2 in Y. *enterocolitica* (Grassl et al. 2003). The 192 amino acid sequence within the carboxy terminal possesses the integrin-binding domain that interacts with five members of β1 class of integrins ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_1$) (Isberg and Leong 1990; Tran Van Nhieu and Isberg 1993; Leong et al. 1990) (Fig. 2b). The abundant expression of β_1 integrins on the apical side of the M-cells makes them the preferred cell types for initial attachment by *Yersinia* as compared to other enterocytes that express integrins on the basolateral side (Clark et al. 1998). Unlike the naturally occurring ECM proteins such as fibronectin and vitronectin that bind to the integrins at the tripeptide RGD site (Ruoslahti 1996), invasin lacks a typical RGD region, but it can still bind to $\alpha_5\beta_1$ integrin with a much higher affinity (Van Nhieu and Isberg 1991; Isberg and Van Nhieu 1995). Asp811 and Asp911 residues found in the C-terminal region along with the 76 amino acid long disulphide loop between Cys907 and Cys982 of *Y. pseudotuberculosis* invasin are known to be responsible for this higher binding affinity (Leong et al. 1995; Saltman et al. 1996; Leong et al. 1993). Based on the information derived from the crystal structure of invasin, it is clear that this protein displays some similarities to fibronectin in terms of receptor recognition. Competitive inhibition studies have further provided evidence that the two ligands bind to the same site (or in close proximity) to β_1 integrins. A single point mutation of Asp130 in β_1 subunit of $\alpha_5\beta_1$ can interfere in the binding of both fibronectin and invasin to this integrin (Takada et al. 1992).

Integrin–invasin interaction initiates internalization of *Yersinia* into the M-cells. Several factors are known to influence this invasin-promoted uptake by these cells as: (a) high affinity of receptor (integrin)–ligand (invasin) interaction, (b) ability of invasin to promote β_1 integrin clustering at the cell membrane, (c) increase in β_1 integrin concentration at this site of interaction (Dersch and Isberg 1999). D2 region of the *Y. pseudotuberculosis* invasin can enhance invasin-mediated uptake by integrins by initiating self-association of this receptor. This self-association causes multimerization of several integrin receptors which in turn is necessary for generating signals for integrin-dependent cytoskeletal rearrangements (Dersch and Isberg 1999). Upon ligand binding, α and β chains of β_1 integrins transmit downstream signals via their cytoplasmic tails. This cytoplasmic domain plays important functions of cytoskeletal association and signal transduction. Besides, cytoplasmic domain of β_1 integrins can also interact with actin-binding cytoskeletal proteins such as talin, kindlin-2, α-actinin, paxillin, ICAP-1, and focal adhesion kinase (FAK) (Gahmberg and Grönholm 2021). They are also known to play an antagonizing role in cytoskeletal association and bacterial internalization. The direct interaction of invasin with β_1 integrins generates a number of intracellular signals, such as the association and activation of tyrosine kinases, small GTPases, cytoskeletal factors, and MAP kinase cascades resulting in bacterial uptake, the expression of collagenase-1 and NF_kB-regulated cytokines (Wong and Isberg 2005; Werner et al. 2001; Kampik et al. 2000; Bohn et al. 2004). Downstream signaling cascades involving tyrosine kinases FAK and Pyk2, the Crk-associated substrate (Cas) and Crk, the integrin-linked kinase (ILK), phosphoinositide 3-kinase (PI3 kinase), the guanosine dissociation inhibitor RhoGDI, Arf6 and phosphoinositol-4-phosphate-5-kinase (PIP5K), as well as the small GTPase Rac-1 and the Arp2/3 complex have been found to play important roles in invasin-mediated uptake of *Yersinia* into host cells (Wong and Isberg 2003, 2005; Wang et al. 2006; Uliczka et al. 2009; Alrutz et al. 2001; Owen et al. 2007; Wong et al. 2006).

3.3.2 Indirect Interaction of YadA and Ail via ECM Molecules with β_1-Integrins

Yersinia adhesin A (YadA), an adhesin found in pathogenic *Yersinia* is expressed by the *yadA* gene present extrachromosomally on the virulence plasmid pYV. YadA is an important virulence factor in *Y. enterocolitica* and its absence results in avirulence in mice. YadA is required for interaction with the intestinal layer and the persistence of *Y. enterocolitica* in the Peyer's patches (Pepe et al. 1995). However, its role is not indispensable in *Y. pseudotuberculosis* and this could be due to the presence of InvA which might compensate for the loss of YadA (Han and Miller 1997). In *Y. pestis yadA* is dysfunctional due to the presence of a frameshift mutation.

YadA belongs to the family of trimeric autotransporter adhesin (TAA). This homotrimeric protein consists of a C-terminal which is anchored in the outer membrane, an intermediate stalk, and an N-terminal globular head providing an overall lollipop like structure (El Tahir and Skurnik 2001; Hoiczyk et al. 2000).

Unlike invasin, YadA mediates indirect attachment to β_1 integrins of the host cell surface by interacting with multiple proteins of the ECM like fibronectin, laminin, collagen (I, II, III, IV, V, and VI) (El Tahir and Skurnik 2001; Leo et al. 2008). The head-stalk structure in YadA. provides binding site to the different host ECM proteins (Nummelin et al. 2004). Even slight changes in the head region can alter the ECM-binding capacity of YadA. This can be seen in case of $YadA_{pstb}$ (YadA of *Y. pseudotuberculosis*), where presence of a "unique domain" in addition to the head domain of $YadA_{ent}$ (YadA of *Y. enterocolitica*) at the N-terminus mediates strong binding to fibronectin-bound $\alpha_5\beta_1$ integrin (Heise and Dersch 2006). Also, a $YadA_{pstb}$ deletion variant lost its adhesiveness to fibronectin but gained adhesion to other ECM proteins like collagen and laminin, similar to that observed in $YadA_{ent.}$ Besides playing an important role as an adhesin, YadA also acts as an invasin helping *Y. pseudotuberculosis* gain entry into the host cells and this involves the recruitment and activation of many signaling molecules to the uptake site that are also implicated in the invasin-mediated uptake process (Uliczka et al. 2009; Heise and Dersch 2006; Eitel et al. 2005).

Attachment-Invasion Locus (Ail) is found in all the three pathogenic species of *Yersinia*. It was first studied in *Y. enterocolitica* for its role in adhesion-invasion to the host cell in a species-specific manner and resistance to killing by host serum (Miller and Falkow 1988; Pierson and Falkow 1993). Of the three most common adhesins found in *Yersinia* spp. Ail is the key adhesion factor in *Y. pestis* due to the absence of functional *inv* and *yadA* genes (Felek and Krukonis 2009). It is a 17 kDa chromosomally encoded outer membrane protein and belongs to the class of omptins that consists of eight strands of antiparallel β barrel with four long extracellular loops and three small intracellular loops spanning the bacterial membrane. In particular loops 2 and 3 seem to contribute to host cell binding, of which the mutant variants F80A (loop 2), S128A, and F130A (loop 3) had very severe and additive defects in host cell binding and Yop delivery (Tsang et al. 2017). The *Y. pestis*-encoded Ail/OmpX protein contains 70% amino acid sequence similarity to *Y. enterocolitica* Ail. Ail binds to proteins of the extracellular matrix like laminin and fibronectin. However, there is no evidence of Ail binding to collagen, which is another protein widely found in the extracellular matrix. Using *E. coli* as an expression system, it has been identified that Ail binds laminin specifically at a 40 kDa fragment on its C-terminal end known as LG4-5 (Laminin G like domain 4 and 5) and this induced β_1-integrin clustering (Yamashita et al. 2011; Dogic et al. 1998). Ail also promotes binding to fibronectin and thus enables host cell adhesion via different ECM molecules very similar to other bacterial adhesins (Tsang et al. 2013). In 2012, it was shown by Tsang *et. al.*, that Ail specifically binds to the ninth fibronectin repeat which is a 120 kDa region located in the center of fibronectin protein (Tsang et al. 2010, 2012). This binding of Ail to fibronectin and laminin is essential for the delivery of Yops into the host cells like phagocytic human monocytes THP-1 cells and non-phagocytic epithelial cells (Felek and Krukonis 2009).

3.4 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) belongs to the family of retroviruses and is transmitted as an enveloped single-stranded, positive-sense RNA virus. It is a causative agent of the acquired immunodeficiency syndrome (AIDS). HIV is divided into two major groups: HIV-1 closely related to the simian immunodeficiency virus originating from chimpanzees (SIVcpz) and HIV-2 closely related to an SIV strain found in sooty mangabeys (Sharp and Hahn 2010). Upon cellular entry, the provided genome is transcribed reversely into double-stranded DNA by the virally encoded reverse transcriptase. Another viral enzyme, integrase, as well as host co-factors further help to import the viral DNA into the cell nucleus where it gets integrated into the host genome.

HIV binds primarily via its surface-expressed glycoproteins gp41 and gp120 to CD4 receptors and its co-receptors CCR5 and/or CXCR4 of memory T cells, dendritic cells, and macrophages. Both gp41 and gp120 are encoded by the viral *env* gene. The primary transcript is the glycoprotein gp160 which is subsequently cleaved by a host cell protease to form gp41 and gp120. Both proteins are important for host cell binding and entry, and gp120 induces several signaling cascades within infected cells, for e.g. phosphorylation of FAK which are also known to be important for bacterial-triggered endocytic processes (Araújo and Almeida 2013). In addition, HIV-1 can bind to an array of cell surface receptors, such as heparan sulfate proteoglycans, C-type lectins, and $\alpha_4\beta_7$-integrins (Nawaz et al. 2011; Cicala et al. 2011) (Fig. 3a). $\alpha_4\beta_7$ integrin, the gut-homing receptor, was also found to be associated with CD4 and CCR5 on T cells at sites where HIV replicates (Cicala et al. 2011). gp120 has been shown to directly bind to $\alpha_4\beta_7$ integrins through the variable loop 2 and 3. Upon infection, $\alpha_4\beta_7$/CD4$^+$ T cells migrate within days from the genital or rectal tissues to the Peyer's patches as well as mesenteric lymph nodes. There, gp120-$\alpha_4\beta_7$ interaction mediates the activation of the adhesion-associated integrin LFA-1, which in turn facilitates the formation of virological synapses and cell-to-cell spread of HIV-1 (Nawaz et al. 2011; Cicala et al. 2011; Liu and Lusso 2020). Notably, in macaques, treatment with anti-$\alpha_4\beta$ integrin antibodies reduced viral load and mucosal transmission of the simian immunodeficiency virus (Frank et al. 2021). Moreover, due to its size of nearly 22 nm, $\alpha_4\beta_7$ integrins are very prominent receptors for efficient virus binding compared to CD4 (~ 7 nm) alone. Furthermore, HIV virions incorporate $\alpha_4\beta_7$ integrins which remain fully functional in terms of their binding to MAdCAM-1. This endothelial cell adhesion molecule is selectively expressed on mucosal endothelial cells and interacts preferentially with the leukocyte β_7 integrin LPAM-1 ($\alpha_4\beta_7$), L-selectin, and VLA-4 ($\alpha_4\beta_1$) on myeloid cells, driving memory T-cell re-circulation through mucosal and inflamed tissues (Tan et al. 1998; Ikeda et al. 2003).

Besides its glycoproteins, the trans-activator of transcription (Tat) of HIV which is released from infected cells, also binds to RGD binding integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ leading to viral entry into host cells via endocytosis. Tat homodimerizes and was shown to engage several receptors, including $\alpha_v\beta_3$ integrin, heparan sulfate

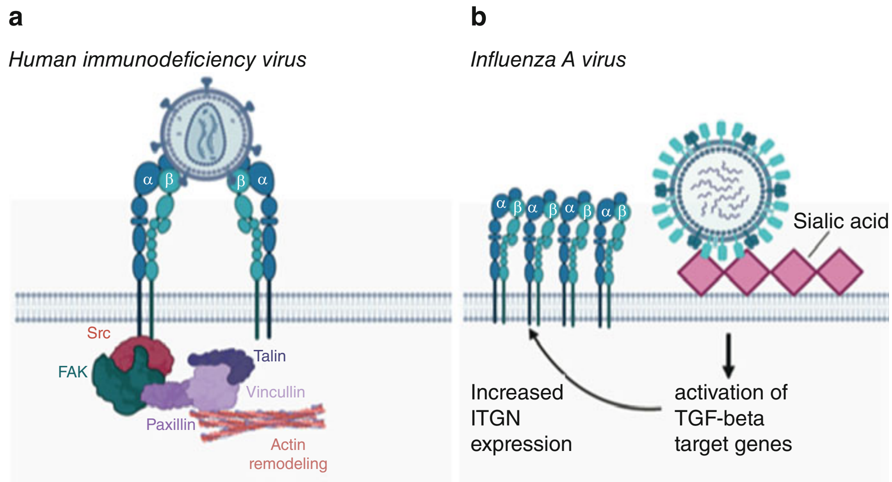

Fig. 3 Examples of integrin-mediated entry or modulation of signaling pathways targeting by viruses. (**a**) The surface glycoprotein gp120 of the human immunodeficiency virus (HIV) promotes direct interaction with $\alpha_4\beta_7$ integrins leading to cell attachment and virus entry, and (**b**) the surface proteins hemagglutinin and neuraminidase (HA/NA) of the influenza virus A trigger a signal transduction pathway leading to TNF-β expression with in turn induces β_1 and β_7 integrin expression with an influence on T cell adherence and motility

proteoglycan (HSPG) syndecan-1, and vascular endothelial growth receptor-2 (VEGFR2). Modeling further suggested that Tat binds to both α_v and β_3 chains (Cafaro et al. 2021). This induces cytoskeletal reorganization, formation of $\alpha_v\beta_3$ focal adhesion plaques, activation of lymphocytes, and polarization of endothelial cells through FAK, Src, and ERK1/2 which leads to endothelial cell dysfunction and increases extravasation of Tat-presenting lymphocytes (Hussein et al. 2015; Urbinati et al. 2021). Interestingly, internalized Tat only seem to promote HIV replication in endothelial cells treated with inflammatory cytokines (Cafaro et al. 2021). Moreover, not only Tat protein alone, but also Tat-Env complexes can be formed which target and promote viral entry by binding integrins on dendritic cells or other cell types (Monini et al. 2012).

3.5 *Influenza Virus*

Influenza virus is an enveloped virus belonging to the family of Orthomyxoviridae. Its segmented negative-sense RNA genome is encapsulated within the viral core consisting of the matrix and membrane proteins M1 and M2, respectively, as well as the surface glycoproteins neuraminidase (NA) and hemagglutinin (HA) (Krammer et al. 2018). To date, 4 different genera of influenza viruses have been reported, of which three mostly infect humans (Influenza A, B, and C viruses) causing severe

illness, seasonal epidemics as well as occasional pandemics (e.g., Spanish flu in 1918).

The infection cycle begins with binding of HA on the viral surface to sialic acid (SA) containing glycan receptors on the host cells such as epithelial cells, type II pneumocytes, dendritic cells, and alveolar macrophages mainly located in the respiratory tract but can also infect retinal epithelial cells (Shim et al. 2017). Furthermore, the NA glycoproteins of H3N2 viruses were found to facilitate viral entry into target cells during infection. Moreover, the sialidase activity of NA is able to remove SA residues from newly synthesized virions during budding, helping to prevent aggregation near the cell surface and thereby enhancing infectivity (Allen and Ross 2018).

Binding of the virions to these cellular receptors activates the PI3K-dependent pathway leading to phosphorylation of FAK at position Y397 which associates with integrins. This facilitates viral uptake via endocytosis as well as viral replication (Elbahesh et al. 2014). Throughout this whole process, influenza viruses have to sustain the host type I interferon response. Interferons initiate the expression of interferon-stimulated genes (ISGs) which encode a variety of antiviral proteins to i) inhibit viral replication in infected cells, ii) alert non-infected cells for potential infections, and iii) attract immune cells (Shim et al. 2017). In addition, the viral NA triggers the expression of TGF-β which activates Smad signaling pathway leading to the upregulation of $\alpha_5\beta_1$ integrin expression and exposure at the cell surface (Li et al. 2015) (Fig. 3b). In addition to TGF-β signaling, attachment and entry of influenza A virus induces an interaction of FAK with cyclophilin A (CypA) inhibiting the degradation of FAK (Bai et al. 2021). This entails that binding and uptake of secondary pathogens, such as *S. aureus*, *S. pneumoniae*, or *S. pyogenes* gets promoted via modulation of integrin-mediated uptake process (see also Sects. 3.1 and 3.2, Table 1). As CypA is also the intracellular receptor for cyclosporine A, a drug used in transplantation medicine, treatment of cells with cyclosporine A clearly reduced secondary bacterial infections of influenza A virus-infected bone-marrow-derived macrophages (Bai et al. 2021). Besides the induction of $\alpha_5\beta_1$ integrin expression on epithelial cell surfaces, an influenza A virus infection also drives resident memory $CD8^+$ T cells (T_{RM}) to express an additional set of integrins: namely CD49a/CD29 (β_1) and/or CD103 (β_7) with an influence on T cell adherence and motility. Following intranasal challenge of mice with an H3N2 strain, a large proportion of memory T cells within the lung parenchyma were positive for CD49a/CD29 (β_1) after three months (Reilly et al. 2020).

3.6 *Corona Virus SARS-CoV-2*

The corona virus SARS (severe acute respiratory syndrome)-CoV-2 emerged at the end of 2019. Since then, it spread very rapidly and caused a severe global pandemic. The clinical spectrum of infected individuals ranges from asymptomatic infections to fatal, life-threatening course of the disease referred to as Covid-19. Patients

experience fever, cough, and upper respiratory symptoms, and in severe cases can develop severe acute respiratory syndromes, including lung edema, surfactant depletion, and alveolar collapse (alveolar collapse acute respiratory syndrome, ARDS). In addition. many vascular and neurological problems and diverse Post-Covid syndromes occur (Cameli et al. 2021).

The predominant receptor of the SARS-CoV-2 virus is the angiotensin converting enzyme 2 (ACE2) on mammalian cells (Zamorano Cuervo and Grandvaux 2020). As this receptor is mainly expressed in the heart, gut, and kidney but less in lungs, other additional receptors may play a role for virus attachment and entry. In fact, recent studies showed that co-receptors such as basigin or neuropilin 1, and integrins also play a role in virus binding (Gahmberg and Grönholm 2021; Park et al. 2021; Sigrist et al. 2020). The viral spike protein contains the integrin-binding RGD motif which enables it to interact with β_1-integrins and likely $\alpha_v\beta_3$ which are highly expressed on human and mouse pulmonary epithelial cell lines (Park et al. 2021; Dakal 2021; Makowski et al. 2021; Carvacho and Piesche 2021). Another report indicates that SARS-CoV-2 also implicates the major endothelial integrin $\alpha_v\beta_3$ to cause vasculature permeability and leakage (Nader et al. 2021). In line with these results, integrin antagonists, which bind to the MIDAS site and stabilize the closed/inactive form, prevent SARS-CoV-2 binding, and RGD-integrin antagonists inhibit SARS-CoV-2 engagement independent of the integrin activation state (Simons et al. 2021). Until now, it is still unknown whether β_1-integrins act as co-receptor or function as independent receptor to strengthen SARS-CoV-2-receptor interaction (s) and facilitate virus entry. Interestingly, recent studies also reported that the SARS-CoV-2 spike protein triggers ECM-integrin reorganization and TGF-β signaling. This, similar to influenza infections could trigger barrier dysfunction and lead to an upregulation of integrin expression, which would also enhance RGD-promoted host cell binding (Carvacho and Piesche 2021; Biering et al. 2021).

4 Concluding Remarks

Cell adhesion and subsequent invasion or manipulation of host cells is of pivotal importance for microbes to initiate infection. Numerous viral and bacterial pathogens have been found to express surface-associated or secreted ligands that interact with integrin receptors or bind to host cell surface proteins (e.g., ECM components) that are bound to integrins. This interaction can trigger the internalization of viruses and bacteria ranging from 100 nm to 2 μm in size. An important attribute that makes integrins a preferred target for host cell interactions is their high prevalence on epithelial, endothelial, and immune cells in mammals. This allows the microbes to adhere to a large set of distinct cell types in various tissues of different hosts. Moreover, integrins are functionally linked to the intracellular cytoskeleton and participate in multiple signal transduction pathways. This not only enables the pathogen to trigger endocytic processes to promote their uptake, but also allows them to manipulate other specific functions (e.g., migration, proliferation, cell death

programs) and subvert defense mechanisms (e.g., manipulation of cytokine- and chemokine expression). Another property of integrins that make them ideal targets for microbes is that their ligand affinity as well as their signal transduction activity can be regulated by extracellular and intracellular components.

Microbes use both direct and indirect interactions (e.g., via ECM proteins) to associate with integrins, and in several cases, both types of integrin engagements are used by distinct adhesion molecules expressed in the same pathogen. Direct interaction may allow binding to integrins in their active and inactive forms, whereas indirect engagements provide host cell adhesion through diverse and well accessible cell surface-exposed host components. The different binding modes allow pathogens, depending on the binding affinity, ligand concentration, and ECM composition of the infected tissues, to stimulate individual sets of signaling events within the targeted host cell that triggers cell attachment, cell invasion, and cell processes to foster their colonization, proliferation, and dissemination in their hosts.

Although much is known about the molecular structure and interaction sites of the microbial adhesins, the ECM components, and different integrins, there is a lack of knowledge on how the cell membrane is reorganized in response to integrin-mediated adhesion to initiate entry of viral and bacterial pathogens. Some studies report a role of membrane microdomains and the lateral mobility of lipid raft components for the entry process (Hoffmann et al. 2010). For instance, bacterial fibronectin-binding factors were found to trigger the formation of membrane microdomains enriched with ganglioside GM1 and GPI-linked proteins, and agents disrupting these platforms impaired uptake of fibronectin-binding bacteria such as *S. aureus* and *S. pyogenes*. Moreover, presence of calveolin-1, which stabilizes ligand-bound receptors in cholesterol-rich domains and slows down microdomain mobility and endocytosis, also reduces the fibronectin/integrin-mediated uptake (Hoffmann et al. 2010, 2011). Until now the composition and dynamics of activation and association of signaling factors to the cytoplasmic domains of integrins and cell/tissue-specific differences are not fully understood. Complex integrin phosphorylation patterns upon integrin engagement regulate the formation of signaling complexes and downstream events, and co-receptors can crosstalk and manipulate this process (Gahmberg and Grönholm 2021; Gahmberg et al. 2019). Unraveling the dynamics of this complex molecular process is one of the most challenging and intriguing tasks in the analysis of host–pathogen interactions.

Several efforts have been made to use virus-mediated interactions with integrins as therapeutic tools. Recently, encouraging results have been generated from experimental studies and clinical trials using adenoviruses which target multiple integrins (Table 1) as vectors for oncolytic virotherapies. As an example, recombinant adenoviral vectors were generated which utilize the capsid protein (CAP) to target the prognostic cancer cell marker $\alpha_v\beta_6$ (Davies et al. 2021; Uusi-Kerttula et al. 2018). Rapid growth of our knowledge in integrin-mediated host cell–pathogen interactions has also led to the development of antimicrobial therapeutics targeting integrin interactions to prevent the attachment and internalization of viruses and bacteria. To date, several integrin antagonists such as synthetic peptides with RGD motif, disintegrins, and antibodies directed against distinct integrins are known to interfere

with integrins and may represent possible preventive and therapeutic tools (Slack et al. 2021). For instance, monoclonal antibody 3G9 blocking the function of integrin $\alpha_v\beta_6$ inhibited the interaction of *Helicobacter pylori* to host cells via CagL (Barden and Niemann 2015), and application of different RGD-containing peptides and antibodies directed against β_1 integrins successfully blocked invasin-mediated cell binding of *Yersinia pseudotuberculosis* (Isberg and Leong 1990; Leong et al. 1995). An effective dual $\alpha_v\beta_6/\alpha_v\beta_8$ ligand has been identified that haltered spread of the Herpes simplex virus (Tomassi et al. 2021). Several potent and selective integrin antagonists, including nonapeptides, cyclopeptides, and peptidomimetics with RGD motifs as well as non-peptidic small-molecule inhibitors have been published and also discussed as possible drugs against Covid-19 (Carvacho and Piesche 2021; Bugatti 2021), and a pulmonary EDTA chelation therapy interfering with SARS-CoV-2 spike-RGD-mediated integrin interaction was suggested (Dakal 2021). However, interfering with pathogen-promoted integrin engagements also comes with some drawbacks as: (i) manipulation of complex integrin-mediated signaling events may have undesirable side effects and risks, (ii) differential expression of adhesion factors at distinct time points of the infection course, and (iii) evolution of mutant variants which escape ligand binding, may hamper the development of these anti-virulence therapies. Nonetheless, considering the threat of emerging fatal pathogens like SARS-CoV-2 and our increasing problems with antibiotic-resistant bacteria, this approach might still be worth the effort and a valuable alternative strategy.

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