Chapter 5
Barrier Enhancing Signals

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Abbreviations

AJs	Adherens junctions
AKAP9	A-kinase anchoring protein 9
ANP	Atrial natriuretic peptide
ARAP3	ArfGAP, RhoGAP, ankyrin repeats and PH domain 3
Arf	ADP ribosylation factor
ARM	Armadillo
C3G	Cyanidin 3-Glucoside
CAR	Coxsackie and adenovirus receptors
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division cycle 42 GTP-binding protein
cGMP	Cyclic guanosine monophosphate
CNB	Cyclic nucleotide binding
DEP-1	Density enhanced phosphatase-1
EC	Extracellular repeat
ECM	Extracellular matrix
ECs	Endothelial cells
Epac	Exchange protein activated by cAMP
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FERM	Four-point-one, ezrin, radixin, moesin binding domain
Gab1	Grb2-associated binder 1
GAP	GTPase activating protein

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GDI	Guanine nucleotide dissociation inhibitor
GUK	Guanylate kinase-like homologues
ESAM	Endothelial cell-selective adhesion molecule
GEF	GTPase exchange factor
GIT1	G-protein-coupled receptor kinase-interacting protein 1
GJs	Gap junctions
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
HGF	Hepatic growth factor
HPAEC	Human pulmonary arterial endothelial cell
IQGAP	IQ motif containing GTPase activating protein
JAM	Junctional adhesion molecule
KRIT1	K-Rev1 interaction Trapped gene 1
MDCK	Madin-Darby canine kidney
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NPR	Natriuretic Peptide Receptor
PAF	Platelet-activating factor
PAK	P21 activated kinase
PI3K	Phosphatidylinositol 3-kinase
PTP	Protein tyrosine phosphatases
Rac1	Ras-related C3 botulinum toxin substrate
Rap1	Ras-related protein1
RGD	Arg-Gly-Asp peptide
Rho	Ras homologue
S1P	Sphingosine-1- phosphate
S1P1	S1P receptor 1
SHP2	Src Homology Phosphatase 2
TGF-β	Tumor growth factor β
Tiam1	T-lymphoma invasion and metastasis 1
TIMP2	Tissue inhibitor of metalloproteinase 2
TJs	Tight junctions
TNF-α	Tumor necrosis factor-α
Vav2	Vav2 guanine nucleotide exchange factor
VEGF	Vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein verprolin homologous
ZO	Zonula occludens
βPIX	Beta-p21-activated kinase-interactive exchange factor

5.1 Introduction

The continuous monolayers of endothelial and epithelial cells lining the luminal surface of pulmonary microvessels and the pulmonary alveoli, respectively, are vital for efficient gas exchange in the lung. Both monolayers are essential for minimizing the plasma leakage of blood (both cells and plasma) into pulmonary interstitium. Stability of the monolayer is highly regulated by a wide range of signaling pathways that ultimately enhance cytoskeleton rearrangement, cell–cell interactions, and cell–matrix attachment. Cell–cell interactions involve a complex network of adhesion proteins that bind to each other and are linked to intracellular cytoskeletal and signaling partners. These proteins are organized into distinct structures and can be categorized into three groups, namely, adherens junctions (AJs), tight junctions (TJs), and gap junctions (GJs). They provide structural support for intercellular connection and maintain membrane integrity. They also take part in actin cytoskeleton remodeling, signaling transportation, and transcriptional regulation. Various signaling proteins and second messengers involved in barrier function have been identified, including small GTPases, tyrosine kinases, serine/threonine kinases, and lipid mediators. In this chapter, we focus on the role of the barrier enhancement signaling pathways and their underlying mechanism. We also discuss their potential development of barrier protective therapeutics.

5.2 Role of Cell–Cell Adhesions Junctions in Regulating Endothelial/Epithelial Barrier Function

Endothelial cells (ECs) and epithelial cells have all three types of cell–cell junctions assembled at cell–cell adhesions that maintain the integrity of endothelium and alveoli (Fig. 5.1).

While AJs and TJs are essential for monolayer permeability, GJs allow changes in transmembrane potential and serve a portal for the selective and direct cell-to-cell transfer of signaling molecules and ions. Therefore, GJs may not directly regulate monolayer permeability. This chapter focuses on the nature of molecular compositions of AJs and TJs in ECs, which are similar to those in epithelial cells (Hartsock and Nelson 2008), with some exceptions.

5.2.1 Structure of AJs

ECs and epithelial cells express different AJs proteins. ECs express mainly vascular endothelial-cadherin (VE-cadherin) and neural cadherin (N-cadherin), whereas epithelial cells harbor epithelial cadherin (E-cadherin). Each cadherin has a relatively small cytoplasmic domain, a transmembrane domain, and a bulky extracellular binding domain. Both VE-cadherin and E-cadherin tend to form homogeneous

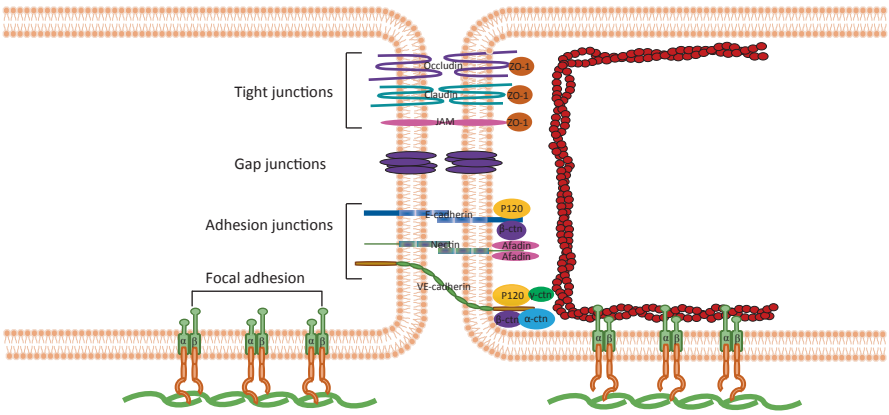

Fig. 5.1 Intercellular and matrix structures maintaining cell barrier function. Typical cell–cell contacts include adhesions junctions, tight junctions, and gap junctions. Occludin, claudins, and JAMs are the backbones of tight junctions. VE-cadherin, E-cadherin, and nectin are components of adhesions junctions for endothelial and epithelial cells. Integrins mediate the interaction between cell and matrix. These structures utilize intracellular binding partners (ZO, p120 catenin, β-catenin, γ-catenin, α-catenin, afadin, vinculin, and talin) to stabilize their localization and interaction with cortical actin cytoskeleton. (*VE-cadherin* vascular endothelial cadherin, *E-cadherin* epithelial cadherin, *JAMs* junctional adhesion molecules, *ZO* zonula occludens)

clusters in a Ca^{2+}-dependent manner. VE-cadherin plays a key role in the maintenance of the vascular barrier function. Most barrier protective agonists promote cadherin stability and enhancement at AJs (Fig. 5.2), however, these agonists initiate distinct signaling pathways. Although both endothelial and epithelial cells play critical roles in maintaining pulmonary permeability, ECs are more intensively investigated for the mechanisms of pulmonary permeability. Therefore, we mainly focus on the mechanisms of barrier enhancement observed in ECs.

5.2.2 VE-Cadherin Structure

Based on domain structure, genomic organization, and protein analysis, there exist five major groups of cadherins namely, the classical type I cadherins, atypical type II cadherin, desmogleins, desmocollins, and BS-cadherin (Nollet et al. 2000). VE-cadherin (CD144, cadherin 5, or type II cadherin) is encoded by the human gene CDH5. The protein is composed of five extracellular cadherin repeats (EC1–5), followed by a single transmembrane region, and a short cytoplasmic tail. It forms calcium-dependent *trans* homophilic interactions, through its EC, with VE-cadherins expressed on adjacent cells. The *trans* interaction is mediated by EC1 (Al-Kurdi et al. 2004; Hewat et al. 2007; Lambert et al. 2005). This is supported by the finding that antibodies to the EC1 region, but not EC3 and EC4, of VE-cadherin inhibit vessel formation and disrupt endothelial barrier function (Corada et al. 2001). *Trans* homophilic interaction is preceded by *cis* homophilic interaction of two adjacent

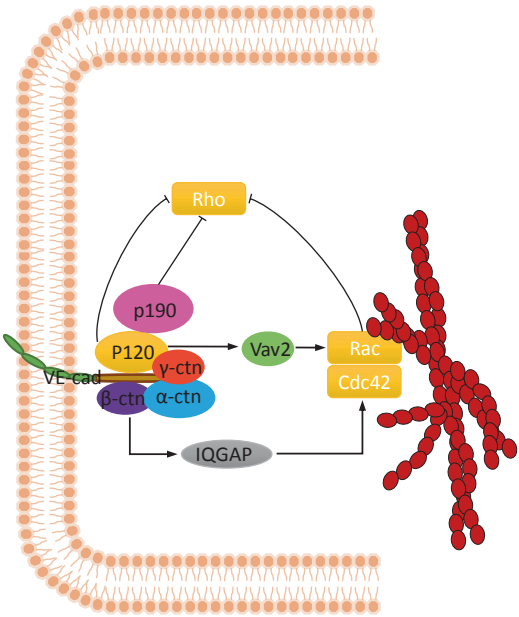

Fig. 5.2 Model of adherens junction (AJ) complex. The AJ complex is formed by homotypic interactions of transmembrane VE-cadherin, which binds to p120 catenin at the juxtamembrane domain and either β-catenin or γ-catenin at the catenin-binding domain. Attachment of actin cytoskeleton to VE-cadherin is mediated by α-catenin. Interaction of VE-cadherin with these plakoglobin proteins conveys signals to activate small GTPases Rac1 and Cdc42, or inactivate Rho, resulting in formation of cortical actin ring and enhanced barrier function. (*VE-cadherin* vascular endothelial-cadherin, *Rac1* ras-related C3 botulinum toxin substrate, *Cdc42* cell division cycle 42 GTP-binding protein, *Rho* ras homologue)

VE-cadherin molecules within the plane of the plasma membrane of one cell. *Cis*-interaction is mediated by lateral interactions between EC1 repeats of VE-cadherin molecules that are on the same cell surface. This interaction occurs via insertion of the side chain of conserved Trp2 and Trp4 residue into a complementary hydrophobic pocket in its partner (Patel et al. 2006). Both *cis*- and *trans* interactions are dependent on the presence of Ca^{2+}.

In addition to homophilic interactions, VE-cadherin interacts directly with proteins of the Armadillo-repeat gene family, including p120 catenin, plakoglobin (γ-catenin), and β-catenin through its cytoplasmic domain. Moreover, VE-cadherin also indirectly associates with other adaptors, cytoskeletal and signaling proteins, such as α-catenin, actin, and p190RhoGAP.

The cytoplasmic domains of VE-cadherin, N-cadherin, and E-cadherin are highly homologous. All of them contain a juxtamembrane domain and a C-terminal catenin-binding domain. Both domains interact with the armadillo-repeat domain (ARM domain) of p120 catenin, β-catenin, and plakoglobin proteins. The ARM repeats create a tertiary structure consisting of a positively charged groove that binds to the negatively charged region of cadherins^{8-} (Anastasiadis and Reynolds 2000). Similar

to p120 catenin, the binding of β-catenin and plakoglobin to VE-cadherin is also mediated through the ARM domains, and these two proteins bind to the CBD in a mutually exclusive fashion. β-catenin has 12 ARM repeats; each repeat is composed of three α-helices in close apposition, forming a superhelix. There is a 130-amino-acid N-terminal extension and a 100-amino-acid C-terminal extension. A 100-amino-acid region of cadherin, spanning residues 625–723, interacts across the entire 12 ARM motifs. The interaction between VE-cadherin and β-catenin occurs in early endoplasmic reticulum (ER) formation (Hinck et al. 1994) and VE-cadherin interacts with plakoglobin at a later stage of AJ formation (Schnittler et al. 1997).

5.2.3 Barrier Enhancement via VE-Cadherin

5.2.3.1 p120 Catenin-Mediated VE-Cadherin Enhancement

VE-cadherin availability at the cell surface is a critical determinant for vascular barrier integrity, therefore, many barrier enhancement signaling pathways exert their roles by regulating the stability and availability of VE-cadherin at AJ. Binding of VE-cadherin with catenins provide structural basis for VE-cadherin stability at AJ. Earlier studies pointed out that manipulating p120 catenin availability exerted significant influence on intracellular VE-cadherin levels. Expression of VE-cadherin mutants that complete for β-catenin resulted in a decrease in endothelial barrier function and a dramatic downregulation of endogenous VE-cadherin (Iyer et al. 2004; Xiao et al. 2003). Further, downregulation of p120 catenin using specific small interfering RNA (siRNA) resulted in a corresponding loss of VE-cadherin (Iyer et al. 2004; Xiao et al. 2003), whereas, overexpression of p120 catenin inhibited VE-cadherin entry into endocytic compartments and caused a corresponding increase in cell surface levels of VE-cadherin (Iyer et al. 2004; Xiao et al. 2003). Interestingly, mutating the p120 catenin binding site on dominant negative VE-cadherin mutants abrogated the ability of the mutant to cause downregulation of the endogenous cadherin (Xiao et al. 2003). Later studies revealed that the direct binding of p120 catenin to VE-cadherin helps to retain VE-cadherin at the cell surface. Serine 879 of p120 catenin was recently identified to be phosphorylated by protein kinase C α in response to lipopolysaccharide (LPS) and thrombin. The phosphorylation of this site mediates p120 catenin binding affinity for VE-cadherin (Vandenbroucke St Amant et al. 2012). Meanwhile, a dual-function motif in VE-cadherin consisting of three highly conserved acidic residues has been identified to alternately serve as a p120 catenin-binding interface and an endocytic signal, and mutation of this motif resulted in resistance to endocytosis (Nanes et al. 2012). Clathrin-mediated endocytosis of VE-cadherin is inhibited as it interacts with the dileucine motif of VE-cadherin and precludes its binding to adaptor proteins of the endocytic machinery (Vaezi et al. 2002; Vasioukhin et al. 2000; Verma et al. 2004). Regulation of VE-cadherin by p120 catenin not only involves the stability of VE-cadherin at the cell surface but also their effects on Ras homologue (Rho) family GTPases. Rho family GTPases (including Ras homologue gene family, member

A (RhoA), Rac, and Cell division cycle 42 GTP-binding protein (Cdc42)) are key mediators of cytoskeletal dynamics. The interaction between p120 catenin with Rho GTPase activating protein (p190RhoGAP) promotes p190RhoGAP recruitment to cell periphery and local inhibition of Rho activity. The inhibition of Rho by p120 catenin interaction with p190GAP accounts for the barrier protective effects of oxidized phospholipids. It was further identified that the interaction between p120 catenin and p190RhoGAP is mediated by a 23-amino-acids stretch within the C-terminal domain of p120 catenin (amino acids 820–842; Zebda et al. 2013). Different mechanisms were proposed based on the cell types. p120 catenin was shown to interact preferentially with the GDP-bound of RhoA or Rho1 (the RhoA homology; Anastasiadis et al. 2000; Magie et al. 2002), as would be predicted for a protein acting as Guanine Nucleotide Dissociation Inhibitor (GDI). A study with highly purified proteins also implied a direct interaction between p120 catenin and RhoA (Anastasiadis et al. 2000). Alternatively, p120 catenin may exert its inhibitory effect on Rho through direct interaction with Vav2, Rho GTPase exchange factor (Rho GEF), which could account for the ability of p120 catenin to activate Ras-related C3 botulinum toxin substrate (Rac1) and Cdc42 because Rac1 can inhibit RhoA in some cells via a mechanism that involves reactive oxygen species, low molecular weight (LMW) phosphatase, and p190 RhoGAP (Nimnual et al. 2003).

5.2.3.2 β-Catenin and Plakoglobin-Mediated VE-Cadherin Enhancement

Interaction of VE-cadherin with β-catenin also regulates the amount of VE-cadherin at AJ. The cytoplasmic C-terminus of VE-cadherin is prone to degradation due to the presence of proline, glutamic acid, serine, and threonine (PEST) domain that is targeted for ubiquitination. β-Catenin binding to this region prevents the exposure of cadherin C-terminal tail (Chen et al. 1999; Huber et al. 2001; Huber and Weis 2001). In addition to p120 catenin that regulates AJ stability by modulating GTPase activity, β-catenin also demonstrates such effect. It acts by recruiting IQRas GTP-activating protein 1 (IQGAP1) at AJ. IQGAP1, however, lacks GAP activity. It binds to Rac1 and Cdc42 and inhibits their intrinsic GTPase activity, thus stabilizing them in the active GTP-bound form (Briggs and Sacks 2003, 2006). A recent study revealed different roles for p120 catenin and β-catenin in AJ formation and its strengthening (Oas et al. 2013). P120 catenin appears to regulate adhesive contact area in a Rac1-dependent manner without affecting adhesion as such. In contrast, binding of VE-cadherin to β-catenin resulted in promoting strong steady-state adhesion strength. Plakoglobin also binds to a similar region in VE-cadherin, but it cannot compensate for β-catenin (Cattelino et al. 2003; Schnittler et al. 1997). Overexpression of plakoglobin in human microvascular endothelial cell increased endothelial barrier function (Venkiteswaran et al. 2002). The converse, inhibition of plakoglobin expression resulted in AJ disassembly in ECs exposed to shear stress (Schnittler et al. 1997). Cortical actin ring, which is characterized by actin accumulation at cell–cell contact area, is one of the architectural features of enhanced barrier function. VE-cadherin is anchored to cortical actin cytoskeleton through β-catenin and plakoglobin thus,

stabilizing VE-cadherin at AJ. Both β-catenin and plakoglobin play a key role in bridging VE-cadherin and actin skeleton through α-catenin and/or desmoplakin/vimentin. α-catenin lacks ARM domains and does not associate with VE-cadherin directly, but can interact with actin cytoskeleton through vinculin and α-actinin. Attachment of VE-cadherin to the actin cytoskeleton reduces the lateral mobility of VE-cadherin, thereby inducing VE-cadherin clustering and a subsequent increase in the strength of AJ (Baumgartner et al. 2003; Waschke et al. 2004a, b). However, a direct interaction of the cadherin-β-catenin–α-catenin complex with actin filament appears to be questionable as α-catenin cannot simultaneously bind to β-catenin and actin and therefore, cannot serve as a link between the actin cytoskeleton and the cadherin complex (Drees et al. 2005; Yamada et al. 2005). Unlike β-catenin, plakoglobin mediates attachment of VE-cadherin to intermediate filaments cytoskeleton through desmoplakin (Kowalczyk et al. 1998; Valiron et al. 1996).

5.3 Protein Tyrosine Phosphatases (PTPs)-Mediated AJ Enhancement

Phosphorylation of AJ complex proteins leads to destabilization of the AJ and increases cell monolayer permeability. Barrier disruptive factors, such as vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), platelet-activating factor (PAF), thrombin, and histamine induce tyrosine phosphorylation of VE-cadherin, p120 catenin, β-catenin, and plakoglobin, suggesting that phosphorylation of these AJ proteins promotes disruption of cell–cell contacts. Protein tyrosine phosphatases (PTPs) interact with AJ either directly or indirectly to regulate dephosphorylation of AJ proteins and maintain the stability of the AJ complex. Several phosphatases have been identified that regulate endothelial cell–cell junction, including PTPμ, Src Homology Phosphatase-2 (SHP-2), PTP1B, VE-PTP, and Density enhanced phosphatase-1 (DEP-1) (Nakamura et al. 2008; Nottebaum et al. 2008; Sui et al. 2005). PTPs are composed of 44 isoforms and can be grouped as the receptor and nonreceptor types. Among the five PTPs expressed in EC, PTPμ, VE-PTP, and DEP-1 belong to receptor-type PTP, and SHP-2 and PTPB1 belong to nonreceptor-type PTP. Receptor-type PTP consists of highly variable extracellular region and one or two intracellular phosphatase domains. PTPμ was observed almost exclusively at cell–cell contacts, bound directly to VE-cadherin and p120 catenin (Sui et al. 2005; Zondag et al. 2000). Overexpression of PTPμ decreased VE-cadherin tyrosine phosphorylation and permeability of the monolayer (Sui et al. 2005). In addition, downregulation of PTPμ by siRNA in ECs impaired the barrier function suggesting a critical role in the regulation of AJ integrity (Sui et al. 2005). It is interesting to note that prostate carcinoma cells lacking endogenous PTPμ show disability to form AJ, though, E-cadherin and the catenin proteins are present. Expression of catalytically dead PTPμ restored cadherin-mediated cell–cell adhesion (Hellberg et al. 2002). This finding suggests that PTPμ can maintain the stability of VE-cadherin-mediated AJ by recruiting regulatory proteins to sites of AJ by virtue

of a scaffold protein thereby, invoking an alternate mechanism. VE-PTP is expressed exclusively in the ECs (Baumer et al. 2006), interacts with the extracellular region in VE-cadherin (Nawroth et al. 2002), and maintains it in a dephosphorylated status. VE-PTP null mice show vascular defects (Dominguez et al. 2007) indicating a critical role for VE-PTP in the stabilization and remodeling of the vasculature. The dissociation of VE-PTP and VE-cadherin is a prerequisite for the destabilization of endothelial cell contacts, in response to VEGF and LPS challenge (Broermann et al. 2011) and leukocyte transmigration (Nottebaum et al. 2008). Nevertheless, VE-PTP maintains VE-cadherin integrity through plakoglobin, and not through β-catenin, as overexpression of VE-PTP leads to its increased association with plakoglobin, but not β-catenin in conjunction with VE-cadherin (Nottebaum et al. 2008). DEP-1 is another receptor-type PTP whose expression and activity are upregulated with increasing levels of cell confluence (Ostman et al. 1994). It indirectly associates with VE-cadherin by binding to p120 catenin, plakoglobin, and β-catenin. It also plays important roles in angiogenesis and vasculogenesis (Takahashi et al. 2006, 2003). On the contrary, DEP-1 may also play a barrier disruptive role. Phosphorylation of DEP-1 at Y1320 leads to Src activation and promotes Src-dependent endothelial permeability (Spring et al. 2012, 2014).

Cytosolic PTPs, PTP1B, and SHP2 also maintain AJ stability in a manner similar to receptor PTPs (Balsamo et al. 1996; Brady-Kalnay et al. 1993; Ukropec et al. 2000). Direct binding of PTPB1 to the cytoplasmic domain of N-cadherin promotes β-catenin recruitment to N-cadherin and cell membrane (Xu et al. 2002). SHP2 specifically associates with β-catenin (Ukropec et al. 2000). Loss of SHP2 from VE-cadherin complex accounts for the increase in phosphorylation of β-catenin, plakoglobin, and p120 catenin in response to thrombin treatment (Grinnell et al. 2010; Ukropec et al. 2000), in support of a key role in the stabilization of AJs. SHP2 is also involved in the recovery of endothelial AJs through control of β-catenin phosphorylation (Timmerman et al. 2012). SHP2-depleted cells showed prolonged elevation in tyrosine phosphorylation levels of VE-cadherin-associated β-catenin after thrombin treatment (Timmerman et al. 2012).

5.4 Small GTPases-Mediated Barrier Enhancement

Rho-GTPases have been the target of intense investigation due to their known roles in cytoskeleton organization and barrier function. This subfamily of Ras consists of 23 members that include well characterized members such as Rho, Rac, and Cdc42 (Wennerberg et al. 2005). Rho proteins share approximately 30% homology with the Ras subfamily of proteins and 80–90% homology with each other (Hall 1998). Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound. The inactive GDP-bound Rho proteins are retained in the cytosol in complex with GDI and are activated by guanine nucleotide exchange factors (GEFs), which accelerate the exchange of GDP for GTP. Further, activated Rho proteins are subject to inactivation by GTPase-activating protein (GAPs) that promote hydrolysis GTP to GDP (Takai

et al. 2001). It is the general concept that Rho proteins affect cell adhesion and barrier function via the remodeling of actin (Jaffe and Hall 2005). On the contrary to the barrier disruptive effects of Rho, Rac and Cdc42 have barrier protective effects.

5.4.1 Rac1-Mediated Barrier Enhancement Signaling

Rac1 is usually placed in the center of signaling pathways leading to barrier enhancement. Many barrier enhancement factors act through activation of Rac1 and its downstream target, p21 activated kinase (PAK). Basal Rac activation has been related to monolayer integrity and maintenance (Waschke et al. 2004a, 2004b; Wojciak-Stothard et al. 2001). However, several studies suggest that junctional stability requires a very fine tuning of Rac1 activity as both dominant-negative and constitutively active Rac1 increase endothelial permeability, and are associated with a loss of VE-cadherin at the cell–cell junction (van Wetering et al. 2002; Wojciak-Stothard et al. 2001). A number of barrier enhancement factors, such as sphingosine-1-phosphate (S1P), atrial natriuretic peptide (ANP), hepatic growth factor (HGF), angiopoietin-1, and prostaglandin D2, are known to regulate barrier integrity at least in part, through Rac- and/or Cdc42-dependent cytoskeleton remodeling. The mechanisms of Rac1 activation vary depending on the stimulants.

S1P generated by the activities of sphingosine kinase 1 or 2 on sphingosine. It is released from activated platelets, erythrocytes, and ECs. S1P is known to exert a diverse range of physiological and pathological effects via the activation of five G-protein coupled receptors, $S1P_1$–$S1P_5$. The barrier enhancement effects of S1P are mediated by $S1P_1$ receptor. $S1P_1$ is a 382-amino-acid protein that contains seven transmembrane spanning domains with significant structural similarities to other G-protein coupled receptors (GPCRs). Binding of S1P to $S1P_1$ stimulates mainly two signaling pathways, both of which contribute to enhanced barrier integrity. In one pathway, Rac1 plays a critical role and is characterized by a marked increase in polymerized cortical actin that represents a common and essential feature of barrier protection and also AJ assembly and stabilization. The other pathway involves focal adhesions and matrix interactions. S1P binding to $S1P_1$ receptor induces Gi signaling, which in turn, activates Rac1-specific GEF T-lymphoma invasion and metastasis 1 (Tiam-1) in caveolin-enriched lipid raft in a Phosphatidylinositol 3-kinase (PI3K)-dependent manner (Singleton et al. 2005). Activated Tiam-1 associates with and activates Rac1, resulting in Rac1 redistribution to areas of cell–cell contact (Lee et al. 1999).

ANP is a 28-amino-acid peptide, released mainly by cardiac myocytes of the atria of the heart and acts through three receptors—Natriuretic Peptide Receptor1 (NPR1), Natriuretic Peptide Receptor2 (NPR2), and Natriuretic Peptide Receptor3 (NRP3). Binding of ANP to its receptors induces intracellular Cyclic guanosine monophosphate (cGMP) and Cyclic adenosine monophosphate (cAMP) formation. Although most vascular effects of ANP are mediated by cGMP, barrier protective effect of ANP, however, appears to be mediated by cAMP, as both cGMP activator and inhibitor have no effects on thrombin-induced endothelial barrier permeability

(Birukova et al. 2008c). cAMP is the upstream effecter of ANP-initiated barrier protective response and induced Rac1 activation through PKA-dependent and PKA-independent pathways. In case of PKA-dependent pathway, PKA may regulate Rac1 through Rac1 GEFs, Tiam1, and Vav2 guanine nucleotide exchange factor (Vav2) as Tiam and Vav2 have consensus PKA phosphorylation sites (O'Connor and Mercurio 2001). Alternatively, PKA acts through direct phosphorylation of Rho resulting in the inhibition of this barrier disruptive signaling molecule (Dong et al. 1998; Lang et al. 1996). ANP achieves its barrier protective effects alternatively through PKA-independent and Epac/Rap1-dependent pathway. Epac functions as GEF for Rap1, which is also a small GTPase acting as GEF for Rac. Epa/Rap1 has emerged as a new mechanism for cAMP-mediated barrier protection that could not be attributed to the established target PKA. ANP-elevated cAMP preferentially binds to the N-terminal cyclic nucleotide binding (CNB) domain of Epac and facilitates the auto inhibitory regulatory domain to shift away from the catalytic region, thereby creating access to Rap1 and its activation (Gloerich and Bos 2010). Activated Rap1, in turn, activates the Rac-specific GEF, Vav2 and Tiam1, promoting Rac and Cdc42 activation (Birukova et al. 2007b, 2008c).

HGF, also known as scatter factor, has potent effects on cell proliferation, motility, morphogenesis, survival, and angiogenesis, in various cell types (Bussolino et al. 1992; Grant et al. 1993; Matsumoto and Nakamura 1996; Zhang et al. 2000). HGF is also known to enhance endothelial barrier function by remodeling cytoskeleton and stabilizing AJ complex (Birukova et al. 2007a; 2008b; Liu et al. 2002). The effects of HGF are mediated through the receptor tyrosine kinase c-Met, which is composed of a 50 kDa extracellular αsubunit and a 145 kDa transmembrane βsubunit (Bottaro et al. 1991). Binding of HGF with the receptor stimulates receptor kinase activity, leading to autophosphorylation of the receptor, followed by the recruitment of adaptor proteins, such as Grb2-associated binder-1 (Gab-1), Grb2, PI3K, phospholipase Cγ, Src, and SHP2 (Ponzetto et al. 1994; Schaeper et al. 2000). The effects of HGF on barrier enhancement also appear to be mediated through Rac1 activation (Birukova et al. 2007a, 2008b). HGF binding to the receptor activates Tiam1 via PI3K pathway. Then, Tiam1 activates Rac1. CD44 is also implicated in HGF-induced Rac1 activation as HGF treatment of EC resulted in c-Met association with CD44v10 and recruitment of c-Met into caveolin-enriched microdomains containing CD44. In addition, dynamin 2 is a vesicular regulator that is recruited into caveolin-enriched microdomains in response to HGF treatment. It also mediates Rac1 activation, as knockdown of dynamin 2 by siRNA reduced HGF-induced Rac1 activation (Singleton et al. 2007). Recently S1P1 and integrin β4 were identified to participate in HGF-induced endothelial barrier enhancement. HGF treatment of vascular ECs induced recruitment of c-Met, integrin β4 and S1P1 to caveolin-enriched lipid rafts and c-Met-dependent transactivation of S1P1 and integrin β4 (Ephstein et al. 2013).

As outlined above, Rac1 is a key common player in several signaling pathways and the signals downstream are common. PAK is the major downstream target of Rac1. Several downstream pathways of PAK have been proposed to mediate barrier integrity (Fig. 5.3).

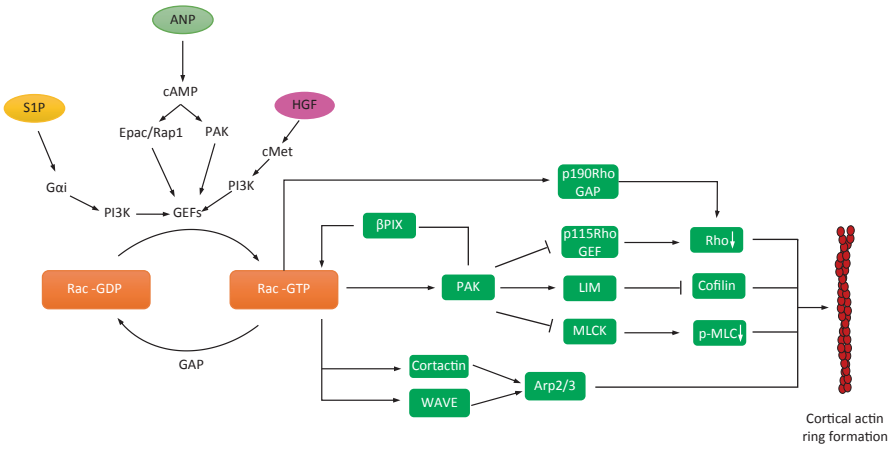

Fig. 5.3 Rac1 plays central role in barrier enhancement signaling. Rac1 acts as molecular switches that stimulate downstream signals which control cellular processes. Barrier enhancement agonists, S1P, ANP, and HGF activate Rac1 through stimulation of Rac1 GEFs by various mechanisms. Activated Rac1 leads to cortical actin ring formation through either activation of cortactin/WAVE/Arp2/3 pathway to induced actin remodeling at cell peripheral or inactivation of Rho signaling to reduce stress fiber formation. (*Rac1* Ras-related C3 botulinum toxin substrate, *S1P* sphingosine-1-phosphate, *ANP* atrial natriuretic peptide, *HGF* hepatic growth factor, *GEF* GTPase exchange factor, *Rho* Ras homologue)

Activated PAK phosphorylates LIM kinase, which then inhibits cofilin activity in the cell periphery (Garcia et al. 2001). The direct consequence of cofilin inhibition is the stabilization of actin at the cell periphery. PAK can also inhibit p115RhoGEF, thus repressing RhoA activity (Rosenfeldt et al. 2006). Furthermore, PAK is known to inhibit myosin light chain kinase (MLCK) that mediates the phosphorylation of MLC in certain types of cells (Sanders et al. 1999). A model of positive feedback loop between Rac and PAK has been proposed. In this model, oxidized PAPC induces Rac1 activation leading to PAK-mediated phosphorylation of paxillin at Ser273, causes assembly of a GIT-Paxillin-PAK1-β-PIX signaling complex, and β-PIX further induces Rac activation (Birukova et al. 2008a). Rac activation is also associated with the translocation of cortactin, an actin-binding protein at AJ (Jacobson et al. 2006; Weed et al. 1998). Cortactin stimulates and stabilizes Arp2/3, an actin nucleator, mediating polymerization of branched actin filaments at peripheral sites (Uruno et al. 2001; Weaver et al. 2001). Alternatively, Rac may exert its effect on Arp2/3 via Wiskott-Aldrich syndrome protein verprolin homologous (WAVE), a protein with significant homology to verprolin, a member of the Wiskott–Aldrich syndrome protein (WASP). P190 Rho GAP plays a critical role in inhibition of RhoA at cell–cell adhesions where it is recruited by p120 catenin. Interestingly, its activity depends on p120 catenin (Wildenberg et al. 2006). Rac can inhibit Rho activity through p120 catenin (Herbrand and Ahmadian 2006; Wildenberg et al. 2006). Binding of Rac to p190RhoGAP results in a conformational change of p190RhoGAP that leads to its autoinhibition status. P190RhoGAP, in

turn, facilitates the hydrolysis of Rho-GTP to Rho-GDP that results in its inhibition at cell–cell adhesions.

5.4.2 Cdc42-Mediated Barrier Enhancement Signaling

In many cases, agonists that activate Rac1 also activate Cdc42 (Baumer et al. 2009; Birukova et al. 2007b, 2008c; Waschke et al. 2006). Therefore, Cdc42 may be equally important for barrier maintenance and enhancement like Rac1. Cdc42 and Rac1 share common pathways leading to enhanced barrier integrity. Nevertheless, Cdc42 appears to be key GTPase that supports barrier restoration, indicating that Cdc42 is different from Rac1. In a thrombin-induced EC barrier dysfunction model, the activation of Cdc42 occurred during the recovery phase that follows loss in barrier integrity, whereas inactive Cdc42 was found only during the RhoA-mediated cell retraction phase (Kouklis et al. 2004). Transfection of EC with dominant-negative Cdc42 mutant delayed the restoration of EC barrier function after thrombin treatment in vivo. The mechanisms underlying Cdc42-mediated restoration of EC barrier function are likely to involve AJ proteins, VE-cadherin and catenins, and actin recruiting at sites in the plasma membrane. Cdc42 may interact with IQGAP1 and intersectin to promote the reannealing of AJs (Mehta and Malik 2006). Furthermore, Cdc42 stabilizes newly formed interactions by promoting actin remodeling through its downstream effector WASP (Mehta and Malik 2006). The mechanisms responsible for the delayed activation of Cdc42 are not well-defined. However, RhoA inactivation at AJ by p190RhoGAP and p120 complex may lead to Cdc42 activation, initiating the recovery phase and AJ reassembly.

5.4.2.1 Rap1-Mediated Barrier Enhancement Signaling

Rap proteins, which include Rap1 (A and B) and Rap2 (A, B, and C) are members of the Ras super family of small GTPases. The landmark paper by Knox and Brown (2002) for the first time linked Rap1 to AJ formation (Knox and Brown 2002). The studies revealed that Rap1 is localized to the AJ. Rpa1 mutant cells displayed aberrant shapes and irregular connection with surrounding cells, indicating a defect in cell–cell adhesion. As Rap1 regulates Rac GEF, Tiam1 and Vav2, as illustrated above, signaling pathways regulated by Rac1 can explain Rap1-mediated barrier enhancement (Fig. 5.4).

ArfGAP, RhoGAP, ankyrin repeats and PH domain 3 (ARAP3), a member of the ARAP family of dual GAPs for ADP ribosylation factor (Arf) and Rho family GTPase, was identified from porcine neutrophils in a screen for PtdIns (3,4,5) P3-binding proteins (Krugmann et al. 2002; Miura et al. 2002). Rap proteins can bind directly to a neighboring Ras binding domain of ARAP3. This activation by Rap is GTP dependent and specific for Rap versus other Ras family members (Bos et al. 2001; Krugmann et al. 2004). Similar to Rho GAP, activated ARAP3 converts

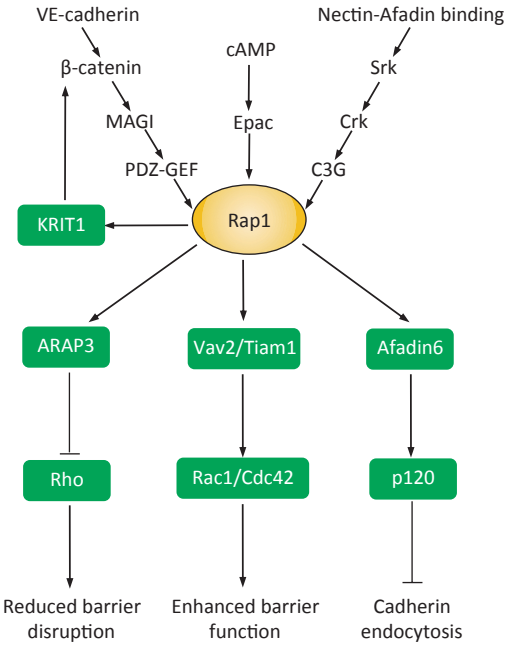

Fig. 5.4 Rap1-mediated barrier enhancement signaling. Establishment of cell–cell contacts initiates signaling to activate Rap1, a small GTPase of Ras superfamily. Also, extracellular stimuli, including those that stimulate production of cAMP which activates Epac, induce Rap1 activation. Several Rap1 effecter proteins might mediate the effect on barrier function. Rap1 activates Rac1/Cdc42 via Vav2/Tiam1. Rap1 mediates the indirect binding of Afadin to p120 catenin, resulting in E-cadherin stabilization. In addition, Rap1 inactivates Rho through activation of Rho GAP ARAP3. (*Rap1* Ras-related protein1, *cAMP* Cyclic adenosine monophosphate, *Epac* Exchange protein activated by cAMP, *Rac1* Ras-related C3 botulinum toxin substrate, *Cdc42* Cell division cycle 42 GTP-binding protein, *Vav2* Vav2 guanine nucleotide exchange factor, *Tiam1* T-lymphoma invasion and metastasis 1, *Rho* Ras homologue, *GAP* GTPase activating protein, *ARAP3* ArfGAP, RhoGAP, ankyrin repeats and PH domain 3)

Rho-GTP into Rho-GDP, and thus downregulates Rho activity, resulting in enhanced barrier integrity. Afadin, an F-actin binding protein found to be a part of AJ, is composed of multiple functional domains, which mediate binding to various proteins. Of these binding partners, nectins are the most important partners that together contribute to cell–cell adhesion. The PDZ domain of afadin binds to the C-terminus of nectins (Aoki et al. 1997; Takai et al. 2008a). The *trans* interactions of nectins at the initial cell–cell contact sites induce the activation of Src (Fukuhara et al. 2004; Takai et al. 2008b) and then recruits Cyanidin 3-Glucoside (C3G), a GEF for Rap1, to nectins through adaptor protein Crk, resulting in the activation of Rap (Fukuyama et al. 2005). Rap1 subsequently binds to afadin and then, afadin indirectly binds to p120 catenin that is associated with *cis*-interacting E-cadherin and prevents the endocytosis of E-cadherin. Stabilized E-cadherin accumulates at the nectin-based cell–cell adhesion sites, leading to the establishment of AJ (Hoshino

et al. 2005; Sato et al. 2006). The Rap1 binding protein, K-Rev1 interaction Trapped gene 1 (KRIT1, also known as CCM1) was shown to be involved in Eapc1/Rap1-induced barrier enhancement of EC (Glading et al. 2007). In confluent EC monolayer, KRIT1 localizes to cell–cell contacts, where it forms a complex with VE-cadherin, p120 catenin, β-catenin, α-catenin, and afadin. Downregulation of KRIT1 by its siRNA-disabled Epac1/Rap1 to rescue thrombin-induced permeability, indicates that KRIT1 might be an effecter of Rap1. It remains to be elucidated how KRIT1 relays the Rap1 signal toward cell–cell contacts. KRIT1 associates with β-catenin and afadin in a Rap1-dependent manner and siRNA targeting KRIT1 disrupts junctional staining of β-catenin (Glading et al. 2007), suggesting KRIT1 might stabilize β-catenin at the AJ.

5.5 Tight Junction-Mediated Barrier Enhancement Signaling

TJs are the most apical structure of a junctional complex bordering the apico-basolateral membrane in both endothelial and epithelial cells. Within the lung tissue, TJs are the major structures that determine the permeability of epithelial cells, while lung vascular ECs rely more on AJs to regulate permeability. TJ is composed of strands of 10 nm fibrils that encircle the apical region of the cell. Three major transmembrane proteins, including occludin, claudin, and junctional adhesion molecule (JAM) are shown to constitute TJ. TJ clusters and cytosolic components form plaque of TJ, where they are associated with zonula occludens (ZO) proteins that link the intracellular domains of TJ with actin binding proteins and cytoskeleton including afadin, cingulin, β-catenin, and p120 catenin.

5.5.1 Occludins

Occludin was the first identified transmembrane component of TJ and was originally isolated from chick livers (Furuse et al. 1993). It has two extracellular loops that appears to mediate localization of occluding to TJ and the paracellular permeability. Synthetic peptides corresponding to a 20-amino-acid sequence within the second extracellular loop of occluding increased barrier permeability and decreased occluding levels, as a result of increased protein turnover, suggesting this sequence mediates the association of occludins from adjacent cells (Wong and Gumbiner 1997). The expression of occluding correlates with barrier properties. The occluding expression from different vascular ECs presents a huge difference. For example, arterial ECs express 18-fold greater occluding protein levels than venous ECs and form a tighter solute barrier (Kevil et al. 1998). Similarly, occluding is highly expressed in brain endothelium which forms a very tight barrier, compared to ECs of non-neuronal tissue, which have lower occluding expression and barrier properties

than brain endothelium (Hirase et al. 1997). As predicted, increased expression of occludin correlates with enhanced barrier function. Hydrocortisone treatment of bovine retinal ECs resulted in increased occluding expression by twofold, and concomitant-enhanced barrier properties (Antonetti et al. 2002). Localization of this protein is also regulated by phosphorylation in both epithelial and ECs (Sakakibara et al. 1997). Non-phosphorylated occludin is localized to both the basolateral membrane and in cytoplasmic vesicles, whereas phosphorylated occluding is localized to TJ (Sakakibara et al. 1997). Specific phosphorylated residues within occludin have been investigated in terms of barrier function. T403 and T404 phosphorylation have been linked to enhanced occludin trafficking to the TJ and paracellular barrier function (Suzuki et al. 2009). In contrast, phosphorylation of Y398, Y402, and S490 is related to barrier disruption and attenuated interactions between occludin and ZO-1 (Elias et al. 2009; Sundstrom et al. 2009). Inhibition of S408 phosphorylation reduced paracellular cation flux by stabilizing occludin-ZO-1 interactions and enhanced subsequent association with claudin-1 and claudin-2, but not claudin-4 (Raleigh et al. 2011).

5.5.2 Claudins

Claudins are a large family of transmembrane proteins that are components of TJs. Claudin-1 and claudin-2 are the first two identified members of the claudin family from chicken liver junctional fractions. So far 27 members of this family have been identified, with each showing a specific organ and tissue distribution (van Wetering et al. 2002). The structure of claudins is similar to occludin with four transmembrane domains, two extracellular loops, and cytoplasmic N- and C-termini. The C-terminal sequences of claudins all share a dipeptide sequence YV motif in the final two amino acids that is required for binding to the PDZ domain of ZO family members. Inhibition of this domain does not affect localization of claudin to TJ, but inhibits the association of ZO-1, -2, and -3 proteins (McCarthy et al. 2000). Extracellular loop regions determine ion selectivity as the regions are charged and exhibit a wide range of isoelectric points. This is, especially true in case of claudin-15, as it is sufficient to change the ion selectivity of the barrier (Colegio et al. 2002). In addition, mutating three positive charges to negative within the same region switched the ion selectivity of the claudin channel from $Na^{2+}$to Cl^{-} (Colegio et al. 2002). Therefore, it seems that the first extracellular loop is sufficient to determine charge selectivity of the ion channel.

Different claudins are expressed throughout the respiratory tract. Claudin 1, 3, 4, 5, 7 have been reported to be expressed in human airway with different cellular localization (Coyne et al. 2003). The role of claudins in barrier integrity is a matter of debate. Some claudins increase paracellular permeability, for example, claudin 2, 3, and 5. IL-13-induced epithelial barrier dysfunction is attributed to claudin 2 induction (Rosen et al. 2013). Claudin 3 may serve as barrier disruptive claudin at baseline for type II alveolar epithelial cell (Mitchell et al. 2011). Human epithelial cell line (IB3.1) from cystic fibrosis airway presents enhanced permeability to different

size dextrans when claudin 5 is overexpressed in this cell line (Coyne et al. 2003). In contrast, claudin 1, 4, and 18 are known to enhance barrier integrity. Madin-Darby canine kidney (MDCK) cells transfected with mouse claudin 1 revealed increased expression of ZO-1 and exhibited enhanced barrier function (Inai et al. 1999). Expression of Claudin 4 is induced in acute lung injury and represents a mechanism to limit pulmonary edema (Mitchell et al. 2011; Wray et al. 2009). Claudin 18 is the only lung-specific TJ protein and the most abundant claudin in type 1 alveolar epithelial cells. Knockdown of claudin18 impaired alveolar epithelial barrier function in vivo and in vitro. Claudin 18 knockout mice developed postnatal lung injury and impaired alveolarization, indicating the importance of claudin 18 in lung epithelial barrier function and maturation (Lafemina et al. 2014). It is important to note that claudins might have different functions depending on the cellular and tissue context in which they are expressed. For example, although claudin 5 represents barrier disruptive property as described above, recent studies also demonstrated a crucial protective role for claudin 5 in lung microvascular ECs during influenza infection (Armstrong et al. 2012).

5.5.3 JAM

JAM are IgG-like transmembrane proteins found at TJ. This family consists of the closely related members JAM-A, -B, and -C, and the more distantly related coxsackie and adenovirus receptors (CAR), endothelial cell-selective adhesion molecule (ESAM) and JAM-4 (Ebnet et al. 2004). JAMs have single pass transmembrane domain and extracellular region that contains two domains with V-type immunoglobin loops. JAMs appear to be important for TJ barrier function, as overexpression of JAM in CHO cells reduced paracellular permeability to 40 kDa dextran by 50 % in a calcium-dependent manner (Aurrand-Lions et al. 2001; Martin-Padura et al. 1998) and anti-JAM antibody reduced blood–brain barrier permeability (Del Maschio et al. 1999).

5.5.4 ZO Proteins

Similar to AJ, TJ transmembrane proteins also require scaffolding molecules to connect with cytoskeleton proteins, such as actin. One important group of TJ scaffolding proteins is ZO proteins ZO-1, ZO-2, and ZO3. They bind directly to claudins via their PDZ1 domain and with occluding via their guanylate kinase-like homologues (GUK) domain, and the proline-rich C terminus mediates their binding to F-actin in vitro (Fanning et al. 2002; Schneeberger and Lynch 2004), and thus may serve to link ZO proteins to the cytoskeleton. As scaffold proteins, they facilitate claudin clustering, strand formation, and barrier function (Umeda et al. 2006). In addition to TJ proteins, ZO proteins also bind to the AJ protein, α-catenin, and the gap junction protein, connexin-43 (Giepmans and Moolenaar 1998; Itoh et al. 1999).

5.6 Focal Adhesion-Mediated Barrier Enhancement Signaling

Endothelial and epithelial cells attach to their underlying matrices through complex transmembrane structures termed focal adhesions (FAs) , which not only provide an anchor point for cells to adhere to the substratum but also selectively recruit various signaling molecules to the sites, allowing cells to monitor their immediate environment. Recent experimental evidence points to the importance of FAs in the regulation of barrier function.

5.6.1 Integrin-Mediated Barrier Enhancement

Cell adhesion to the extracellular matrix (ECM) is mainly mediated by the integrin family of cell-surface receptors. Integrins are composed of noncovalently linked α and β subunits, each of which is a transmembrane glycoprotein with a single membrane-spanning segment and generally, a short cytoplasmic tail (Clark and Brugge 1995; Hynes 2002). In humans, a total of 18 integrin α chains and 8 β chains have been identified. They assemble in parallel arrays to form more than 24 heterodimers (Hynes 2002), each of which has a distinct, nonredundant function, binding to specific ECM components, and soluble protein ligands. Integrin-ECM interaction initiates the occupancy and clustering of integrins and in turn, promotes the recruitment of cytoskeletal and cytoplasmic proteins, such as talin, paxillin, and α-actinin to form focal complexes and focal adhesions (van der Flier and Sonnenberg 2001). As a bridge between ECM and cytoskeleton, integrins play essential roles in establishment and stabilization of barrier function. Synthetic peptides that compete for the Arg-Gly-Asp (RGD)-binding sequence (the targeting sequence in matrix proteins recognized by integrins) or antibodies against α5β1 induce a dramatic increase in barrier permeability (Curtis et al. 1995; Qiao et al. 1995; Wheatley et al. 1993). Integrin may also enhance barrier function through cell–cell adhesion. Several lines of evidence show that integrin participates in VE-cadherin mediated AJ. For instance, cAMP-activated Epac triggers the activation of A-kinase anchoring protein 9 (AKAP9), which plays a central role in microtube growth. AKAP9 promotes integrin α_v accumulation at the cell–cell border in ECs plated on fibronectin-coated plate, as α_v integrin has a relatively lower affinity for fibronectin than α_5. Conversely, on collagen, α_5 is enriched at the cell–cell interface. The ligand(s) at the cell–cell border for integrins are extracellular matrix proteins such as fibronectin, laminin, and collagen type IV, which are produced by the endothelium and concentrated at lateral cell–cell contacts (Sehrawat et al. 2011) . Integrin β4 is known to associate with S1P receptor 1 specifically in caveolin-enriched lipid rafts in human lung EC after HGF treatment. Reduced integrin β4 expression attenuated HGF-induced c-Met activation, c-Met/S1PR1 interaction, and decreased S1P- and HGF-induced EC barrier enhancement (Ephstein et al. 2013). Another study supports the protective role of simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A-reductase inhibitor,

in LPS-induced pulmonary hyper-permeability and the injury that is mediated by integrin β4 (Chen et al. 2012). The protective effects of simvastatin are reversed by co-treatment with an integrin β4 blocking antibody. These results support a critical role for integrin β4 in regulating EC barrier integrity and function. Integrins regulate EC barrier function through cAMP as well (Kim et al. 2012). The mammalian tissue inhibitor of metalloproteinase 2 (TIMP2) binds to the surface of EC via interaction with the integrin α3β1. Treatment of EC with TIMP2 causes cytosolic cAMP elevation and increase in VE-cadherin association with actin cytoskeleton. α3β1 is revealed to mediate cAMP induction by TIMP2 as both α3- and β1-blocking antibodies abrogates TIMP2-mediated increase in cAMP production (Kim et al. 2012). The effects of integrin in barrier function are highly dependent on the subunit type and combination. Integrins β1 and β5 are known to regulate EC contractility and lung vascular permeability (Faurobert et al. 2013; Su et al. 2007) .

5.6.2 Focal Adhesion Kinase-Mediated Barrier Enhancement

Focal adhesion kinase (FAK) is a critical non-receptor tyrosine kinase involved in the engagement of integrins and assembly of FA. It contains four principal regions mediating interactions with other adaptor and signaling proteins. The N-terminus contains a four-point-one, ezrin, radixin, moesin binding (FERM) domain, which acts as an auto inhibitory site by interacting with the kinase domain (Frame et al. 2010). The catalytic tyrosine kinase domain is localized in the middle. At the C-terminus is the C-terminal focal adhesion targeting (FAT) domain providing additional sites for FAK binding partners that include paxillin and members of the Rho family proteins. Between FAT and the central catalytic domain is the proline-rich region, which provides binding sites for FAK binding partners along with FAT domain (Schaller 2010).

Multiple studies have demonstrated an important role for FAK in modulating EC barrier function (Quadri et al. 2003; Yuan et al. 2012; Zebda et al. 2012). The importance of FAK in endothelial barrier function is highlighted by the fact that EC-specific deletion of murine FAK results in early embryonic lethality due to impaired vascular development (Braren et al. 2006; Shen et al. 2005). Furthermore, EC isolated from FAK knockout mice exhibit increased permeability as compared to wild type EC (Zhang et al. 2010). Similarly, reduction of FAK in human pulmonary artery EC (HPAEC) results in delayed barrier recovery after thrombin (Mehta et al. 2002). The underlying mechanism is that thrombin activates FAK via Gβγ and Fyn tyrosine kinase to associate with AJ complexes and stimulate their reassembly by phosphorylating p190RhoGAP to inhibit RhoA activity (Holinstat et al. 2006; Knezevic et al. 2009). Multiple tyrosine phosphorylation sites have been proposed to involve in S1P-induced barrier enhancement (Lee et al. 2000; Miura et al. 2000; Shikata et al. 2003a, b). Y576 of FAK, located in the activation loop of the catalytic domain, has been identified as the major phosphorylation target for S1P (Lee et al. 2000). The phosphorylation of Y576 is mediated by Src as inhibition of Src by PP2 blocks this event and attenuates S1P-induced translocation of FA proteins to the

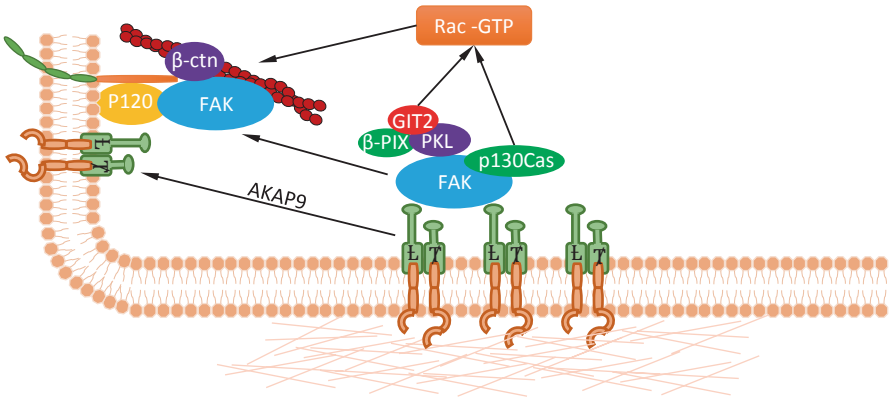

Fig. 5.5 Focal adhesion protein complex cross talk with adhesions junctions (AJs). There is potential for crosstalk between cell–cell and cell–matrix adhesions through integrin shuttling between these distinct adhesion sites. AKAP9, which is activated by Epac, may mediate this process. FAK, a tyrosine kinase localized at FA, undergoes disassembly from FA after S1P treatment, dissociated FAK is then translocated to the EC periphery in association with the *PKL/GIT2*-paxillin complex, where cortical actin ring is facilitated by these proteins. (*AKAP9* A-kinase anchoring protein 9, *Epac* Exchange protein activated by cAMP, *FAK* Focal adhesion kinase, *S1P* Sphingosine-1-phosphate)

EC periphery (Shikata et al. 2003a, b). FAK-regulated barrier function may also be through its role in actin cytoskeleton reorganization via Rac activation (Fig. 5.5).

Evidence reveals that FAK translocates to EC periphery, where it may help regulate Rac activity. FAK activation leads to phosphorylation of the scaffolding protein, p130Cas, which can facilitate Rac activation (Chan et al. 2009; Tomar and Schlaepfer 2009). Alternatively, FAK phosphorylates the adaptor proteins β-PIX and PKL/GIT2 to form a complex and in turn, recruits and activates Rac (Chang et al. 2007; Yu et al. 2009). Additionally, FAK associates with AJ complexes to help cortical actin rearrangement. FAK localized at FA undergoes disassembly after S1P treatment, cytosolic FAK is then translocated to the EC periphery in association with PKL/GIT2-paxillin complex, where cortical actin ring is facilitated by these proteins (Shikata et al. 2003a, b). Furthermore, EC barrier enhancement coincides with increased association between FAK and AJ components. S1P significantly stimulates FAK association with VE-cadherin and β-catenin in HPAEC (Sun et al. 2009). In addition to S1P, thrombin treatment of EC also causes FAK redistribution to the cell peripheral where it interacts with p120 catenin in Y397/Y576 phosphorylation-dependent manner, leading to AJ reannealing during the time in which EC barrier function is restored (Knezevic et al. 2009).

Whether FAK functions to promote or disrupt endothelial barrier function is still a matter of debate, as depletion of FAK was observed to decrease response of S1P (Zhao et al. 2009). Furthermore, FAK phosphorylation and increased activity also occur in association with endothelial barrier-disrupting stimuli such as TGF-β (Lee et al. 2007), PMN (Guo et al. 2005), and VEGF (Wu et al. 2003). Thus, effects of FAK on EC barrier function depends on the stimulus involved.

5.7 Conclusions

As described in this chapter, normal barrier integrity is achieved by multiple cell structures. Most of the investigations have focused on cell–cell and cell–matrix contacts, and cytoskeleton remodeling as the major factors that regulate barrier integrity. Cell–cell contact may represent the most important regulation of barrier integrity as it is most dynamic. There are cross talks between cell-matrix components, however, the detailed mechanisms are unknown and therefore, represents an attractive future research direction. Regulation of barrier integrity by small GTPases is an active area of investigation. Many more GTPase regulators have been identified. In principle, direct targeting small GTPases using small molecular inhibitor would be a desirable approach to regulate barrier integrity for therapeutic purposes. Rho kinase inhibitor (HA-1077) has been used for the treatment of vascular disease. Mechanistic investigation of known barrier-enhancing agents are important areas for future research. These studies may lead to the discovery of new therapeutic targets and concepts for diseases associated with increase barrier permeability.

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