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Review

Focal adhesions: what's new inside

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Abstract

The cytoplasmic side of focal adhesions is comprised of large molecular complexes that link transmembrane receptors, such as integrins, to the actin cytoskeleton and mediate signals modulating cell attachment, migration, proliferation, differentiation, and gene expression. These complexes are heterogeneous and dynamic structures that are apparent targets of regulatory signals that control the function of focal adhesions. Recent studies using genetic approaches in invertebrate and vertebrate systems have begun to reveal the structure and function of these complexes in vivo.

Keywords: Focal adhesion; Cartilage; Knockout mouse; Mutant fly

Introduction

Focal adhesions were first identified by electron microscopy by Abercrombie et al. (1971) as electron-dense regions of the plasma membrane that make intimate contact with the substratum in cultured cells. This physical interaction allows cells to communicate with their outside environment and respond appropriately, leading to cell attachment, migration, proliferation, differentiation, death, and gene expression. At molecular level, focal adhesions are formed around a transmembrane core of an α−β integrin heterodimer, which binds to a component of the extracellular matrix (ECM) on its extracellular region, constitutes the site of anchorage of the actin cytoskeletons to the cytoplasmic side of the membrane, and mediates various intracellular signaling pathways (Burridge et al., 1988; Hynes, 2002; Jockusch et al., 1995; Schwartz et al., 1995). In tissues, focal adhesions do not display prominent structures like others, such as gap junctions, tight junctions, desmosome, and hemidesmosome under electron microscopy. Therefore, some speculated that focal adhesions were artificial structures found in cells cultured on rigid surface. Nonetheless, by immunoelectron microscopy, it is clear that focal adhesions are present in vivo at cell matrix junctions (Fuchs et al., 1997). Because integrins do not contain actin-binding or enzymatic activities, all of the structural and signaling events are presumably mediated by proteins associated with the integrin cytoplasmic tails and molecules they recruit. Multiple molecular linkages connecting integrins to actin cytoskeletons and mechanisms involving focal adhesion assembly have been established primarily based on biochemical and cell culture data (Fig. 1) (Brakebusch and Fassler, 2003; Dedhar, 2000; DeMali et al., 2003; Geiger et al., 2001; Hynes, 2002; Martin et al., 2002; Schwartz et al., 1995; Zamir and Geiger, 2001b). Whether these linkages and mechanisms represent the events occur in vivo are under investigation. This review attempts to provide an overview of newly discovered players and genetic analyses of molecules in the cytoplasmic side of focal adhesions and reveals the advantages of using cartilage as a tissue model for dissecting the architecture and function of vertebrate focal adhesion complexes.

New molecular components of focal adhesions

There were more than 50 focal adhesion proteins identified prior to 2001 (Zamir and Geiger, 2001a), and the list of focal adhesion molecule continues to grow in the last few years. These molecules can be divided into three groups according to

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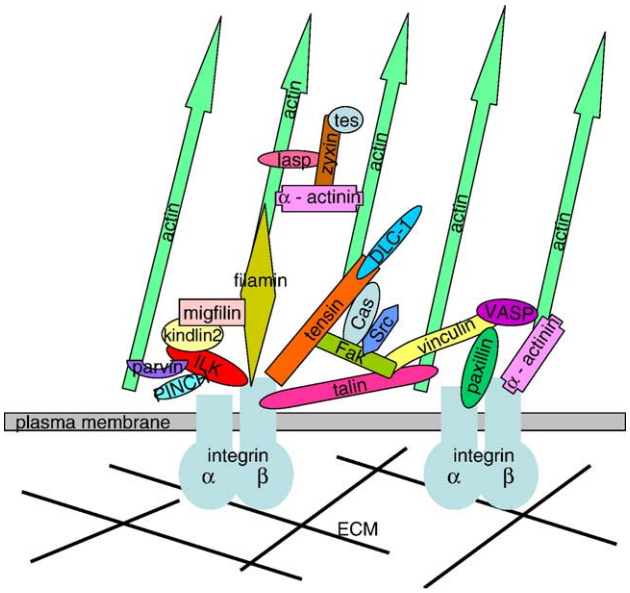

Fig. 1. A scheme illustrating interactions between several components of focal adhesions linking integrin receptors to the actin cytoskeleton. The molecules are not drawn to scale.

their location: extracellular, transmembrane, and cytoplasmic (Table 1). There are excellent reviews on more "senior" members of focal adhesions, including β1 integrin (Brakebusch and Fassler, 2005), integrin-linked kinase (Grashoff et al., 2004; Legate et al., 2006), parvin (Legate et al., 2006; Sepulveda and Wu, 2006), Src (Frame, 2004), focal adhesion kinase (Avraham et al., 2000; Mitra et al., 2005; Parsons, 2003), paxillin (Brown and Turner, 2004; Schaller, 2001), zyxin (Renfranz and Beckerle, 2002; Wang and Gilmore, 2003), talin/vinculin (Campbell and Ginsberg, 2004; Critchley, 2004, 2005), tensin (Lo, 2004), vinexin (Kioka et al., 2002), α-actinin (Otey and Carpen, 2004), PINCH (Wu, 2004), profilin (Witke, 2004), PTP1B (Bourdeau et al., 2005), Rho (Burridge and Wennerberg, 2004), and ERM family (Bretscher et al., 2002). The more recently identified molecules include previously undocumented family members of focal adhesion components, known molecules, but until recently their subcellular localization revealed, and newly identified genes. These newcomers are briefly discussed in this review.

Tensin2, tensin3, and cten

Tensin-related molecules are major contributors to the expansion of the focal adhesion family. Three additional family members were recently identified (Chen et al., 2002; Cui et al., 2004; Lo and Lo, 2002). Human tensin2, tensin3, and cten genes localize to chromosome 12q13, 7p13, and 17q21, respectively. The domain structures of tensin (or tensin1), tensin2, and tensin3 are very similar (Fig. 2). The N-terminal region displays a PTEN-related protein tyrosine phosphatase (PTP) domain. The same region contains actin-binding (ABD-1) and focal adhesion-binding (FAB-N) activities (Chen and Lo, 2003; Lo et al., 1994). The PTP domains of tensin1 and tensin2 are considered to be catalytically inactive due to the lack of a conserved "signature motif," and although tensin3 does contain the "signature motif," its enzymatic activity remains to be tested. All four members contain the SH2 (Src homology 2) and PTB (phosphotyrosine-binding) domains at the C-termini, which also includes the second focal adhesion-binding activity (FAB-C) (Chen and Lo, 2003). Although the PTB domain represents a binding module of phosphotyrosine, as implied by its name, it has been shown that the tensin's PTB domain binds to integrin β tails independent of tyrosine phosphorylation (Calderwood et al., 2003). The middle regions of tensins do not share any sequence homology. Like tensin1, tensin2 also regulates cell migration (Chen et al., 2002), whereas tensin3 participates in the epidermal growth factor (EGF) signaling pathway. Upon EGF stimulation, tensin3 binds to EGF receptor through the SH2 domain and is tyrosine phosphorylated primarily by Src kinase (Cui et al., 2004). Tensin3 null mice are growth retarded and die in 3 weeks after birth. Mutant mice display abnormalities in lung, small intestine, and bone (Chiang et al., 2005). These phenotypes are similar but less severe than those of EGF receptor knockout mice (Sibilia and Wagner, 1995; Threadgill et al., 1995), consistent with the idea that tensin3 is a downstream molecule of the EGF signaling pathway. Cten, C-terminal tensin like, is somewhat unique to other tensins. The molecule is much smaller, lacks the conserved N-terminal regions found in other tensins, and the tissue expression is relatively restricted to the prostate and placenta (Lo and Lo, 2002). Recent data suggest that cten may play a role in preventing prostate cell transformation and regulating apoptosis (Lo and Lo, 2002; Lo et al., 2005).

Talin2

Talin2 is the second member of the talin family (Monkley et al., 2001). The amino acid sequences are highly homologous, and intron/exon boundaries are completely conserved. Talin2 contains a four-point-one ezrin, radixin, moesin (FERM)

Table 1
Focal adhesion proteins

Location		Focal adhesion proteins
Extracellular		Collagen, fibronectin, heparan sulfate, laminin, proteoglycan, vitronectin
Transmembrane		Integrins 18 α and 8 β (24 combinations in humans), LAR-PTP receptor, layilin, syndecan-4
Cytoplasmic	Structural	Actin, α-actinin, EAST, ezrin, filamin, fimbrin, kindling, lasp-1, LIM nebulette, MENA, meosin, nexilin, paladin, parvin, profilin, ponsin, radixin, talin, tensin, tenuin, VASP, vinculin, vinexin
	Enzymatic	Protein tyrosine kinase: Abl, Csk, FAK, Pyk2, Src Protein serine/threonine kinase: ILK, PAK, PKC Protein phosphatase: SHP-2, PTP-1B, ILKAP Modulators of small GTPase: ASAP1, DLC-1, Graf, PKL, PSGAP, RC-GAP72 Others: calpain II, PI3-K, PLCγ
	Adapters	p130Cas, caveolin-1, Crk, CRP, cten, DOCK180, DRAL, FRNK, Grb 7, Hic-5, LIP.1, LPP, Mig-2, migfilin, paxillin, PINCH, syndesmos, syntenin, tes, Trip 6, zyxin

Known focal adhesion components.

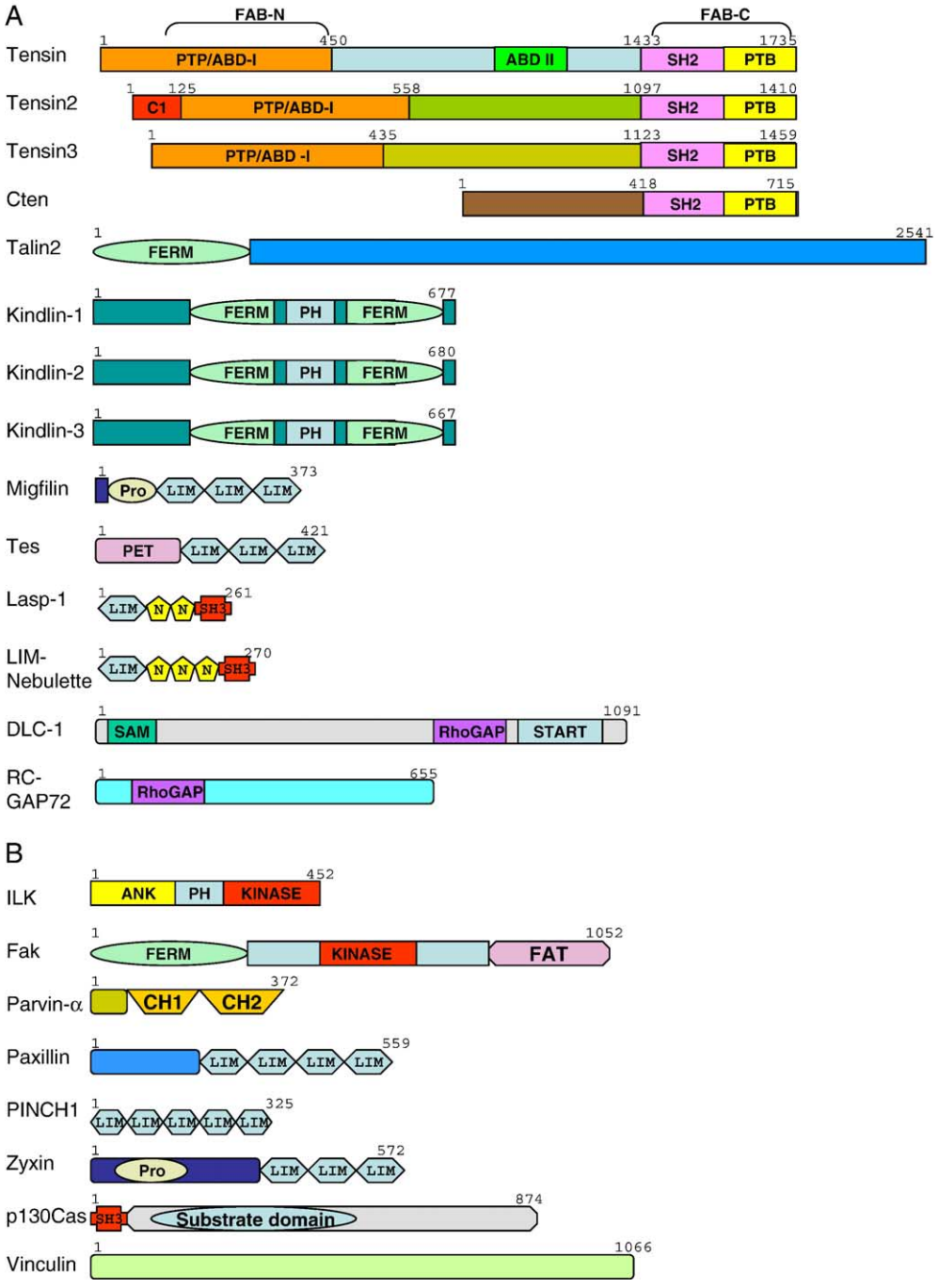

Fig. 2. Domain structures of recently identified (A) and commonly found (B) focal adhesion molecules. The numbers shown are the number of amino acids in human proteins. PTP, PTEN-related protein tyrosine phosphatase domain; ABD, actin-binding domain; SH2, Src homology domain 2; PTB, phosphotyrosine binding; FAB, focal adhesion binding; C1, protein kinase C conserved region 1; FERM, four-point one, ezrin, radixin, moesin; PH, pleckstrin homology; Pro, proline-rich region; LIM, lin-11, isl-1, and mec3; PET, prickle espinas, testing; N, nebulin-like repeat; SH3, Src homology domain 3; SAM, sterile alpha motif; START, StAR-related lipid transfer; ANK, ankyrin repeat; CH, calponin homology.

domain and the human gene is located at 15q15–21. Due to much larger introns in talin2, the entire talin2 gene is about 6 times larger than talin1 gene in mammals. Because all the domains in talin1 are conserved in talin2, they most likely bind to the same group of molecules. For example, talin2 is shown to bind to PIP kinase 1γ (Di Paolo et al., 2002) and actin (Senetar et al., 2004). Nonetheless, there are many potential alternative splice forms of talin2 and a more restricted expression pattern, suggesting that talin2 may have a unique function in specific tissues. A talin2 mutant mouse line generated from a gene-trapped ES clone has been established (Chen and Lo, 2005). The homozygous mutant mice still expressed the N-terminal half (1–1295) of talin2 fused to β-galactosidase. Under these circumstances, it was predicted that deletion of the C-terminal half of talin2 and in some splice forms the majority of protein (for example, testis form) would impair the dimerization, and

disrupt the integrin-, vinculin-, and actin-binding activities located at the deleted C-terminus. In addition, the remaining N-terminal half might function as a dominant-negative fragment to elicit a gain of function phenotype in heterozygous and homozygous mice. Surprisingly, the talin2 mutant mice are normal and fertile, suggesting that talin2 is not as essential as talin1 in mice (Chen and Lo, 2005). Alternatively, it is possible, although less likely, that the N-terminal region is responsible for all talin2's in vivo function.

Kindlin-1/kindlerin/URP1, kindlin-2/Mig-2, kindlin-3/URP2

The kindlin family is a newly organized focal adhesion family, which includes kindlin-1/kindlerin/URP1 (UNC-112-related protein 1), kindlin-2/Mig-2 (mitogen-inducible gene-2), and kindlin-3/URP2. Kindlins contain a bipartite FERM domain and a pleckstrin homology (PH) domain. They are the human homologues of *Caenorhabditis elegans* gene UNC 112, which is an essential component for the recruitment of ILK (Pat-4) to the muscle attachment structure in worms, and has been implicated in linking the actin cytoskeleton to the ECM (Rogalski et al., 2000). Kindlin-1/kindlerin has been linked to Kindler syndrome, a rare autosomal-recessive genodermatosis characterized by bullous poikiloderma with photosensitivity (Jobard et al., 2003; Siegel et al., 2003). The expression of kindlin-1 is regulated by transforming growth factor-β1 (Kloeker et al., 2004) and is often upregulated in lung and colon cancers (Weinstein et al., 2003). In addition, kindlin-1 binds to integrin β tails and regulates cell spreading (Kloeker et al., 2004). Mig-2 was initially isolated as a serum-inducible gene (Wick et al., 1994). Downregulation of Mig-2 in mammalian cells by siRNA leads to a more rounded cell shape, suggesting a role of Mig-2 in cell adhesion (Tu et al., 2003) that may represent a common function for kindling family members. In addition, Mig-2 binds to migfilin and serves as a docking molecule recruiting migfilin to focal adhesions. Not much is known about kindlin-3 other than its expression appears to be confined to the immune system-related tissues (Weinstein et al., 2003). Kindlin-1, kindlin-2, and kindlin-3 genes localize to human chromosome 20p12.3, 14q22, and 11q12, respectively.

Migfilin/FBLP-1/Cal

Migfilin/FBLP-1 (filamin-binding LIM protein-1)/Cal (CSX-associated LIM protein) was isolated independently by three groups using the yeast two-hybrid screen. Migfilin was isolated as a molecule binding to Mig-2 that colocalized with Mig-2 at focal adhesions (Tu et al., 2003). FBLP-1 was identified by using filamin B (repeats 10–18) as bait (Takafuta et al., 2003). Cal was isolated also by a yeast two-hybrid screen using a cardiac homeobox transcription factor, CSX/NKX2.5, as bait (Akazawa et al., 2004). The human gene is located at chromosome 1p36. siRNA knockdown experiment shows migfilin affects cell shape, a phenotype similar to the Mig-2 knockdown (Tu et al., 2003). Overexpression of migfilin promotes actin stress fiber formation (Akazawa et al., 2004). Migfilin contains a proline-rich region and three LIM domains. The C-terminal LIM domain binds to Mig-2. In addition, migfilin interacts with filamin and VASP via its N-terminus and the proline-rich region, respectively (Wu, 2005). These interactions may allow migfilin and Mig-2 to link to the actin cytoskeleton via filamin and regulate the cell shape. Furthermore, migfilin shuttles between focal adhesions and the nucleus, where it interacts with cardiac transcriptional factor CSX/NKX2.5 through its LIM domains, and promotes the transcriptional activity of CSX (Akazawa et al., 2004).

Tes

Tes(tin) was identified as a candidate tumor suppressor. The human TES gene is located at 7q31 and falls within the fragile chromosomal region FRA7G, a locus that shows loss of heterozygosity in many human tumors (Tatarelli et al., 2000). Tes contains a PET (prickle espinas, testin) domain at N-terminal region and three LIM domains at the C-terminal half. Its N-terminal region binds to α-actinin and paxillin, whereas the LIM domains interact with mena, zyxin, talin, VASP, actin, and spectrin (Garvalov et al., 2003; Rotter et al., 2005). Tes overexpression enhances cell spreading and decreases cell motility (Coutts et al., 2003). Tes knockdown by siRNA leads to a loss of actin stress fibers (Griffith et al., 2005). The focal adhesion localization of tes is zyxin dependent and is regulated by the interaction between the N- and C-terminal halves of tes (Garvalov et al., 2003). Tes null mice appear to be normal but are more susceptible to carcinogen induced gastric cancer, consistent with its proposed role as a tumor suppressor (Drusco et al., 2005).

Lasp-1

Lasp-1 (LIM and SH3 protein 1) was originally identified as a phosphoprotein that migrated on SDS–PAGE gels with an apparent molecular mass of 40 kDa (Chew and Brown, 1987). The phosphorylation of this protein was enhanced by elevation of cAMP and the cloning data identified the molecule as lasp-1 (Chew et al., 1998). Lasp-1 contains an N-terminal LIM domain, two nebulin-like repeats, and a C-terminal SH3 domain (Li et al., 2004). It is structurally related to lasp-2/LIM nebulette and also shares the function of binding to the N-terminal of zyxin through the SH3 domain. In addition, it interacts with actin filaments in a serine phosphorylation-dependent manner (Chew et al., 2002). Human lasp-1 gene localizes to chromosome 17q21.

Lasp-2/LIM nebulette

Lasp-2/LIM nebulette was originally identified in silico (Katoh, 2003) and later as a protein binding to zyxin (Li et al., 2004) and F-actin (Terasaki et al., 2004). It contains a LIM domain, three nebulin-like repeats, and a C-terminal SH3 domain. It is a splice variant of nebulette, which is a 105-kDa sarcomeric protein only expresses in muscle cells. Nebulette contains 23 nebulin repeats, an SH3 domain, but no LIM domain. LIM nebulette is expressed in nonmuscle cells (Li et

al., 2004). The SH3 domain of LIM nebulette binds to the N-terminal of zyxin. The role of LIM nebulette is currently unknown but is proposed to play a role in the assembly of focal adhesions similar to the function of nebulette in the assembly of the sarcomeric Z-disks. The human gene is located at chromosome 10p12.

DLC1/p122RhoGAP/ARHGAP7

DLC-1 (deleted in liver cancer 1) gene was isolated from a primary human hepatocellular carcinoma by representational difference analysis (Yuan et al., 1998). The gene is localized on chromosome 8p21–22, a region of loss of heterozygosity in a number of human cancers. Genomic deletion of DLC-1 was found in many cancer cell lines and tissues. Downregulation of DLC-1 was also observed in human cancer samples. In addition, DLC-1 is able to inhibit tumor cell growth in human liver, breast, and lung cancer (Ng et al., 2000; Yuan et al., 2003, 2004). A rat homologue of the DLC-1, p122RhoGAP, was cloned as a phospholipase Cδ1-binding protein from a rat brain library (Homma and Emori, 1995). It contains a RhoGAP domain in the middle region that enhances the phosphatidylinositol(4,5)-bisphosphate-hydrolyzing activity of PLCδ1. In addition, there is a SAM (sterile alpha motif) domain at the N-terminus and a START (StAR-related lipid transfer) domain at the C-terminus. Overexpression of the C-terminal region of p122 RhoGAP inhibits the lysophosphatidic acid (LPA)-induced formation of actin stress fibers and focal adhesions by inhibiting the GTP-bound-activated form of Rho and leading to a concomitant increase in intracellular $Ca2^{+}$ levels (Sekimata et al., 1999). P122 RhoGAP was found to localize to caveolin and focal adhesions (Kawai et al., 2004; Yamaga et al., 2004) and to interact with tensin (Liao, Si, and Lo, unpublished results). Deletion of DLC1 in mice led to embryonic lethality (Durkin et al., 2005).

RC-GAP72/ARHGAP24

RC-GAP72 (Rac1/Cdc42-specific GAP with a predicted molecular mass of 72 kD)/ARHGAP24 was identified by a bioinformatics search and microscopy-based screen (Lavelin and Geiger, 2005). It contains a RhoGAP domain near the N-terminus and enhances GTPase activity of Rac1 and Cdc42 but not RhoA. The C-terminal region of RC-GAP72 interacts with actin fibers, focal adhesions, and cell–cell contacts. Overexpression of RC-GAP72 induces cell rounding with disruption of actin stress fibers. It is proposed that RC-GAP72 affects cellular morphology by targeting-activated Cdc42 and Rac1 GTPases to specific subcellular sites, triggering local morphological changes (Lavelin and Geiger, 2005). Human RC-GAP72 gene is at 4q21.

Genetic analysis in fly

Drosophila melanogaster provides an excellent model system for studying integrin function and the focal adhesion complex. This is because integrin-dependent cell adhesion is required for proper organization of multiple embryonic and adult tissues, and also the *Drosophila* genome is not as complex as that of vertebrate. There are 5 α (αPS1–5) and 2 β (βPS and βν) integrin subunits identified in *Drosophila*, whereas 18 α and 8 β subunits have been reported in mammals. *Drosophila* αPS1βPS is a laminin-binding integrin, corresponding to vertebrate α3β1, α6β1, and α7β1, whereas αPS2βPS is an RGD-binding integrin, corresponding to vertebrate α5β1, αVβ1, and α8β1. Almost all null mutations in PS integrins cause lethality in the embryo of first instar larva (Bloor and Brown, 1998; Brower, 2003; Bunch et al., 1992). Two of the phenotypes highlight the contribution of integrin adhesion: the detachment of the muscles from the epidermis in null embryos, and the formation of wing blisters in homozygous somatic clones. As noted above, the pool of focal adhesion components is smaller in *Drosophila* than in vertebrates. For example, *Drosophila* has one talin, PINCH, Fak, and tensin gene; whereas two talin, two PINCH, two Fak, and four tensin genes are present in mouse. The lower diversity of focal adhesion components will make data interpretation simpler by reducing the potential redundancy and compensation from other family members, as is often observed in knockout mice. Therefore, studies in relatively simple organisms such as *D. melanogaster* have the potential to reveal more details about the basic, conserved molecular linkages and mechanisms related to the architecture and function of focal adhesions.

To identify potential integrin effectors in *Drosophila*, two laboratories have performed genetic screens for mutants displaying wing blisters, an adult phenotype resulting from the disruption of the integrin-mediated basal junctions that hold the two wing surfaces together (Prout et al., 1997; Walsh and Brown, 1998). About 25 new loci were identified. Among them, the *steamer duck* (*stck*) locus encodes PINCH. PINCH null mutations cause early larval lethality due to defects in muscle attachment. The mutants hatch but fail to grow. Mutation clones in wing tissue lead to blister formation. The actin cytoskeleton was disrupted and detached from integrins adhesion sites, whereas integrin and ILK (integrin-linked kinase) localization was not affected in mutants, demonstrating that the proper localization of integrin and ILK is independent to PINCH but is not sufficient to stabilize the actin cytoskeleton. On the other hand, the appropriate localization of PINCH requires integrins (Clark et al., 2003).

The same screen for potential integrin effectors also identified another locus, *rhea*, which corresponds to the single *Drosophila* talin (Brown et al., 2002). Talin null embryos display a failure in germband retraction and strong muscle detachment phenotypes, which are very similar to integrin (βPS) null embryos. Clones of mutant cells in the wing do not attach to the other cell layer of the wing. One of the key functions of talin is to connect ECM-bound integrins to the actin cytoskeleton because integrins remain at the cell surface and localize normally in the absence of talin. Localization of talin to integrin adhesion sites requires integrins. However, talin is recruited to gonadal mesoderm by a mechanism unrelated to integrins.

ILK is a Ser/Thr kinase that binds to integrin β tail. ILK mutations in flies cause embryonic lethality and have a muscle detachment defect. Clones of cells lacking ILK in the adult wing lead to blister formation (Zervas et al., 2001). These results indicate that ILK is required to link the actin cytoskeleton to the integrin sites. However, the protein kinase activity of ILK is not required for this function because the phenotypes can be fully rescued by ILK kinase-dead mutant. This is also the case in *C. elegans*. ILK (PAT-4) is essential for integrin-mediated adhesion during muscle development in the *C. elegans* and kinase dead of ILK can rescue the lethal phenotype to a normal lifespan (Mackinnon et al., 2002). On the other hand, the kinase domain, even without the kinase activity, is required for ILK's normal function in integrin-mediated attachment, demonstrating that the main function of ILK is to serve as a structural adaptor in invertebrates. Interestingly, although ILK binds to β integrin tail and PINCH, *Drosophila* ILK localizes to muscle ends in the absence of integrins or PINCH (Zervas et al., 2001). Therefore, there must be another mechanism for recruiting ILK to the adhesion sites.

Mutations in *Drosophila* tensin are responsible for the blistery (by) allele mutant, which displays a viable blistered wing phenotype (Lee et al., 2003; Torgler et al., 2004). Because the wing blisters in tensin mutant flies appeared shortly after eclosion and were localized at the distal end of the wings, it was speculated that the mechanical shear stress from normal motion of the back legs helps the expansion and flattening of the wings caused the wing blister. Interestingly, the wing blister phenotype was rescued by gluing the legs of mutant flies to a glass slide, just as the wings started to unfold after eclosion (Torgler et al., 2004). Therefore, tensin is required to stabilize adhesion in the wing so that it can resist the normal mechanical abrasion associated with wing flattening after eclosion. In addition, the genetic approaches also demonstrated that tensin interacts with integrin and the JNK signaling pathway during wing development. The blistered wing phenotype and rate were significantly enhanced in tensin (*by*) and integrin (*if*), JNK (*bsk*), or MKK7 (*hep*) double mutants (Lee et al., 2003). Further experiments using other mutant flies have revealed the localization of tensin to integrin adhesion sites requires integrins, talin, and integrin-linked kinase, but not PINCH (Torgler et al., 2004). It is worthwhile noting that at amino acid sequence level, *Drosophila* tensin is not very similar to vertebrate tensins. The only conserved regions are the SH2 and PTB domains. *Drosophila* tensin is much shorter than tensin1, tensin2, and tensin3. It is more similar to vertebrate cten, which only shares sequence homology with the SH2 and PTB domains. Nonetheless, the N-terminal regions of cten and *Drosophila* tensin are totally different. For some time, it was questioned whether this *Drosophila* tensin represents the true ortholog of tensin. Because there is no other gene in *Drosophila* that looks like tensin, which localizes to the integrin complex and shares functional similarity, this is most likely the *Drosophila* ortholog of tensin.

Flies lacking Fak56, the only Fak gene in *Drosophila*, are viable and fertile (Grabbe et al., 2004), demonstrating that Fak is not essential for integrin function in adhesion, migration, or signaling. This is also the case in *C. elegans* because worms with a large deletion in the Fak (kin-32) open reading frame or with RNAi treatment appear to be normal (www.wormbase.org). The localization of integrin, talin, tiggrin, and actin to integrin complexes does not require Fak56 (Grabbe et al., 2004). Interestingly, muscle-specific overexpression of Fak56 resulted in a potent muscle detachment phenotype (Grabbe et al., 2004), similar to the lack of αPS2 (Brown, 1994). Further experiments showed that αPS2 integrin remains at the ends of the detached muscles indicating its dissociation from the extracellular matrix. The localization of talin and ILK at muscle attachment sites was not affected by Fak56 overexpression. These results suggest that Fak56 overexpression does not lead to the disassembly of the integrin–actin link but results in a detachment of the plasma membrane via dissociation of the integrins from the ECM, indicating that Fak56 overexpression may negatively regulate integrin ligand-binding affinity (Grabbe et al., 2004). It will be interesting to test whether the kinase activity of Fak56 can account for the overexpression phenotype. Because Fak56 and kin-32 are the only Fak genes in *Drosophila* and *C. elegans*, it is clear that Fak is not required for integrin-mediated adhesion or signaling in fly and nematode. Similarly, vinculin mutant flies are viable and fertile (Alatortsev et al., 1997), although vinculin (DEB-1) mutant worms were paralyzed and had disorganized muscle (Barstead and Waterston, 1991). Nonetheless, both genes play more critical roles in vertebrates: both Fak and vinculin knockout mice are embryonic lethal (Furuta et al., 1995; Xu et al., 1998).

In summary, analyses of these fly mutants have confirmed the involvement of several cytoplasmic proteins, including talin, ILK, PINCH, and tensin, in integrin-dependent adhesion. Although not yet completed, the localization studies in various mutant flies have revealed that (1) integrin is required for the proper localization of talin, PINCH, tensin, and actin filament, but not ILK; (2) talin is required for tensin and actin filaments, but not integrin; (3) ILK is required for tensin and actin filaments; and (4) PINCH is not required for tensin and ILK. Meanwhile, several surprises were observed. ILK apparently is essential for integrin function, but the kinase activity is not required. Two major focal adhesion molecules, focal adhesion kinase and vinculin, which are essential for mouse development, are dispensable in fly. Tensin null flies only display a viable wing blister phenotype. These results show the discrepancy between flies and mice, and that some focal adhesion molecules gain more critical function in vertebrates. The systematic use of genetic approaches to characterize the involvement of the focal adhesion molecules in fly should lead to the identification of the essential molecules required and provide the mechanism in assembly and regulating focal adhesions in *Drosophila*.

Genetic analysis in mouse

Ablation of focal adhesion genes leads to various phenotypes during development, ranging from apparently normal mice to early embryonic lethality. There are excellent reviews discussing the ECM and integrin knockout mice (Bouvard et al., 2001;

Table 2
Focal adhesion gene knockout mice

Gene		Phenotypes	Reference
Abl	L, neonatal	Lymphopenia	Tybulewicz et al. (1991)
Caveolin-1	V, F	Loss of caveolae, vascular and pulmonary abnormalities	Drab et al. (2001); Razani et al. (2001)
Cdc42	L, E6.5	Ectoderm defect	Chen et al. (2000)
Csk	L, E9–10	Neural tube defect	Imamoto and Soriano (1993)
DLC1	L, E10.5	Defects in the neural tube, brain, heart, and placenta	Durkin et al. (2005)
Ezrin	L, before wean	Essential for epithelial organization and villus morphogenesis in the developing intestine	Saotome et al. (2004)
Fak	L, E9.5	Mesodermal defect	Furuta et al. (1995)
ILK	L, <E5.5	Peri-implantation lethality; abnormal epiblast polarization, impaired cavitation, detachment of endoderm, and epiblast from basement membrane	Sakai et al. (2003)
MENA	V	Hippocampal commissure defects, MENA−/− Profilin+/− die in utero	Lanier et al. (1999)
Moesin	V, F	No apparent phenotype; no compensatory upregulation of erzin or radixin	Doi et al. (1999)
p130Cas	L, E12.5	Heart and blood vessel defects	Honda et al. (1998)
Paladin	L	Neural tube closure defect	Luo et al. (2005)
Parvin-β	V, F	No apparent phenotype; parvin-α upregulated	ELSO 2005 abstract
Parvin-γ	V, F	No apparent phenotype	Chu et al. (2006)
Paxillin	L, E8.5	Mesodermal defect	Hagel et al. (2002)
PINCH1	L, E6.5	Abnormal epiblast polarity, impaired cavitation, detachment of endoderm, and epiblast from basement membrane	Li et al. (2005); Liang et al. (2005)
PINCH2	V, F	No apparent developmental defect, PINCH1 upregulated	Stanchi et al. (2005)
Profilin 1	L	No detectable mutant blastocysts	Witke et al. (2001)
PTP1B	V, F	Hypersensitive to insulin and resistant to obesity	Elchebly et al. (1999); Klaman et al. (2000)
PYK2	V, F	No apparent developmental abnormal macrophages, obesity, and insulin resistance under high-fat diet	Guinamard et al. (2000); Okigaki et al. (2003); Yu et al. (2005)
Rac1	L, E9.5	Abnormalities in three germ layers	Sugihara et al. (1998)
Radixin	V, F	Mild liver injury in older mice hyperbilirubinemia	Kikuchi et al. (2002)
Src	V	Osteopetrosis	Soriano et al. (1991)
Talin1	L, E8.5	Incomplete gastrulation	Monkley et al. (2000)
Talin2*	V, F	No apparent developmental phenotype	Chen and Lo (2005)
Tensin1	V, F	Kidney and muscle regeneration defects	Ishii and Lo (2001); Lo et al. (1997)
Tensin3	L, postnatal	Growth retardation	Chiang et al. (2005)
Testin	V, F	No apparent phenotype, higher susceptibility to induced carcinogenesis	Drusco et al. (2005)
VASP	V	Megakaryocyte hyperplasia in bone marrow and spleen, enhanced platelet activation	Hauser et al. (1999); Massberg et al. (2004)
Vinculin	L, E8–10	Heart and brain defect	Xu et al. (1998)
Zyxin	V, F	No apparent phenotype	Hoffman et al. (2003)

V indicates viable; F, fertile; L, lethal.
* Not a complete knockout, see text.

Hynes, 1996). Here, we focus on the mice carrying mutations in cytoplasmic components of focal adhesions (Table 2). By now, we have learned that it is difficult to "predict" the phenotypes prior to generating mutant mice, but generally they range from embryonic lethal to apparently normal with mild defects. Knockout animals of ILK, talin1, Fak, Csk, PINCH1, vinculin, p130Cas, paxillin, DLC1, Cdc42, Rac1, and paladin die at various stages during embryogenesis, reflecting the critical function of these genes in cell adhesion and migration during embryogenesis. Zyxin, Tyk2, Tes, moesin, talin2, and parvin-beta null mice were normal and fertile. Only very mild abnormalities were found in mice lack of MENA, VASP, and radixin. Molecules with adult phenotypes are Src, tensin1, tensin3, and ezrin. Generally, if the gene has no other known family member, it is required for development and survival. If the gene is a member of a family, one member tends to be more critical than the other(s). For example, in the cases of Fak versus Tyk2, PINCH1 versus PINCH2, and talin1 versus talin2, knockout of the former gene results in a more severe phenotype than that of the later. It seems reasonable because other members may compensate or have overlapping function, although this is apparently not due to the upregulation of gene expression in most cases.

It is clear that a gene is not essential for the development of a specific tissue/organ, if the gene is eliminated and the tissue still develops normally. The question is why the animals do not need the gene. As mentioned, overlapping functions and compensation by other family members or related molecules are two apparent possibilities. On the other hand, it is strange that an expressed gene has no unique function in an animal. Mutant mice are routinely examined for the gross morphology and histology of major tissues/organs. Many other functions, such as sensory systems, are not routinely analyzed. For example, it is later found that VASP−/− mice were more sensitive to noise (Schick et al., 2004). One other possibility is that these "no phenotype" genes may play roles involving in aging, repair, regeneration, or defense processes, which are not often occurred in most experimental setups. This is true for tyk2, tensin1, and tes mice. When tyk2 null mice were fed with high-fat diet, the mutant mice had higher obesity and insulin resistance (Yu et al.,

2005). In addition to kidney defects (Lo et al., 1997), the incident of central nuclei was significantly higher in tensin1 skeletal muscle and the regeneration process of damaged muscle induced by cardiotoxin was severely delayed (Ishii and Lo, 2001). Tes null mice were more susceptible to *N*-nitrosomethyl-benzylamine (NMBA)-induced carcinogenesis (Drusco et al., 2005). This is consistent with tes' proposed role as a tumor suppressor. I am convinced that with the generation of compound gene knockout mice, systematic analysis of all expression tissues in various conditions, sooner or later many of the undetected phenotypes will be revealed.

From the analyses of the null mice, we have learned that some targeted genes are essential for embryonic development, some are important for specific tissues, and some appear dispensable. These studies are very informative in terms of the function of each individual gene. However, because of the early lethality and/or defects in various tissues, it is difficult to establish a functional linkage and the relationships among focal adhesion molecules from these data. It will require other approaches, for example, the systematic analysis of a specific mutant tissue in constitutive or conditional knockout mice to dissect the functional network of these molecules in vivo. To this end, the cartilage may provide an excellent tissue model because chondrocytes, the primary cell type of cartilage, are completely surrounded by ECMs, they form functional and histological distinct zones in the growth plate, and cartilage development is not essential for the embryonic survival.

Cartilage as a tissue model for analyzing focal adhesion molecules

The skeleton is essentially developed through two mechanisms and both involve the transformation of a preexisting mesenchymal tissue into bone tissue. The direct conversion of mesenchymal cells into flat skull bones is called intramembranous ossification. The other mechanism to build bones is endochondral ossification, which involves a two-stage process in which mesenchymal cells differentiate into cartilage that is later replaced by bone. Cartilage is the blueprint for subsequent bone morphogenesis, the location of tendon and ligament insertions, and morphogenesis of the joints. During endochondral bone development, chondrocytes form the growth plate, in which several zones are established (Fig. 3). At the top of growth plate, round chondrocytes no longer proliferate rapidly and are called resting zone, which is followed by orderly columns of proliferating chondrocytes (proliferating zone). The proliferating cells then stop proliferating and form the hypertrophic zone, which is the primary engine of bone growth. The hypertrophic chondrocytes undergo apoptosis and the deposited matrix provides a scaffold for osteoblasts and blood vessels invasion (invasion zone). The cartilage development is well regulated by local signals, including Wnt, Hedgehog, fibroblast growth factor, transforming growth factor-β, and bone morphogenetic protein families, and by transcriptional factors such as Sox and Runx families (Ballock and O'Keefe, 2003a; de Crombrugghe et al., 2001; Kronenberg, 2003; Leinders-Zufall et al., 2004).

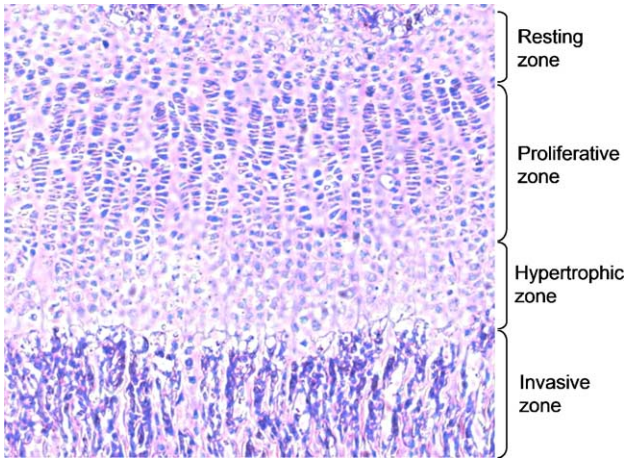

Fig. 3. Histological appearance of the mouse growth plate.

On the other hand, chondrocytes are completely surrounded by ECM with no cell–cell contacts. Therefore, the interactions between chondrocytes and ECMs may regulate, in concert with factors mentioned above, their proliferation and differentiation. Likewise, ECM receptors (mainly integrins) on chondrocytes may modulate cell adhesion and the assembly of ECM molecules in cartilage. The ECM network is dynamic and coordinated with proliferation stages. When resting chondrocytes switch to prehypertrophic chondrocytes, the synthesis of ECM molecules is also switched. Whereas proliferating chondrocytes express and secrete type II collagen, hypertrophic chondrocyte produce type X collagen. Chondrocytes express a characteristic set of integrins including receptors for collagen II (integrins α1β1, α2β1, and α10β1), fibronectin (integrins α5β1, αvβ3, and αvβ5), and laminin (integrin α6β1) and other focal adhesion molecules such as ILK, talin, tensin, vinculin, Fak, tyk2, and paxillin (Vinall et al., 2002). It is not surprising that mutations in these genes may lead to human diseases. For example, collagen II mutations cause a spectrum of chondrodysplasias ranging from mildly affected patients of normal stature and premature osteoarthritis to severely affected patients with short stature and the lethal forms of achondrogenesis type II (Ballock and O'Keefe, 2003b).

From the available constitutive and conditional knockout mouse models, the involvements of several ECM and focal adhesion components in cartilage development can be defined. For ECM components, collagen II null mice die around birth with disorganized cartilage and lack of growth plate (Aszodi et al., 1998). Collagen IX null mice show degenerative changes of articular cartilage (Fassler et al., 1994). On the other hand, chondrocyte-specific knockout of fibronectin, or tenascin-C, matrilin1/2/3, cartilage oligomeric matrix protein (COMP) null mice show no bone defect (Aszodi et al., 1999, 2003; Brandau et al., 2002; Forsberg et al., 1996; Saga et al., 1992; Svensson et al., 2002). For integrin receptors, loss of integrin α1, α2, α6, αv, β3, or β5 expression in mice shows no skeletal phenotypes (Bouvard et al., 2001). Loss of α10 integrin expression leads to moderate dysfunction of the growth plate (Bengtsson et al., 2005). The mutant mice had a normal lifespan and were fertile but developed a growth retardation of the long bones, a

disturbed columnar arrangement of chondrocytes, an abnormal chondrocyte shape, and a reduced proliferation. Loss of β1 integrin in chondrocytes results in a severe chondrodysplasia, characterized by the complete lack of chondrocyte columns in growth plates, distorted collagen fibrillar network in the cartilage matrix, and reduced proliferation (Aszodi et al., 2003). For focal adhesion molecule binding to integrin cytoplasmic tails, ILK conditional knockout mice also develop chondrodysplasia and die at birth (Grashoff et al., 2003; Terpstra et al., 2003). The proliferative and hypertrophic zones in mutant growth plate were reduced and the column was slightly disorganized. Tensin3 null mice are growth retarded and die before weaning. The mutant long bones were shorter but the resting zone was larger in mutant growth plate (Chiang et al., 2005). However, conditional knockout mice from another integrin-binding protein, Fak, show no bone defect (Chen, Liao, and Lo, unpublished results), despite the fact that Fak null constitutive knockouts are embryonic lethal. These results support the idea that focal adhesions are structurally and functionally heterogeneous. It is clear that chondrocyte-specific ECM, collagen II, is required for overall cartilage development and is essential for growth plate formation. Integrin β1 is essential for chondrocyte column organization to modulate other zone formation. ILK regulates proliferative and hypertrophic zone development, whereas tensin3 may regulate the transition process from resting to proliferative zones. From the severity of the phenotype, the degree of involvement could be established as collagen II > integrin β1 > ILK > tensin3. With this approach, we should be able to define the singular and collaborative roles of focal adhesion molecules in cartilage development and function in the near future.

Conclusions and future directions

Three decades after the discovery of focal adhesions, we have learned a lot of this dynamic organelle. Numerous associated molecules, molecular linkages connecting ECM to the actin cytoskeleton, and mechanisms that regulate signal transduction pathways and modulating focal adhesion assembly have been identified. With a growing number of null mice available, the physiologic role of each individual focal adhesion molecule is demonstrated. The future challenges include determination of how these molecules work together as a complex, and how focal adhesion heterogeneity contributes to various biological responses in different tissues. Genetic analysis of the less intricate focal adhesion systems in invertebrates will lead to discovery of the minimal set of conversed components necessary to form and regulate focal adhesions. In mice, the combination of multiple and conditional knockout approaches will provide a powerful tool in dissecting the focal adhesion network in vertebrates.

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