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Focal Adhesions: a personal perspective on a half century of progress

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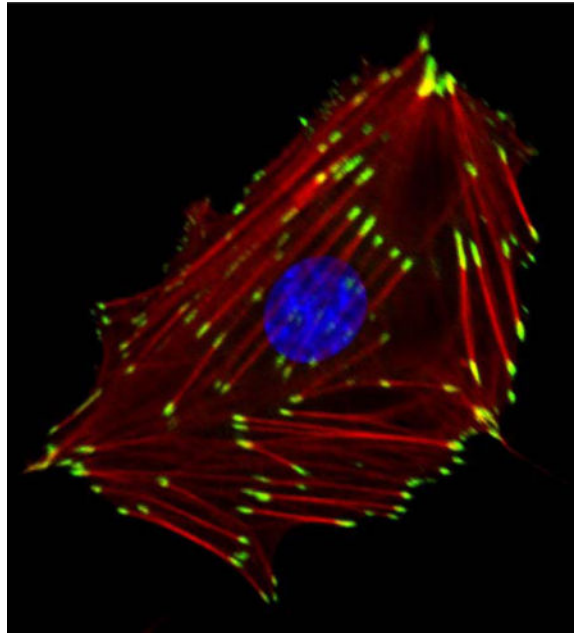
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Abstract

Focal adhesions are specialized sites within the cell where clustered integrin receptors interact with the extracellular matrix on the outside of cells and with the actin cytoskeleton on the inside. They provide strong adhesion to the matrix and transmit mechanical tension generated within cells across the plasma membrane to the external environment. Additionally, they act as scaffolds for many signaling pathways triggered by integrin engagement or mechanical force exerted on cells. Here I describe my personal perspective on focal adhesion research which I have witnessed since the initial discovery and description of focal adhesions as electron dense regions of the ventral plasma nearly half a century ago.

Graphical abstract

A REF52 fibroblast, plated on a collagen-coated glass coverslip, reveals focal adhesions stained for vinculin (green), F-actin (red) showing stress fibers, and the nucleus labeled in blue. In this Discovery-in-Context Review, Keith Burridge provides a personal account on focal adhesion research since its initial discovery and description nearly half a century ago.



Keywords

Focal adhesions; adhesion plaques; focal complexes; nascent adhesions; RhoA; FAK; stress fibers; cell migration

During my first weeks as a graduate student in the autumn of 1971, I went to a lecture by Michael Abercrombie at the Cambridge Philosophical Society. With hindsight, this seems like an unlikely venue for a memorable research presentation, but Abercrombie had been invited to share his recent work investigating cell migration in tissue culture. He showed a series of light and electron microscopic images of migrating fibroblasts, and several of the images stand out in my memory. Some were electron micrographs (EMs) illustrating lamellipodia at the front of migrating cells, but the most memorable images revealed bundles of filaments approaching and possibly inserting into dense plaques on the ventral plasma membrane. These regions came close to the underlying substratum. The filaments were not identified, but Abercrombie speculated they might be actin, which had only recently been identified in non-muscle cells. The plaque structure providing attachment of filaments was discussed and a similarity noted to the *zonula adherens* and *macula adherens* of epithelial cells, structures already believed to function in cell adhesion and filament anchoring. These images appear in the paper by Abercrombie et al. [1], although now when I look at them again they are less impressive than my memory of them as presented in his talk. Little did I anticipate that much of my own career would be directed towards studying these structures which we now refer to as focal adhesions (FAs). I have witnessed research on FAs progress from being a small and obscure corner of the cytoskeletal field to becoming a huge field in itself. It is a field that includes multiple signaling pathways that influence cell growth and behavior in many ways. Some of these pathways are initiated in response to engagement of the extracellular matrix (ECM), whereas others are triggered by mechanical tension or in response to the rigidity or other physical characteristics of the matrix. FAs are also sites

where mechanical force generated within the cell is transmitted to the surrounding ECM, influencing its organization but also contributing to cell migration. In this brief review, I hope to give my personal perspective on the early history of FA research and to highlight some of the key developments that have driven this field.

Early microscopy studies

In Abercrombie's classic paper, he referred to the electron dense regions on the ventral plasma membrane as "plaques" and from this the term "adhesion plaque" became widely used to describe these structures. A few years after Abercrombie's study, Izzard and Lochner applied Interference Reflection Microscopy (IRM) to investigate how close the underside of fibroblasts comes to the glass coverslip on which they are moving [2]. With IRM the more closely apposed the membrane is to the glass, the darker the resulting image. The darkest structures, which they termed "focal contacts", had an elongated oval shape about 2–10 μm in length, and 0.25 to 0.5 μm wide. They calculated that the plasma membrane at focal contacts was separated by 10–15 nm from the substratum [2]. Heath and Dunn combined both EM and IRM to show that adhesion plaques and focal contacts were the same structures [3]. They used the term "focal adhesion" once in the summary of their paper and this was subsequently adopted by Couchman and Rees [4] who were studying cardiac fibroblasts migrating out of heart explants. These authors made the interesting observation that during the initial period of rapid migration the fibroblasts displayed few if any FAs; however, as cell migration slowed down, FAs developed. It is often forgotten that cell migration does not require FAs. Indeed, many cells migrate effectively without them; in these cells adhesion to the ECM is still occurring via integrins but adhesion is too transient and unstable to permit large clusters of integrins to develop into identifiable FAs. In their study, Couchman and Rees made a distinction between FAs and focal contacts, with the former term being used for the darkest structures seen by IRM. Not only were focal contacts less dark, but they were more transient than FAs. They also concluded that both structures were equivalently close to the substratum but they interpreted FAs being darker because of increased protein recruitment leading to a higher refractive index. Subsequent work has indeed supported the idea that as adhesions mature more proteins are recruited to these sites.

For about a decade the terms focal adhesion, adhesion plaque, and focal contact were used synonymously by many labs. Gradually, the term focal adhesion became the most common term used to describe these sites of tight adhesion to the ECM and the other terms are now less commonly used. However, the idea that many newly formed adhesions are less stable, with many disassembling and only a few maturing to become the relatively stable structures, was important. Nobes and Hall introduced the term "focal complex" to differentiate the smaller, more transient adhesions, only some of which mature into FAs [5]. Although focal complexes are smaller than FAs, they are still large macromolecular arrays that have developed from still smaller adhesions, for which the term "nascent adhesion" is now frequently used. In general, the terminology and temporal sequence of nascent adhesions progressing to focal complexes and then maturing to FAs has been widely adopted. Finally, the term "fibrillar adhesion" was developed to describe adhesions made to extracellular matrix (ECM) fibrils, such as fibronectin [6]. Fibrillar adhesions often grow out of FAs and typically involve a subset of integrins ($\alpha 5\beta 1$) and are enriched in specific components such

as tensin. These terms describing the different types of matrix adhesion are valuable but in reality there must exist a continuum of adhesions reflecting the integrins expressed by different cell types and distinct conditions in which the adhesions develop.

Fluorescence microscopy ushers in a revolution

Although EM and IRM were important in the initial discovery of FAs, it has been immunofluorescence microscopy that has driven the field more than any other technique. In the mid 1970s a revolution in cell biology was initiated by the application of immunofluorescence to visualize actin in cells in culture [7]. There was a rush to generate antibodies against many muscle proteins so as to investigate their existence and distribution in fibroblasts and other cell types. As a student in Dennis Bray's lab, my primary focus was on non-muscle myosins, but I was also interested in how actin filaments attach to membranes. One of the few proteins known to be involved in attachment of F-actin to any structure was α -actinin, which had been implicated in attaching F-actin to striated muscle Z-discs. This prompted me to develop an antibody to look for α -actinin in non-muscle cells. Arriving at Cold Spring Harbor laboratory for my postdoc, I discovered that Elias Lazarides, a graduate student in Jim Watson's group, had similarly developed an antibody against α -actinin. Rather than competing, we decided to pool our results and publish together [8]. We noted that α -actinin was prominently localized along stress fibers with a periodic distribution reminiscent of its localization in muscle myofibrils. Significantly, it was also concentrated in plaques at the ends of actin filament bundles (i.e. in FAs, although this term had not yet been coined). We speculated that these sites corresponded to the dense structures involved in adhesion described by Abercrombie and colleagues and that α -actinin might serve to attach actin to the plasma membrane at these regions.

The seminal breakthrough in the field came a few years later with Benny Geiger's discovery of vinculin [9]. With striking images he showed that vinculin was a protein uniquely concentrated in FAs. Unlike α -actinin, it was not distributed along stress fibers [9]. Geiger discovered vinculin as a contaminant while purifying α -actinin from chicken gizzard smooth muscle. Rather than discarding this contaminant, he went on to purify and generate an antibody against the protein. These gave the eye-catching images of FAs. I recall my own chagrin at failing to be the first to publish on vinculin because at Cold Spring Harbor, Jim Feramisco and I had similarly stumbled onto this previously unknown protein while developing a purification of α -actinin from chicken gizzards. Sensing no rush, our characterization of this "new" protein had remained as a back-burner project. We had begun to immunize rabbits with vinculin, when in the summer of 1979 Benny Geiger came to a cytoskeletal conference at Cold Spring Harbor and electrified us and most of the attendees with his images of vinculin in FAs. Accelerating our own effort on this protein, Feramisco exploited his expertise at microinjection, introducing a fluorescently-derivatized version of the protein into cells. The results were striking as we observed fluorescent vinculin beautifully targeting FAs but also co-aligning with fibronectin fibrils on the cell surface [10].

Geiger's discovery of vinculin lifted FA research out of obscurity and pushed FAs to center stage in both the cytoskeleton and adhesion fields. At the personal level, our failure to be first with vinculin motivated my research for several years. I no longer hesitated about

generating antibodies against purified proteins for localization studies. Indeed, it became my strategy to pursue and purify “new” proteins from smooth muscle, which turned out to be a rich source of FA components, and to generate antibodies against these proteins to probe their localization in fibroblasts. This approach resulted in our discovery of talin, another prominent FA component [11]. Even with talin, however, we later discovered that the protein had previously been isolated from human platelets, although in that work it had not been shown to be a FA component [12]. We went on to demonstrate that talin interacts with vinculin [13], and then, collaborating with the labs of Rick Horwitz and Clayton Buck, we showed that isolated integrins could bind talin [14]. Notably, in subsequent work talin was discovered to be critical for activating integrins, thereby promoting their association with the ECM [15]. Given that both talin and vinculin bind actin, the interaction of talin with integrins provided a long sought link between the cytoskeletal components of FAs and the plasma membrane. Integrins had been discovered a few years earlier by multiple groups investigating cell adhesion in several different cellular contexts, ranging from platelets and leukocytes to myoblasts and fibroblasts. The work converged to identify integrins as a family of transmembrane cell adhesion proteins, potentially connecting the ECM on the outside of the cell to the actin cytoskeleton on the inside [16]. The localization of these ECM receptors revealed them to be concentrated in FAs [17, 18]. Integrins are not the only transmembrane proteins in FAs, but they are the dominant ones. Indeed, FAs can be defined as regions of tightly clustered integrins, providing strong adhesion to the underlying matrix, while simultaneously serving as sites for attachment of stress fibers, as well as being platforms on which many signaling molecules assemble. A representative image of a fibroblast adhering to a collagen-coated glass coverslip revealing FAs, prominently stained with an anti-vinculin antibody, is shown in Figure 1.

Over the years, a vast number of additional components have been discovered in FAs and this has given rise to ever more complicated models of FA structure. With hindsight it is amusing to look at the evolution of these models as more proteins and interactions were incorporated into diagrams of FA organization. An early model that I drew from ~1984 is shown in Figure 2A. At that time, integrins had not yet been identified and no transmembrane FA proteins were known. Twelve years later, not only were integrins prominent, but many more proteins and associations had been identified. More elaborate models could be constructed (Figure 2B). Progress in light microscopy has culminated with the application of interferometric photoactivated localization microscopy (iPALM), a form of super-resolution light microscopy, to determine the relative 3D organization of major FA components by Clare Waterman’s group (Figure 3) [19]. Mapping the distribution of 9 different components through the thickness of the FA revealed three distinct layers [19]. Closest to the membrane, where integrin cytoplasmic tails emerge, they have defined an “integrin signaling layer” where FAK and paxillin are located. Above this, there is a “force transduction layer” containing talin and vinculin. Farthest from the membrane, an “actin-regulatory layer” contains zyxin, VASP, α -actinin and actin filaments. More than just assigning the relative locations of these proteins to different strata within FAs, the authors were able to determine the orientation of talin. By selectively labeling either the N- or C-terminus, they revealed that this elongated molecule is oriented with its N-terminal domain close to integrins and its C-terminus extending into the actin-regulatory layer. Notably, this

latter domain contains an actin-binding site, whereas the N-terminal FERM domain binds to integrins. Finding α -actinin in the actin-regulatory layer, separated by a significant distance from the integrin cytoplasmic domains, argues against it contributing to the membrane attachment of stress fibers. This is interesting because some years earlier we had discovered that α -actinin, like talin, can interact with integrins [20]. Although the spatial separation of α -actinin from the plasma membrane in the iPALM images indicates that it cannot be serving an attachment function in these FAs, nevertheless, evidence has been presented elsewhere that α -actinin may play an attachment role in FAs based on $\beta 3$ integrins [21].

Signaling

Whereas the 1980s were a time for discovering structural proteins within FAs and establishing some of the critical interactions, the 1990s revealed that FAs are major sites of signal transduction. Much of the interest in signaling emanating from FAs was driven by the discovery of the focal adhesion kinase (FAK) [22, 23], together with the observation made by many of us that integrin clustering or engagement with ECM triggers tyrosine phosphorylation of multiple FA proteins. FAK and Src family kinases acting in FAs have been implicated in diverse signaling pathways. These pathways in turn influence cell growth and survival, cell migration, as well as the assembly and disassembly of FAs themselves.

Coinciding with the work on FAK and tyrosine phosphorylation in FAs, Ridley and Hall made the key discovery in 1992 that active RhoA drives the assembly of FAs and stress fibers [24]. The initial interpretation was that this was due to stimulating tyrosine phosphorylation. Indeed, tyrosine phosphorylation was seen to be elevated in response to RhoA activation, but whether this drove assembly or was downstream of assembly was not determined at that time. A different model came from the discovery that RhoA activates myosin [25, 26] and that myosin activity contributes to the assembly of FAs and stress fibers [25]. Not only does RhoA promote assembly of FAs, but integrin engagement also regulates RhoA activity in a complex manner. Initial engagement is associated with decreased RhoA activity but over time, as cells spread and develop FAs, GEFs become activated and RhoA activity is stimulated [27, 28].

Mechanotransduction

It has been known for a long time that cells respond to mechanical forces. Many signaling pathways that are stimulated by integrin engagement are also activated by, or even require mechanical force. Mechanical tension exerted on cells affects their growth and survival, but also their differentiation [29]. Research into mechanotransduction has intensified in the past two decades, with much of the interest in this area being directed toward FAs [30]. Many lines have converged on this research area. For example, not only are internally-generated forces exerted on FAs [31], but FA assembly is affected by the physical state and mechanical properties of the external environment. On soft substrata, FA formation is greatly reduced or inhibited all together [32]. It is striking that FAs are seen most clearly in cells cultured on rigid glass or plastic coverslips. Mechanical tension has been implicated in the growth of FAs [33] and recruitment of proteins to these structures. It is relevant that RhoA activity is elevated on rigid substrata [34, 35] and that tension on integrins leads to activation of RhoA

[36], suggesting a positive feedback loop that most likely contributes to the robust assembly of FAs on rigid surfaces. RhoA-stimulated tension can affect the strength of bonds between interacting proteins and it can expose cryptic binding sites within proteins. Investigating how RhoA-mediated contractility can promote fibronectin matrix assembly, we discovered that tension on fibronectin exposes a cryptic site involved in assembly [37]. Similarly, with respect to mechanical tension promoting assembly of components at the cytoplasmic face of FAs, Michael Sheetz' group has shown that stretching talin exposes multiple cryptic vinculin binding sites that are buried within talin [38]. Given the prominence of talin and vinculin in FAs, this tension-induced interaction is likely key to FA assembly and growth. It will be interesting to learn how many other protein interactions within FAs are similarly regulated in this way by mechanical force.

Future directions

The field of FA research appears to be in a robust and exciting phase. With the influx of physicists, mathematicians and biomedical engineers into the field, perhaps there are even parallels with the origins of molecular biology in the 50s and 60s. It has been argued that the revolution in molecular biology came about as a result of the infusion of crystallographers and physicists into biology. Their efforts led to elucidating the structure of DNA and continued with pursuit of the genetic code. With respect to FAs, new techniques such as super-resolution light microscopy and tension-based measurements have introduced a more quantitative perspective. Now the strength of bonds mediating the interactions of key FA components is being considered, along with how mechanical forces affect these associations. When I look at the field I believe I get a glimpse of the future from recent work using super-resolution microscopy [19, 39]. One of the intriguing results from the iPALM analysis of FAs was that, whereas most of the proteins examined were confined to one of three layers, vinculin was detected in all 3 strata. Investigating the basis for this distribution, Case and coworkers demonstrated that vinculin in the membrane proximal layer was recruited to this region in an inactive closed state via binding to phosphorylated paxillin [39]. As FAs matured in response to tension, they were able to show that vinculin was activated and transitioned to higher levels in these FAs, likely through binding to the tension-exposed, cryptic sites in talin, mentioned above [38]. The ability to visualize the relative positions, interactions and activities of various structural and signaling components within FAs as these mature or disassemble will surely lead to a greater understanding of how these structures are regulated and how they, in turn, signal to other activities within cells.

Acknowledgments

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Abbreviations

FA	Focal Adhesion
EMs	electron micrographs

ECM	Extracellular matrix
IRM	Interference reflection microscopy
iPALM	Interferometric photoactivated localization microscopy
FAK	Focal Adhesion Kinase
GEFs	Guanine nucleotide exchange factors

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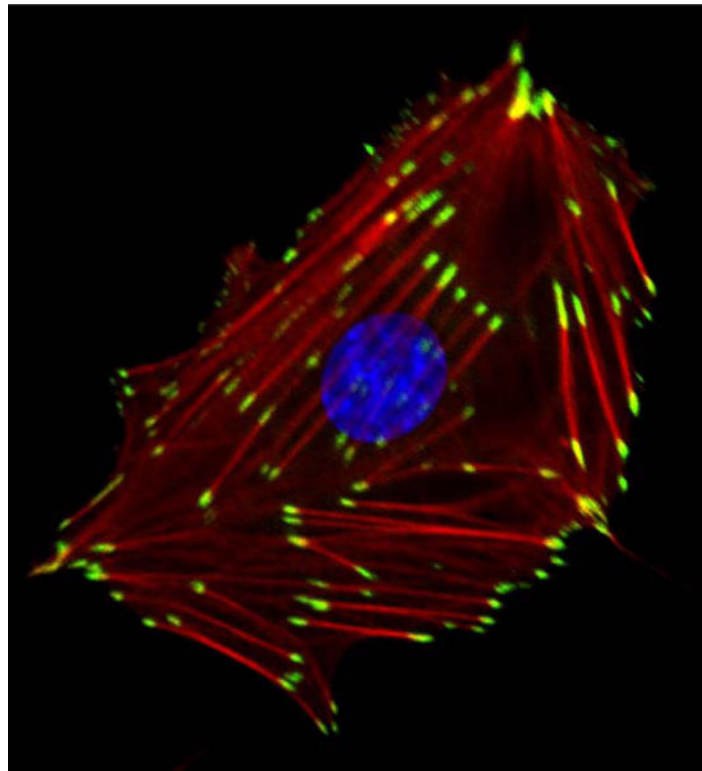


Figure 1.

A REF52 fibroblast plated on a collagen-coated glass coverslip, reveals focal adhesions stained for vinculin (green), F-actin (red), showing stress fibers and the nucleus labeled in blue. Image was kindly provided by David Graham.

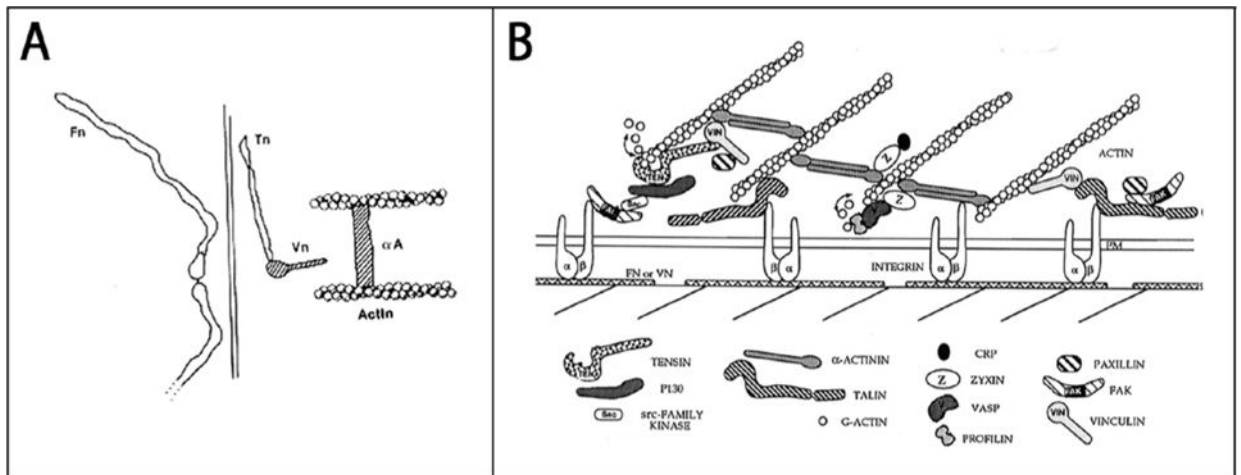


Figure 2.

Early models of focal adhesion structure. In A, a diagram of a FA is shown that I made in ~1984. It is noteworthy both for how few components are represented and the lack of any transmembrane proteins. By 1996, the time of the model shown in B, many more proteins had been identified and a more complex FA architecture is illustrated. This figure is reproduced from Burrige and Chrzanowska-Wodnicka [40].

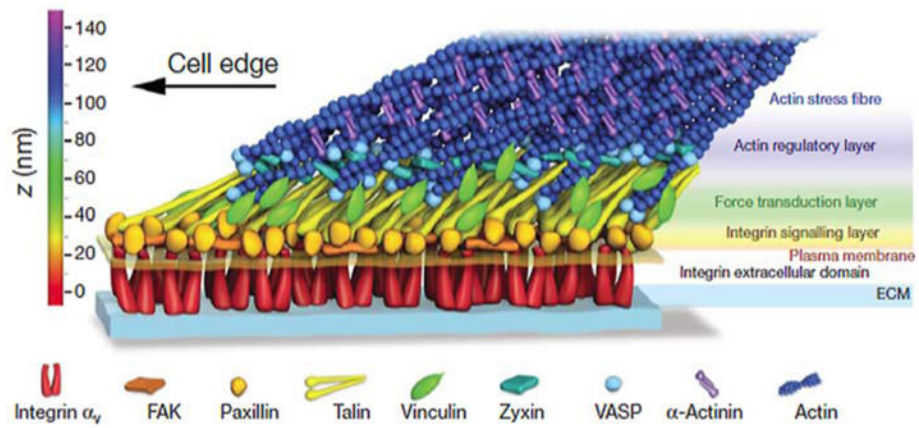


Figure 3.

A schematic model of focal adhesion molecular architecture based on iPALM analysis. Figure reproduced with permission from Kanchanawong et al. [19].