Utilizing Fibronectin Integrin-Binding Specificity to Control Cellular Responses

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Significance: Cells communicate with the extracellular matrix (ECM) protein fibronectin (Fn) through integrin receptors on the cell surface. Controlling integrin–Fn interactions offers a promising approach to directing cell behavior, such as adhesion, migration, and differentiation, as well as coordinated tissue behaviors such as morphogenesis and wound healing.

Recent Advances: Several different groups have developed recombinant fragments of Fn that can control epithelial to mesenchymal transition, sequester growth factors, and promote bone and wound healing. It is thought that these physiological responses are, in part, due to specific integrin engagement. Furthermore, it has been postulated that the integrin-binding domain of Fn is a mechanically sensitive switch that drives binding of one integrin heterodimer over another.

Critical Issues: Although computational simulations have predicted the mechano-switch hypothesis and recent evidence supports the existence of varying strain states of Fn *in vivo*, experimental evidence of the Fn integrin switch is still lacking.

Future Directions: Evidence of the integrin mechano-switch will enable the development of new Fn-based peptides in tissue engineering and wound healing, as well as deepen our understanding of ECM pathologies, such as fibrosis.

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Submitted for publication December 3, 2014.
Accepted in revised form March 12, 2015.

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SCOPE AND SIGNIFICANCE

CELL INTERACTIONS WITH the extracellular matrix (ECM) can drive not only cell behaviors such as adhesion and migration but also contribute to wound healing and embryonic morphogenesis. Fibronectin (Fn), a cell-adhesive ECM protein, has been studied for several decades, yet still offers a rich variety of new information regarding its interaction with cells and other components of the extracellular environment. This review focuses on Fn–integrin interactions and their subsequent effect on cell and tissue behavior. Recent advances

in engineering recombinant Fn-based protein fragments are also discussed.

TRANSLATIONAL SIGNIFICANCE

Application of the useful information that has been found on Fn is relevant to many different fields. Molecular dynamic simulations have suggested that when applied forces are present, Fn's 10th III repeat can partially unfold. The intermediate state that is then represented has a distance between the 9th and 10th type III repeat leading to downstream effects on cell behavior. This

can implicate mechanotransduction effects, which have been seen elsewhere. Engineered recombinant Fn fragments are attractive as examples of other peptide fragments, particularly those similarly sensitive to force mediated translocation.

CLINICAL RELEVANCE

ECM proteins are of particular interest due to their effects on cell behavior. Fn is known to be present in the ECM of injured tissues undergoing regeneration. Bone and wound healing can be promoted by controlling Fn's integrin-binding domain. Fn's integrin-binding domain has been studied, and recombinant Fn fragments that contain this region are of particular interest due to their relevance to cellular responses. Engineered Fn fragments have also been shown to bind growth factors, control epithelial to mesenchymal transition (EMT), and influence stem cell differentiation.^{1–3} This makes Fn fragments a desirable candidate for tissue engineering and wound care.

BACKGROUND

The ECM comprises the noncellular component of tissues, including water, proteins, and polysaccharides. Although the basic components remain the same, the ECM of each tissue has a unique composition and organization that lead to a wide diversity of ECM in the body. In addition, the ECM of a tissue is highly dynamic and undergoes remodeling by cells through both enzymatic and non-enzymatic processes. Furthermore, the structure of the ECM can be modulated by post-translational modifications to several ECM proteins. Fibrous ECM proteins such as collagen and Fn, as well as glycosaminoglycans such as hyaluronic acid (HA), form complex three-dimensional scaffolds that mechanically support cells and form the basis of tissue shape.⁴ This support can exert signaling cues through a process known as mechanotransduction.

For example, varying the rigidity of ECM substrates can influence cell behaviors such as adhesion, migration, and differentiation.^{5–7} However, the role of the ECM is not limited to mechanical support. ECM components also provide numerous biochemical cues to cells through the presentation of specific amino acid-binding sequences. Cells bind to ECM proteins through ECM receptors such as syndecans, discoidin domain receptors, and most importantly for this review, integrins.^{8–10} Integrins binding to ECM proteins initiate signaling responses in cells that can affect cell behavior and direct coordinated physiological responses.¹¹

Fn is an excellent example of an ECM protein that is intricately involved in cell behavior. With binding sites for integrins, other ECM proteins, growth factors, and itself, Fn is a mediator in many cellular interactions with the ECM.¹² As a result, Fn plays a key role in cell behaviors, such as adhesion, migration, and differentiation, as well as more coordinated tissue behaviors such as morphogenesis and wound healing. Fn is capable of binding a number of different integrins, and growing evidence suggests that the selectivity of integrin engagement can drive distinct cell responses. In this review, we discuss in detail the fundamentals of Fn–integrin interactions, the mechanical properties of Fn that may contribute to the specificity of integrin engagement, and the physiological consequences of integrin specificity.

DISCUSSION

Fundamentals of integrin binding to Fn

Fn structure. Fn is a dimeric glycoprotein containing two identical ~250 kDa subunits that are covalently linked through a pair of disulfide bonds near their C-termini. Each subunit is composed of three types of repeating modules: Type I (12 modules), Type II (2 modules), and Type III (15–17 modules) (Fig. 1). Type I and Type II modules are structurally stabilized by two intrachain disulfide

Figure 1. Modular structure of fibronectin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

bridges, which are not present within Type III modules. Type I modules have a hydrophobic core made up of highly conserved aromatic residues and a disulfide bond contained within stacked β -sheets.¹³ Type II repeats are made up of perpendicular antiparallel β -sheets linked together through the previously described disulfide bonds.¹⁴ Type III repeats are ~ 90 amino acids long and lack intrachain disulfide bonds. These repeats are composed of antiparallel β -sheets linked together with flexible loops and only hydrogen bonding stabilizes their structure.^{15,16} As a result, Type III repeats are highly sensitive to force-mediated unfolding.¹⁷

Fn is a known ligand for many integrin receptors, linking the ECM with the intracellular cytoskeleton. Although there are many different integrins that bind to Fn, the classic receptor is the $\alpha 5 \beta 1$ integrin receptor. Specific integrin-recognition sites have been recognized within the Fn molecule.¹² Identification of integrin-specific recognition sites was preceded by the characterization of Fn's interaction with cells and it was determined that the Fn-receptor interaction has only moderate affinity ($K_d = 8 \times 10^{-7}$ M) relative to other ECM protein receptors.^{18,19} An isolated peptide sequence arginine-glycine-aspartic acid-serine (RGDS) has been identified as a key cell-binding site within Fn, and a second, distant synergistic site within the cell-binding domain provides recognition effectively mediating adhesion and cytoskeletal organization.²⁰

Background on integrins. Cells interact with their surrounding ECM, including Fn, through transmembrane cell surface receptors known as integrins. Integrins are heterodimeric proteins consisting of one α -subunit and one β -subunit.¹⁰ The ECM is a protein scaffold that defines part of the extracellular microenvironment and constitutes the noncellular component of tissues. The functions of the ECM are diverse and include both mechanical support as well as biochemical signaling.⁴ Integrin interactions with their ECM ligands facilitate a host of cellular responses, including cell spreading, migration, proliferation, and differentiation, and can contribute to more orchestrated cellular events, such as development and wound healing.^{10,21} A variety of ECM protein ligands can be bound by integrin receptors and these are reviewed elsewhere.²² Integrins serve as the link between structural intracellular protein complexes and the extracellular environment, effectively physically coupling the intracellular and extracellular physical network.

The α - and β -subunits of integrins combine to form at least 24 unique heterodimers. Certain integrins can bind multiple ECM proteins, and some ECM proteins can interact with several different integrin heterodimers.^{23,24} The presentation of specific adhesive ligands direct which integrins bind and correspondingly alter intracellular signaling events.²⁴ The ligand-binding sites within integrins, particularly for the ligand Fn, can be classified into two different groups of ligands: arginine-glycine-aspartic acid (RGD)-binding and leucine-aspartic acid-valine (LDV)-binding type integrins. The active site pocket located between the α - and β -subunits binds ligand peptides and provides ligand specificity.¹⁰ Integrin binding to Fn's cell-binding domain offers an excellent example of the complexity of controlling integrin specificity and is a continuing area of investigation both from the perspective of how biochemical specificity is determined as well as the cell biological consequences of that specificity.

RGD and synergy sites in integrin-binding interactions. The major cell-binding site for many integrins is the three amino acid sequence RGD on the 10th type III repeat of Fn. This tripeptide, originally identified in Fn by Erkki Ruoslahti, is an integrin-binding site present in many adhesive ECM, blood, and cell surface proteins.²⁵ Importantly, RGD is the primary cell attachment site for Fn binding by integrins.²⁶ Integrins that engage Fn through RGD include $\alpha IIb \beta 3$, $\alpha V \beta 3$, $\alpha V \beta 6$, $\alpha V \beta 1$, $\alpha 5 \beta 1$, and $\alpha 8 \beta 1$.¹⁰ Mimicking adhesion proteins with RGD peptides has been a useful way to enable cell adhesion; however, RGD peptides alone are not selective for specific integrins as they mimic sites in a number of adhesion proteins and are capable of binding to numerous different integrins.²⁶

Recognition of RGD by integrins is highly sensitive to changes in Fn conformation and the presentation of neighboring amino acid sequences. For example, nearby amino acid residues can inhibit or suppress the activity of RGD due to conformational restrictions that shield integrin binding.²⁷ Integrin binding can also be modulated by the activity of peptide sequences on neighboring domains. A classic example is $\alpha 5 \beta 1$ integrin binding to Fn. This interaction requires RGD in combination with a second recognition sequence (PHSRN) on the adjacent ninth type III repeat. This second sequence, commonly known as the synergy site, promotes the specific binding of $\alpha 5 \beta 1$ integrin to Fn through interactions with the $\alpha 5$ subunit.^{28,29} The synergy site is separated from the RGD loop on the 10th

Figure 2. Proposed model of FnIII domain unfolding by Erickson in 1994.³⁴ As tension is applied to the relaxed molecule (**A**), the domains first align (**B**), followed by unfolding of certain domains (**C**). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

type III repeat by about 32 Å. Due to the elasticity of type III repeats, whose β -sheet structures are stabilized only by hydrogen bonds, the 9th and 10th type III repeats can present in a number of different conformations. Steered molecular dynamics (SMD) simulations suggest that tension on the order of 10 pN is sufficient to partially unfold the 10th type III repeat. As FnIII10 unfolds, the RGD loop is subsequently pulled away from the synergy site on FnIII9. The distance between RGD and synergy thus increases from 32 Å to ~55 Å.¹⁷ The spatial conformation of RGD and synergy has critical implications for integrin binding and, as a result, cell behavior. The affinity of integrin $\alpha 5 \beta 1$ for Fn is dependent on simultaneous interaction with RGD and synergy sites. Although a 23 Å increase in separation between RGD and synergy may seem small, it greatly diminishes the ability of $\alpha 5 \beta 1$ to bind Fn.³⁰

These results suggest that the conformation of these two domains may be used to drive which integrins cells use to bind Fn. For example, by stabilizing the 9th type III repeat by a Leu-Pro mutation at amino acid 1408, there is an increased affinity for $\alpha 5 \beta 1$ over integrin $\alpha v \beta 3$. Alternatively, by increasing the linker region between the RGD and synergy sites, there is a reduction in $\alpha 5 \beta 1$ binding.³ Furthermore, $\alpha 3 \beta 1$ integrin binding to Fn may also be promoted by the 9th type III repeat (unpublished data). Integrins that do not bind the synergy site, such as $\alpha v \beta 3$, are not affected by this separation in adhesive domains.³¹ This is of particular interest because it suggests that the ratio of $\alpha 5 \beta 1$ binding to $\alpha v \beta 3$ binding can be modulated by changing the conformation of these sites of Fn. This hypothesis forms the basis of Fn conformation-driven integrin specificity and has significant implications in cell behavior, discussed later in this review.

Molecular dynamics of the integrin-binding domain

Erickson and Carrell first noted Fn as a structurally dynamic protein in 1983. These researchers used electron microscopy and sedimentation analysis to observe the effect of ionic strength on Fn conformation. At low ionic strength (0.05 M KCl), Fn was observed as a more compact irregularly coiled strand. At increasing ionic strength (0.2 M KCl), Fn was found to take on a more extended conformation with fewer intrachain bends.³² These observations suggested that electrostatic interactions influence Fn molecule conformation and introduced the possibility that other forces, such as mechanical tension on Fn fibers, may alter Fn structure as well. This discussion focuses primarily on the force-induced changes in the integrin-binding domain of Fn, composed of FnIII repeats.

Mechanics of the FnIII domain. About a decade later, the elasticity of another large protein, titin, was explored after observing that titin molecules extend up to four times their length at their I-band region. Titin is composed of many immunoglobulin- and FnIII-like domains, and the extraordinary elasticity of titin is, in part, due to the unfolding of these FnIII-like domains.³³ FnIII domains are composed of seven β -strands arranged into two sheets and are connected in series in an Fn molecule. Inspired by these findings, a structural model for the unfolding of FnIII domains was proposed in 1994 (Fig. 2), but this was not directly validated on Fn fibers.³⁴ The comparisons to titin continued when researchers studied the intramolecular dynamics of FnIII10 (where the integrin-binding RGD motif is located) and an analogous FnIII-like domain on titin. Using ¹⁵N nuclear spin relaxation, Carr *et al.* found that FnIII10 is more flexible than

the corresponding titin domain, especially on the loop containing the RGD sequence. At the time, these results were discussed in the context of an induced fit model for Fn integrin-binding reactions and were used as support for the idea that the RGD motif on FnIII10 was not as specific to distinct integrins as the analogous RGD motif on titin was.³⁵ If this idea was correct, we would not expect to see specific integrins engaged with Fn in different structural contexts. However, previous work has shown that mutations affecting the mechanical stability and structure of FnIII9–10 can cause distinct integrins to bind.³⁶ An alternative explanation to these results might be that the flexibility of FnIII10 allows for greater mechanical sensitivity, through which Fn can tailor tension-modulated integrin specificity.

Knowledge on the molecular dynamics of FnIII domains was accelerated by the use of atomic force microscopy (AFM). AFM allows for single-molecule measurements of force versus extension, providing myriad insights into the mechanical properties of fibrous proteins. The first protein with FnIII that repeats to be investigated by AFM was tenascin. Tenascin is an ECM protein important in embryonic development and tissue repair. Notably, tenascin contains multiple FnIII repeats along its chain. Single-molecule AFM was used to study the modular elasticity of tenascin and suggested that unfolding of FnIII domains was responsible for the sawtooth pattern of force versus extension curves observed in tenascin hexabrachions.³⁷ While this work focused on tenascin elasticity, the importance of FnIII domain unfolding has applications to other ECM molecules, especially Fn.

The first study to specifically investigate the molecular dynamics of FnIII10 utilized SMD simulations to describe the sequence of events of FnIII10 unfolding and the resulting effects on RGD loop accessibility to integrin binding. This study predicted that the β -strand attached to the RGD loop would be the first to break away from the module under tension and would, therefore, pull the RGD loop, which is normally located between two β -strands, closer to the surface of the β -sheet structure that comprises the bulk of the module (Fig. 3). This conformational change was suggested to decrease the affinity of the loop for integrin receptors on the cell surface. The authors discussed that this property of Fn may be a mechanism of mechanosensitive control of ligand recognition.³⁸ Further work with SMD showed that when compared to other FnIII domains such as FnIII7, FnIII8, and FnIII9, FnIII10 has the lowest force threshold for the early stages of unfolding.

Figure 3. SMD simulation of early stages of FnIII10 unfolding under force. RGD loop is shown in red between the F and G strands. As the G strand of FnIII10 (shown in **(A)**) is pulled **(B)**, the RGD loop is brought closer to the bulk of the module **(C)**. SMD, steered molecular dynamics. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

This conclusion is important when discussing the dynamics of the integrin-binding domain since it predicts that FnIII10 will be one of the first domains to unfold under tension.³⁹ This simulation was later confirmed by AFM measurements of FnIII domain unfolding.⁴⁰ The incorporation of the neighboring synergy site in SMD simulations of FnIII10 provided a structural model for the increase in distance between the synergy site (PHSRN, on FnIII9) and RGD and the effect on integrin binding.¹⁷ These simulations gave structural confirmation to earlier studies, which noted that an increase in the linker region between synergy and RGD led to a decrease in $\alpha 5 \beta 1$ binding in favor of $\alpha v \beta 3$ binding.⁴¹ These findings form the basis of the theories on the conformational sensitivity of integrin specificity to Fn.

Simulations on the unfolding of FnIII domains were supported by *in vitro* experiments involving fluorescence resonance energy transfer (FRET). This technique involves labeling Fn with two fluorophores: one donor and one acceptor. Excita-

tion energy from the donor fluorophore is transferred to the acceptor fluorophore, resulting in excitation of the acceptor. Energy transfer decays rapidly with increasing distance between the fluorophores, resulting in a signal that varies in strength directly with increasing distance. In Fn, the fluorophores labeled are FnIII7 and FnIII15, which encompass the integrin-binding domain (FnIII9–10). Thus, the FRET signal can be used as an indicator of integrin-binding domain extension and unfolding. This technique was first utilized to suggest unfolding of Fn in response to varying concentrations of a denaturant and was then applied to show this effect in response to cytoskeletal tension.^{42,43} Together with the SMD simulations discussed earlier, these results strongly suggest that cell-mediated forces can partially unfold the integrin-binding domain of Fn.

Until recently, the existence of varying strain states in Fn fibers had not been observed in native tissue. However, using phage display against fragments of Fn that mimic different spatial orientations of the Fn integrin-binding domain, peptide-based molecular probes were developed that could distinguish between low-strain and high-strain regions of Fn fibers. These probes were used to stain *ex vivo* mouse lung slices and discriminate between varying strain states in the tissue.⁴⁴ These results give strong evidence to the hypothesis that distinct force-induced structural states of Fn exist *in vivo*. It is tempting to think of the integrin-binding domain of Fn as a mechanically sensitive switch for specific integrin engagement, and the SMD simulations discussed earlier support this idea. Moving forward, the challenge will be experimental detection of the conformational changes to the integrin-binding domain that confirm the validity of the switch model and its relevance to mammalian physiology.

Exposure of cryptic binding sites. Concurrent with the advances in knowledge of FnIII unfolding, researchers had begun to investigate the process of Fn matrix assembly, which involves the polymerization of multiple Fn molecules together into fibers. This process requires the accessibility of Fn–Fn binding sites on the Fn molecule. Treatment of Fn with Fn fragments containing a cleaved form of the first type III repeat (FnIII-1C) was found to stimulate spontaneous disulfide crosslinking of Fn molecules into multimers resembling matrix fibrils. It was hypothesized that III-1C represented a binding site within native Fn that became revealed during matrix assembly.⁴⁵ Shortly after, another group demonstrated that heat-denaturing III-1

allowed for it to bind the amino-terminal portion of Fn, as in matrix assembly. The authors noted that since Fn matrix assembly is a cell-dependent process, the exposure of the Fn-binding site in III-1 could result from tension generated by the cytoskeleton through integrin–Fn connections.⁴⁶ These sites, exposed through cleavage of FnIII domains, heat denaturation, or tension, became known as cryptic binding sites.

In the following years, other cryptic self-association sites were discovered in Fn.⁴⁷ Further research into this area confirmed that Rho-mediated cell contractility exposes cryptic binding sites in Fn and can induce Fn matrix assembly. Interestingly, it was also discovered that even III-10 contains a cryptic binding site for III-1 and could potentially serve as an assembly site for stretched Fn fibrils in focal adhesions.^{48,49} About a decade later, it was shown that tension-mediated exposure of cryptic binding sites on Fn leads to an increase in fiber rigidity due to increased Fn–Fn binding. This strain-hardening property allows Fn fibers to limit how much they can be extended by cell forces and accordingly alter local ECM environments by increasing fiber rigidity, which can be sensed by cells.⁵⁰

Engineering approaches to control Fn–integrin interactions

FnIII9 and FnIII10 affect integrin specificity. It is believed that integrin specificity is related to the binding domains within Fn and their spatial relationships, which are controlled by the flexibility between the two sites of integrin binding within the FnIII9 and III10 domains. It has been directly observed that integrin specificity between the RGD and the synergy site, PHSRN, within the cell-binding domain of Fn can direct cells down a migratory/repair phenotype necessary for wound healing processes.³¹ These critical sites regulate the epithelial cell phenotype by their ability to direct integrin specificity.

Some ECM proteins contain the ability to specifically engage certain integrins, while other sequences within ECM proteins are known to be promiscuous for multiple integrins. It has been indicated that RGD, for example, engages α_v integrins; however, due to the ability of several ECM proteins, particularly Fn, to be responsive to cellular and environmental changes such as cell adhesion and spreading forces, the distance between these two synergistic sites can have downstream effects on cellular expression. For example, peptides incorporating FnIII10 only (containing just the RGD site) lead lung epithelial cells down a path of EMT; whereas peptides, including the synergy

site, such as FnIII9–10, maintain the epithelial phenotype of the same cells. Interestingly, substrate mechanics plays an important role in this interaction. Stiff substrates (~ 32 kPa) spontaneously induce EMT in lung epithelial cells, but this response can be overcome by engagement with FnIII9–10. Taken together with the studies of RGD and synergy as they relate to specific integrin engagement (discussed earlier in the RGD and Synergy Sites in Integrin-Binding Interactions section), these results suggest interplay between substrate rigidity sensing, stiffness-mediated cell phenotypes, and integrin specificity.⁵¹

The 12th through 14th type III repeats of Fn serve an important role in the delivery of growth factors to cells. FnIII12–14 has been shown to bind most growth factors from the platelet-derived growth factor, vascular endothelial growth factor (VEGF), and fibroblast growth factor families in the nanomolar range, without inhibiting the growth factor activity.⁵² Building on this finding, the Hubbell group designed Fn fragments incorporating both the integrin-binding domain (FnIII9–10) and the growth factor-binding domain (FnIII12–14), along with a Factor XIIIa substrate fibrin-binding sequence as a method of enhancing the effects of various growth factors in wound and bone healing strategies.¹ Coupling the cell-binding and growth factor-binding domains of Fn shows promise in tissue engineering and wound healing approaches.

Engineering Fn fragments. Particular Fn fragments have thus far been designed to initiate desired responses from cells such as cell adhesion, motility, spreading, proliferation, and differentiation. In particular, it has been noted that these ECM protein changes can engage different integrin switches, which are guided by integrin specificity. These fragments have been designed with different length and flexibility to induce the desired cellular outcomes. It could be proposed that by engaging even more drastically different conformations, there could be unique cellular responses that may not be as predictable.

The work of Mardon and Grant has focused on further understanding integrin specificity within the cell-binding domain of Fn.²⁸ This work has outlined the initial cell attachment and downstream cell adhesion events as significantly affected by the addition of short flexible linkers between the 9th and 10th type III domains. These short linkers are primarily designed with a series of glycine residues to provide a tensile character and distance between the two synergistic sites.⁴¹ This work inspired further exploration of recom-

binant FnIII9 and 10 domains explored by Martino *et al.* Engineered integrin-specific Fn fragments were designed from Fn's 9th and 10th type III repeats since this is a known cell-binding domain. A stabilized fragment Fn 9*10 is designed with a Leu to Pro mutation, which provides a greater $\alpha 5\beta 1$ -integrin specificity based on the conformational stability of the RGD and synergy sites.^{3,17} This recombinant Fn fragment, Fn 9*10, has a greater degree of $\alpha 5$ clustering than the wild-type Fn 9 and 10 fragment, which due to conformational change is believed to contribute to specificity alternatively to the wild-type fragment, which has a poor specificity for the integrin-binding type.

Physiological relevance of integrin specificity

As discussed in the RGD and Synergy Sites in Integrin-Binding Interactions section of this review, Fn can bind multiple integrins, dependent on the conformation of two adjacent FnIII domains. Integrin $\alpha 5\beta 1$ has a high affinity for RGD (located on FnIII10) when the synergy site (PHSRN, located on FnIII9) is conformationally close. As distance between RGD and synergy increases, the affinity for $\alpha 5\beta 1$ is decreased, while the affinity for synergy-independent integrins, such as $\alpha v\beta 3$, remains unchanged. As a result, Fn conformational change can drive integrin specificity. To illustrate the importance of this mechanism, it is useful to consider the consequences of integrin specificity within the context of more organized cell behaviors and even diseases.

Cell adhesion, spreading, migration, and growth. Evidence for integrin-specific cell behaviors was first noted in the ability of $\alpha 5\beta 1$ integrins to promote migration to a greater degree than $\alpha 4\beta 1$.^{20,53} This concept was bolstered by research performed by the Ruoslahti group in 1993. Starting with a cell type that lacks the $\alpha 5$ subunit, Zhang *et al.* expressed αv subunits in these cells to obtain an $\alpha v\beta 1$ integrin. Cells with the $\alpha v\beta 1$ integrin adhered to an Fn substrate, but did not migrate or assemble an Fn matrix. Transfecting the cells to produce the $\alpha 5$ subunit rescued these two behaviors.⁵⁴ These results were particularly striking because the β -subunit remained the same in both heterodimers ($\beta 1$), suggesting that changing one subunit of the integrin is sufficient to affect cell behavior. Two years later, the same group took the experiment one step further and showed that cells lacking $\alpha 5$ undergo apoptosis under conditions of serum starvation, whereas reintroducing $\alpha 5$ protected against apoptosis under these same conditions.⁵⁵ Taken together, these studies show the dramatic effect of integrin specificity on basic cell behavior.

About a decade later, researchers explored the effects of varying biomaterial surface chemistries on integrin specificity. While $\alpha 5 \beta 1$ integrins preferentially bound to surfaces rich in hydroxyl groups (-OH), $\alpha v \beta 3$ integrins preferred surfaces with carboxyl groups (-COOH). Interestingly, this variation in surface chemistry not only influenced which integrins cells use to adhere but also modulated the composition of the resulting focal adhesion complex.⁵⁶ These results were strong evidence that integrin specificity could drive distinct cellular behaviors by altering focal adhesion composition. Integrin specificity has recently been shown to influence the assembly of ECM proteins such as collagen and Fn.⁵⁷

Integrin specificity is highly sensitive to the conformation of RGD and PHSRN. Having identified these sequences as important for $\alpha 5 \beta 1$ ligation, it is tempting to reduce bioadhesive material design strategies to incorporate solely these two binding epitopes. However, research has shown that the structural context of these sites in Fn is beneficial for cell adhesion, focal adhesion assembly, and cell proliferation. All of these behaviors were enhanced in cells exposed to FnIII7–10, a fragment of Fn containing both RGD and PHSRN in their native form, versus cells exposed to an RGD-PHSRN oligopeptide with a polyglycine linker between the two sites. Importantly, FnIII7–10 also promoted specificity for integrin $\alpha 5 \beta 1$ over $\alpha v \beta 3$ to a much greater degree than the RGD-PHSRN oligopeptide.⁵⁸ Other work in this area has suggested that RGD-containing peptides such as GRGDSP can direct integrin specificity depending on the presentation of the peptide. Linear GRGDSP was bound primarily by $\alpha 5 \beta 1$ integrins, whereas binding to GRGDSP in a loop conformation was mediated by $\alpha v \beta 3$.⁵⁹

Cancer. The tumor microenvironment is complex, with many of the properties of the ECM distinct from those in surrounding healthy tissue. One of the major concerns in understanding cancer biology is metastasis, a process that involves cell migration and, by extension, integrin engagement. Anti- $\alpha 5 \beta 1$ antibodies were found to inhibit experimental metastasis in human breast carcinoma cells, suggesting that $\alpha 5 \beta 1$ -mediated migration is a critical process in metastatic disease.⁶⁰

Recent work has determined that Fn conformation in breast tumors influences the proangiogenic capability of tumor stromal cells by modulating integrin specificity. Angiogenesis, or the formation of new blood vessels, is crucial for tumor progression. Fn is unfolded to a greater degree in tumor tissue than surrounding tissue. Unfolded Fn favors $\alpha v \beta 3$ engagement over $\alpha 5 \beta 1$ which, in turn, en-

hances the secretion of VEGF, a proangiogenic growth factor, in tumor stromal cells.⁶¹

Stem cell differentiation and renewal. The binding of $\alpha 5 \beta 1$ to Fn was shown to be critical for the proliferation and differentiation of progenitor cells into both osteoblasts and myoblasts.^{62,63} Using the biomaterial surface chemistry modification technique described in the Cell Adhesion, Spreading, Migration, and Growth section, it was found that integrin specificity for the $\beta 1$ subunit over the $\beta 3$ subunit was critical for osteoblastic differentiation of progenitor cells.⁶⁴ Further studies on mesenchymal stem cells (MSCs) revealed similar results regarding the importance of the $\alpha 5$ subunit in promoting osteoblast differentiation.⁶⁵

Combining these results with the knowledge of FnIII unfolding events, researchers have engineered approaches to tailor MSC differentiation using FnIII9–10 variants with different mechanical stabilities. Variants that specifically promoted $\alpha 5 \beta 1$ binding over $\alpha v \beta 3$ resulted in enhanced osteogenic differentiation of MSCs.³

Integrin specificity has also been explored as a method of promoting stem cell self-renewal. By designing a defined, synthetic three-dimensional matrix, researchers stimulated specific combinations of integrins to promote the renewal of embryonic stem cells.⁶⁶

Wound healing. The process of cutaneous wound healing involves a close coordination between cells and the surrounding matrix. Fn is abundantly present in the ECM of regenerating injured tissues. After the initial fibrin clot forms a provisional matrix, inflammatory cells and fibroblasts migrate to the wound area. Fibroblasts proliferate and assemble ECM proteins before differentiating into myofibroblasts to contract and organize the new ECM. This example of matrix assembly and remodeling has the potential to expose cryptic binding sites, as discussed in the Exposure of Cryptic Binding Sites section. The importance of $\beta 1$ integrins in wound healing was first noted in keratinocyte migration during cutaneous wound repair in mice.⁶⁷ Later, the $\beta 1$ integrin was found to be critical for wound closure and ECM deposition in cutaneous wounds.⁶⁸ Recently, the Hocking group designed Fn matrix mimetic proteins that preferentially engage either $\alpha 5 \beta 1$ or $\alpha v \beta 3$ integrins, or an equal contribution of both.⁵⁷ Initial testing of these proteins in a diabetic mouse wound model has shown the capability of Fn matrix mimetics to promote wound healing, although the impact of integrin specificity is uncertain.⁶⁹

Tissue engineering. Integrin-specific peptide sequences are an obvious candidate for tissue engineering approaches due to their ability to modulate cell behavior. One example of these approaches is the use of FnIII7–10, an $\alpha 5\beta 1$ -specific fragment, as a coating on titanium bone implants to improve osseointegration.⁷⁰ Augmentation of bone regeneration has also been achieved by delivering bone morphogenetic protein-2 (BMP-2) in a HA hydrogel modified with FnIII9*–10, a fragment known to bind $\alpha 5\beta 1$.⁷¹

Controlling cell behavior in engineered tissues is critical for successful tissue implantation and function. EMT is an example of a cellular process that could be beneficial to regenerative medicine strategies or lead to pathological progression if left uncontrolled.⁷² Although mechanical cues such as tissue stiffness are known to drive EMT, recent research has shown the capability of integrin specificity to overcome mechanical signals. Utilizing integrin-specific Fn fragments on polyacrylamide gels of varying stiffness, it was shown that while stiff substrates induce spontaneous EMT, integrin $\alpha 3$ or $\alpha 5$ engagement could mitigate this effect. By contrast, fragments that engage αv integrins promote EMT in a Rho/ROCK signaling-dependent manner.

SUMMARY

The ECM provides both mechanical support and biochemical cues that influence cell behavior. Fn is an example of an ECM protein that contains binding sites for other ECM proteins, growth factors, and cells. Interactions between cells and Fn are mediated by transmembrane integrin heterodimers. Integrin engagement can affect cell behaviors, such as adhesion, migration, and differentiation, as well as direct more coordinated behaviors like wound healing. Over the past decade, several groups have developed recombinant Fn fragments that engage specific integrins leading to distinct cellular responses. The mechanics of the Fn molecule is thought to be an important regulator of integrin specificity. Moving forward, the field will benefit from experimental evidence of this mechanosensitive integrin switch hypothesis as well as further engineering advances in Fn-based tissue engineering strategies.

ACKNOWLEDGMENT AND FUNDING SOURCE

This work was supported by the National Institutes of Health (R01EB11566 and R01HL127283).

TAKE HOME MESSAGES

- Fn is a cell adhesive ECM protein, which interacts with transmembrane integrin receptors commonly through the RGD sequence on Fn's 10th type III repeat.
- The synergy site on Fn's 9th type III repeat is involved in specific integrin-binding interactions, such as $\alpha 5\beta 1$, but is irrelevant for $\alpha v\beta 3$.
- Fn type III repeats comprised stacked β -sheets, which are stabilized only by hydrogen bonding and are thought to be susceptible to force-mediated unfolding.
- Recombinant Fn fragments have been developed that engage specific integrins by mimicking strain states of the integrin-binding domain.
- Integrin specificity as a result of Fn engagement has consequences on cell behaviors such as adhesion, migration, and differentiation.
- The integrin-binding domain is hypothesized to act as a mechanosensitive integrin switch, but this is yet to be experimentally validated.

AUTHOR DISCLOSURE AND GHOSTWRITING

No competing financial interests exist. The contents of this article were expressly written by the authors listed. No ghostwriters were used to write this article.

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Abbreviations and Acronyms

AFM	= atomic force microscopy
BMP-2	= bone morphogenetic protein-2
ECM	= extracellular matrix
EMT	= epithelial to mesenchymal transition
Fn	= fibronectin
FnIII	= fibronectin type three repeat
FnIII9	= ninth type three repeat of fibronectin
FRET	= fluorescence resonance energy transfer
HA	= hyaluronic acid
MSC	= mesenchymal stem cell
SMD	= steered molecular dynamics
VEGF	= vascular endothelial growth factor