Published in final edited form as:
Regen Med. 2009 July ; 4(4): 593–600. doi:10.2217/rme.09.30.

Emerging concepts in engineering extracellular matrix variants for directing cell phenotype

Ashley E Carson and **Thomas H Barker**†

Abstract

Directing specific, complex cell behaviors, such as differentiation, in response to biomaterials for regenerative medicine applications is, at present, a mostly unrealized goal. To date, current technological advances have been inspired by the reductionist point of view, focused on developing simple and merely adequate environments that facilitate simple cellular adhesion. However, even if extracellular matrix (ECM)-derived peptides, such as Arg–Gly–Asp (RGD), have largely demonstrated their utility in supporting cell adhesion, their lack of biological specificity is simply not optimal for controlling more integrated processes, such as cell differentiation. These more complex cellular processes require specific integrin-signaling scaffolds and presumably synergistic integrin and growth factor-receptor signaling. This article will introduce some current efforts to engineer ECM variants that incorporate additional levels of complexity for directing greater integrin specificity and synergistic ECM growth factor signaling toward directing cell phenotype.

Keywords

cell phenotype; differentiation; extracellular matrix; integrins; protein engineering; regenerative medicine

The ability to direct specific cell behavior in biomaterials, in particular differentiation, would be of particular use for regenerative medicine applications, but until now this has been a mostly unfulfilled goal in the fields of biomaterials and regenerative medicine [1,2]. Current technological advances have been inspired by the reductionist point of view and have focused on developing simple and merely adequate environments that facilitate simple cell processes, such as cellular adhesion. For example, the integrin-binding trimeric peptide motif, Arg–Gly–Asp (RGD), derived from the extracellular matrix (ECM) protein fibronectin (FN) has been abundantly tested and used as an 'ECM mimetic' in the context of synthetic biomaterials [3]. However, even if ECM-derived peptides have largely demonstrated their utility, such as supporting cell adhesion, the lack of biological specificity of many of these peptide motifs is simply not optimal for controlling more integrated processes, such as differentiation [4]. These complex cellular processes require more specific integrin-signaling scaffolds, such as those naturally presented in the native ECM. However, the modification of materials with full-length ECM proteins, whilst displaying the molecule in its native form, presents significant complications in working with macromolecules that present complex biology. For example, the ECM protein FN is capable of binding at least 20 distinct integrins [5]. In addition, FN, as well as many other structural ECM proteins, are also capable of binding a significant number of polypeptide growth factors (GFs) [6-8]. For example, active TGF-β has been shown to

†Author for correspondence: The Wallace H Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, 313 Ferst Drive, Atlanta, GA 30332−0535, USA Tel.: +1 404 385 5039; Fax: +1 404 894 4243; thomas.barker@bme.gatech.edu.

interact with decorin, FN, thrombospondin and collagen type IV, while BMP-2 and -7 have both been shown to bind collagen type IV. TGF-α has been observed in the context of FN, and PDGF has a well-documented interaction with the matricellular protein SPARC [8]. Although this is not an exhaustive list, it highlights the prevalence of these interactions. This inherent capacity of the ECM to bind multiple and diverse integrins and GFs represents a significant challenge to instructing specific cell behaviors with the native proteins. For this reason, a number of investigators have begun developing engineered ECM variants that display only a fraction of the native ECM protein complexity, yet still attempt to provide sufficient instruction for directing cell phenotype. The following article addresses a few emerging topics in the biology of the ECM that may hold promise in engineering new ECM variants that direct cell phenotype, and describes some early approaches taken in the field to begin controlling integrin–ECM interactions and, thus, cell behavior.

Complexity of cell–ECM interactions

Complexity in ECM–integrin interactions can be regulated by at least two different mechanisms: proteolytic processing and mechanochemical translation of integrin-binding domains. FN, collagen and laminin (LN) processing by proteases, such as matrix metalloproteinases (MMPs), and/or conformational changes due to cell-induced mechanical strain, liberate fragments and unmask 'cryptic' binding sites and/or disturb conformation-specific receptor binding, resulting in biological activities that are not observed in the intact, unmodified molecules. One thought is that cryptic sites are possibly a means for positioning instructional cues used by cells during tissue organization, repair or remodeling processes [9]. Such an evolutionarily developed system offers the advantage that ECM cues can be kept masked until their presence is required, precluding the need for active, energy-dependent inhibition of an activity that is not required during most time points [10].

Proteolytic processing of ECM

Proteolysis of ECM proteins can significantly affect integrin specificity, as well as release sequestered GFs, cytokines and a new class of bioactive ECM-derived fragments, termed 'matrikines' [11]. Cryptic sites and cryptic matrikines have been implicated in many events governed by cell–ECM interactions, such as migration, invasion, adhesion and differentiation. Moreover, the unmasking of cryptic sites and cryptic matrikines is a tightly controlled process, reflecting the importance of cryptic ECM functions [9,11-13]. Cryptic domains are probably a means for positioning instructional cues to be used by cells in a time-sensitive manner, such as during tissue organization, repair or remodeling processes. Evolutionarily, employing a relatively small repertoire of structural ECM molecules with numerous and, perhaps, time-sensitive functional roles imparts a high degree of efficiency in controlling diverse extracellular and cellular processes [9]. Prototypical matrikines include EGF-like repeats of tenascin-C and LN, the leucine-rich region of decorin and the triple helical structure of collagen [13]. For example, proteolysis within the coiled-coil structure of the γ_2-chain of LN liberates a putative cryptic matrikine, containing EGF-like repeats that are capable of activating the EGF receptor, leading to cell phenotypic changes via EGF-receptor signaling of Src-family kinases. Specifically, human γ_2-chain can be cleaved by membrane type 1-MMP, releasing DIII fragments (25 and 27 kDa). The 27-kDa fragment contains two EGF-like domains that engage the EGF receptor and promote cell migration of various epithelial cells [9,14,15].

In addition to the release of matrikines from the backbone of the structural ECM, proteolysis has been shown to regulate integrin-specific interactions and concomitant cell phenotype. A classic example of this concept is the proteolysis-sensitive, integrin-selective nature of LNs [16]. LN-5 (LN-332), comprised of the α_3-, β_3- and γ_2-LN chains, principally recognizes three integrins in the epidermis ($\alpha_3\beta_1$ [16], $\alpha_6\beta_1$ and $\alpha_6\beta_4$ [17]) through its laminin globular (LG) domains, situated at the C-terminus of the α_3-chain. *In vivo*, LN-332 influences stable

epidermal layers via interaction of the LN-α_3-chain with integrin-$\alpha_6\beta_4$ and promotion of hemidesmosome structures at the basal cell surface and, yet, the LG3 module of LN-α_3 binds integrin-$\alpha_3\beta_1$ through the PPFLMLLKGSTR motif and promotes cell migration; an apparently paradoxical function for LN-α_3 [18]. This elegant regulation of epithelial cell motility has been observed in wounded skin. Intact, full-length LN-332 is secreted at the wound edge where it engages integrin-$\alpha_3\beta_1$ via the LG3 domain, inducing cell motility to initiate wound closure. LN-α_3-chain processing between the LG3 and LG4 domains by serine proteolysis [19] at Gln^{1337}–Asp^{1338} results in the exposure of an $\alpha_6\beta_4$-preferred ligand, thus inducing hemidesmosome formation and stable epithelial cell–cell adhesion, downregulating cell motility (Figure 1) [20-23].

Interestingly, in a recent report it was shown that the unprocessed LN-α_3-chain does indeed prevent the formation of mature hemidesmosomes but not the nucleation of hemidesmosomal plaques [24]. Furthermore, it showed that keratinocytes migrate equally well on both processed and unprocessed LN-332, whereas mature hemidesmosomes can only be formed after the α_3-chain of LN-332 is processed. Nevertheless, mainly unprocessed LN-332 is present underneath the leading edge of keratinocytes within a skin wound. It is possible that in the case of excessive production of LN-332 following wounding, the efficiency of processing will be reduced and the formation of mature hemidesmosomes inhibited, favoring a migratory phenotype [17]. In addition, other LNs have been shown to have differing roles in wound repair and regeneration, for example, LN-10 is critical for hair follicle development [25].

Mechanochemical translation of cell-binding ECM domains

In addition to proteolysis of the ECM, mechanical forces have been shown to expose cryptic domains and alter potentially conformationally sensitive binding regions within the ECM, including integrin-binding domains. Although there have been implications of this behavior in numerous ECM proteins, the classic example is mechanical alteration of FN type III (FNIII) repeats. Biochemically, FN is a soluble dimeric glycoprotein composed of two nearly identical 230−270-kDa monomers linked covalently near their C-termini by a pair of disulfide bonds [5,26]. Each monomeric subunit consists of three types of repeating modules: types I, II and III. These modules comprise functional domains that mediate interactions with other ECM components, cell surface receptors and FN itself. Whereas type I and II repeats are structurally stabilized with two intrachain disulfide bonds in each repeat, type III repeats contain no disulfide bonds and, therefore, are highly sensitive to force-mediated unfolding.

Fibronectin is extremely sensitive to cell-derived mechanical forces, a fact that is exemplified by the requirement of mechanical exposure of cryptic sites during its own polymerization into fibrillar form [26-28]. Recent studies on cell-mediated FN molecular unfolding illustrate that loss of *in vitro* cellular contraction through inhibition of actin polymerization results in significant relaxation of FN into a more compact conformation [29], while inhibition of cell contractility in excised (*ex vivo*) frog embryo tissue results in a completely collapsed FN conformation [30]. Furthermore, extraction of cells from fibroblast-derived FN ECMs and quantification of the FN molecular collapse indicates that cells strain FN fibers (and unfold FNIII repeats) between 200 and 700% [31].

The best-defined model demonstrating that FN unfolding at the secondary level has a significant impact on integrin engagement and subsequent cell fate is domain unfolding of the RGD-containing tenth FNIII repeat. Owing to the elasticity of type III repeats in the FG loop (the loop between the F- and G-β strands of FNIII repeats), the ninth and tenth FNIII repeats are together capable of presenting multiple conformations that direct integrin specificity to this region (Figure 2). The synergy site is located on the ninth FNIII repeat, approximately 32 Å from the RGD loop on the tenth FNIII repeat in solution. In this conformation, the two motifs act synergistically to bind integrin-$\alpha_5\beta_1$ [32]. Under small forces (tens of pN), the tenth FNIII

repeat, prior to complete unfolding, assumes an intermediate state characterized by the RGD loop translocating away from the ninth FNIII repeat. In this state, the synergy–RGD distance is increased from 32 Å to approximately 55 Å; a distance too large for both sites to cobind the same receptor. This is further evidenced by experiments demonstrating that increasing the length of the linker chain between the two repeats by insertion–mutation reduces $\alpha_5\beta_1$ binding [33]. These data suggest that integrin binding attributed to the synergy site can be 'turned off' mechanically by stretching these domains into this intermediate state or beyond. The effect of force-mediated unfolding of FN on integrin engagement and activity levels may be further explained by studies demonstrating that the degrees of conformational stability of the ninth (or tenth) FNIII repeat can modulate integrin accessibility of the RGD motif. A stabilization of the ninth FNIII repeat via a Leu–Pro point mutation at amino acid 1408 [34], or stabilization of the hydrogen bonding within the tenth type-III repeat [35], increases affinity for $\alpha_5\beta_1$ over $\alpha_V\beta_3$.

Importantly, the exact composition of FN isoforms, with respect to its FNIII repeats, possibly plays a significant role in determining the overall stability of the primary integrin-binding domain, since the stability of FNIII repeats is critically dependent on neighboring domains [36]. For example, extra domain A (EDA) FN contains an extra FNIII repeat; the EDA domain (inserted following the 11th FNIII repeat), has been shown to exhibit extraordinary instability. The nuclear magnetic resonance spectra of the EDA domain demonstrates that it exists in a predominately unfolded state at 37°C and can be completely unfolded upon the removal of four amino acids at its N-terminus [37]. These data suggest the EDA FNIII repeat is highly sensitive to mechanical forces and is predicted to cause local conformational alterations through potential destabilization of adjacent FNIII repeats [38,39]. This example is particularly interesting, since the presence of EDA could result in destabilization of the ninth and tenth FNIII repeats, resulting in preferential engagement of certain integrins over others.

Integrin specificity is clearly linked to cell fate determination, including differentiation and proliferation processes. For example, the integrin-β_1 subunit enhances the progression of differentiation of precursor cells [40,41], while integrin-α_v, such as $\alpha_v\beta_3$, correlate with increased cell adhesion [42], enhanced cell proliferation and decreased differentiation [43]. FN has been shown to cause a switch between stimulation of proliferation and differentiation of mouse C_2C_{12} myoblasts to myotubes [44] and MC3T3-E1 osteoblast-like cells [41], by a yet-to-be-identified structural change that results in an increase of the $\alpha_5\beta_1$–$\alpha_v\beta_3$ binding ratio. Thus, the engineering of protein variants with differing contributions of FNIII repeats, and modifications of FNIII repeats that alter their relative sensitivity to both proteolysis and mechanical forces offers an attractive means to dynamically regulate integrin specificity and, thus, cell phenotype.

Engineered ECM variants toward directing integrin specificity

Integrin binding can ultimately direct cell processes and phenotype, providing an interesting mechanism for controlling cell fate. If integrin binding can be controlled specifically, cell fate could in turn be controlled. Integrin binding is determined by specific binding sequences within an ECM molecule. The most abundant of these sequences is the RGD sequence and is found in a variety of ECM proteins including FN, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, LN, entactin, tenascin, osteopontin and several others [3]. The RGD sequence is promiscuous in integrin binding, including $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ [3]; therefore, this sequence alone is not capable of directing integrin specificity. There are examples, however, of adjacent sequences affecting integrin specificity, particularly in FNIII repeats [5]. As described previously, the RGD sequence is found on the tenth FNIII repeat, immediately adjacent to the ninth FNIII repeat, which contains the synergistic PHSRN sequence. It has been shown that RGD, in the presence of PHSRN, strongly

increases the affinity for $\alpha_5\beta_1$ binding compared with other integrins (namely α_v-containing integrins) [45-47]. These responses have also been shown in 3D FN matrices; Mao and Schwarzbauer produced normal and chimeric FN matrices containing no RGD or PHSRN sites and found that they could control Chinese hamster ovary cell integrin attachment, with Chinese hamster ovary cells attaching preferentially to wild-type matrices via $\alpha_5\beta_1$ and to the PHSRN-null matrices via $\alpha_v\beta_3$ [48].

Various groups have shown that cellular responses can be controlled by producing FN variants that display the tenth FNIII repeat (RGD alone) or the ninth and tenth FNIII repeat (RGD and PHSRN). Garcia *et al.* demonstrated that presenting the FNIII7−10 fragment on titanium surfaces enhanced osteoblastic differentiation of bone marrow stromal cells, and improved function implant osteointegration compared with titanium surfaces functionalized with RGD alone [4,49]. These responses were found to be the result of differential integrin binding, such that cells bound to the FNIII7−10 functionalized surfaces via integrin-$\alpha_5\beta_1$ and to RGD functionalized surfaces via integrin-$\alpha_v\beta_3$. A similar response has been shown by Martino *et al.* with human mesenchymal stem cells and has been shown to apply in 3D culture conditions [50]. Human mesenchymal stem cells plated in 2D on FNIII fragments and in a 3D fibrin ECM containing the same FNIII fragments demonstrated increased specificity for $\alpha_5\beta_1$ over $\alpha_v\beta_3$ and, subsequently, enhanced osteogenic differentiation when presented with FNIII9−10 rather than FNIII10 alone. Although these examples focus on osteoblastic differentiation, $\alpha_5\beta_1$ is also heavily implicated in other processes such as angiogenesis and, therefore, one could easily envision these same fragments being used to control angiogenic processes.

While the significant majority of investigations have simply presented FN as truncated fragments of the FNIII repeats containing the native structure and sequence, there has been a recent focus on mutated FNIII fragments that enhance and potentially modulate integrin binding. Richards *et al.* produced an FNIII10 fragment with an RGDWXE sequence that exhibited enhanced affinity and specificity to integrin-$\alpha_v\beta_3$ [51], while Martino *et al.* enhanced $\alpha_5\beta_1$ binding by producing an FNIII9−10 fragment that contains the previously discovered Leu1408–Pro mutation to ‘stabilize’ the conformation of the ninth and tenth domains [50, 52]. These variants not only open many possibilities for regenerative medicine applications by allowing one to potentially control cell fate by directing integrin binding, but also have the potential to serve as valuable tools for exploring cell biology and integrin signaling in response to physiologically relevant conformations of FN. As described earlier, FN is a mechanically dynamic molecule that shows great flexibility within the FG loop of FNIII repeats, and has been shown to undergo conformational changes in response to forces that extend the ninth and tenth FNIII domains enough to disengage the PHSRN site from the RGD site such that they can no longer be bound simultaneously [32]. These mutants could provide experimental biologists with new ways to study these interactions. In addition, these simplified systems allow not only for the comparison of different conformations of FN, but also for the comparison of cellular responses to other RGD-containing proteins that do not contain PHSRN, and could provide insight into the markedly different responses to these proteins when compared with FN.

While the use of RGD with and without PHSRN provides a very interesting way of directing integrin specificity, there are a number of other systems that potentially allow for specific integrin binding, including the GFOGER sequence found in residues 502−507 of type I collagen that directs integrin-$\alpha_2\beta_1$ binding [53,54]. It has been shown that integrin recognition of this sequence is entirely dependent on the conformation of the sequence; it must be presented in a triple-helical conformation similar to that of native collagen [35,55]. Tong *et al.* produced a series of collagen-mimetic peptides containing the GFOGER sequence and showed that while the GFOGER sequence is critical for cell binding, the triple-helical context was critical for integrin recognition; cells were found to bind more tightly to peptides with higher triple-helix

stability. Both Tong and Bellis have shown that this sequence can direct cell fate in vastly different systems, for example liver and bone [35,56]. By incorporating another triple-helical collagen mimetic onto the surface of microspheres, Khew *et al.* were able to increase cell binding, spreading, cell proliferation, viability and the cell bridging of hepatocyte cells. Garcia *et al*. produced a triple-helical collagen variant containing this GFOGER sequence for directing $\alpha_2\beta_1$ binding [57]. By using these variants alone to direct $\alpha_2\beta_1$ binding, or by using them in conjunction with FNIII fragments to direct $\alpha_5\beta_1$ binding, this group was able to explore the synergistic effects of integrin binding and subsequent signaling [55]. It was found that engagement of integrin-$\alpha_2\beta_1$ of murine osteoblast-like cells upon binding to the triple-helical GFOGER molecule resulted in osteoblastic differentiation and matrix mineralization.

These variants, while not an exhaustive list, have been shown to be extremely useful in directing cell fate *in vitro* as well as *in vivo* and, therefore, show much promise in regenerative medicine applications in the areas of stem cell technology and biomaterials. These ECM variants can also serve as powerful tools for studying the effects of differential integrin binding/signaling as well as potential synergistic effects. Although the examples reviewed in this article focus mainly on FN and collagen variants, there are many other ECM variants that could be engineered to direct integrin specificity and, ultimately, cell fate. The only limitation is the knowledge of integrin-binding sequences within the ECM molecule and the dynamics controlling integrin specificity. Thus, the challenge moving forward is to obtain a more complete description and understanding of matrix biology, a task not easily accomplished owing to the inherent complexity of the ECM described previously.

Conclusion

Directing specific cell behaviors, in particular differentiation, in response to biomaterials for regenerative medicine applications remains a challenge. While current technological advances have been predominately inspired by the reductionist point of view, with exhaustive use of the integrin-binding trimeric-peptide motif RGD for supporting cell adhesion, the lack of biological specificity is not optimal for directing differentiation and cell phenotype. The native ECM directs these behaviors through a number of integrated mechanisms, including ECM–GF complexes (reviewed elsewhere; [58-60]), and time-resolved exposure of cryptic sites through proteolytic processing and mechanical unfolding. Several attempts have been made to harness these complexities, most notably with respect to the integrin-binding domain of FN, where mechanical stabilization of the ninth and tenth FNIII domains has demonstrated significant influence on integrin specificity and concomitant cell differentiation and phenotype. However, these early attempts to engineer novel ECM domains represent only the tip of the iceberg. Investigation into the dynamics of LN-mediated integrin specificity alone yields interesting design concepts for engineered LN variants that are capable of displaying complex, time-resolved integrin specificity that could direct cell phenotype in the regeneration of complex tissues. As our understanding of the nuances of matrix biology emerges, so too will the number of potential strategies for engineering ECM variants with specific design criteria to exquisitely direct cell phenotype in space and time.

Future perspective

Our future abilities to engineer ECM variants critically depends on the ECM biology community's efforts toward investigating and understanding the complexity of the native ECM. While there is an inherent appreciation by all biologists and engineers of both the tremendous complexity of and fundamental role that the ECM has on directing cell and tissue behavior, more significant efforts and ideally collaborations between biologist and bioengineers toward characterizing and defining this complexity are required. Appropriately, the stem cell biology field has begun a significant push toward defining the stem cell ‘niche’ – the microenvironment

that maintains the 'stemness' of these cells – and continued exploration of these unique ECMs and their eventual integration with the emerging concepts in ECM biology reviewed previously (e.g., proteolytic processing and mechanochemical translation) provide excitement about our potential to direct stem cell phenotype, differentiation and potentially regeneration by engineering highly specific ECM variants that engage cells in a predicted and designed fashion. Apart from our general lack of understanding of the complexity of ECM, biotechnological advances are also desperately require, which would allow the construction of such complex polypeptide domains in a routine manner. Depending on the level of modification and complexity of the ECM variants, the 'easy-to-use' and relatively cheap bacterial yeast systems may not be sufficient to generate the desired protein or protein domains. Both of these challenges represent exciting areas of research with significant opportunity for new discovery.

Executive summary

Complexity of cell–extracellular matrix interactions

■ Complexity in extracellular matrix (ECM)–integrin interactions can be regulated by at least two different mechanisms: proteolytic processing and mechanochemical translation of integrin-binding domains.

Proteolytic processing of ECM

■ Proteolysis of ECM proteins can significantly affect integrin specificity, as well as release of sequestered growth factors, cytokine and matrikines; a new class of molecules that direct many cellular phenotypes.

■ Proteolysis has been shown to regulate integrin-specific interactions and concomitant cell phenotype, most notably through the destruction of and/or exposure of integrin-binding domains; laminin is a classic example of this.

Mechanochemical translation of cell-binding ECM domains

■ Mechanical forces expose cryptic domains and alter conformationally sensitive binding regions within the ECM, including integrin-binding domains.

■ The type III repeats of fibronectin (FN) are highly sensitive to mechanical forces that result in alterations to the conformation of integrin-binding sites and, thus, modifications that alter the mechanical sensitivity of these domains may hold promise.

Engineered ECM variants toward directing integrin specificity

■ Engineered ECM variants that direct integrin specificity, either through regulation of proteolysis or mechanosensitivity, can be used to direct cell fate.

■ FN variants have been produced to direct integrin binding through Arg–Gly–Asp (RGD) and PHSRN sequences by presenting either truncated fragments of the FNIII repeats containing the native structure and sequence, or mutated FNIII fragments that enhance and potentially modulate integrin binding through enhanced mechanical stabilization.

■ Collagen variants that incorporate the GFOGER sequence are capable of directing integrin specificity and cell fate. This is dependent on the triple-helical structure detail.

Acknowledgement

The authors would like to acknowledge M Martino for his helpful insights with the concepts regarding proteolytic processing of the extracellular matrix.

Financial & competing interests disclosure

Funding was provided by NIH T32-GM008433 (AE Carson) and NSF ERC EEC-9731643 (TH Barker). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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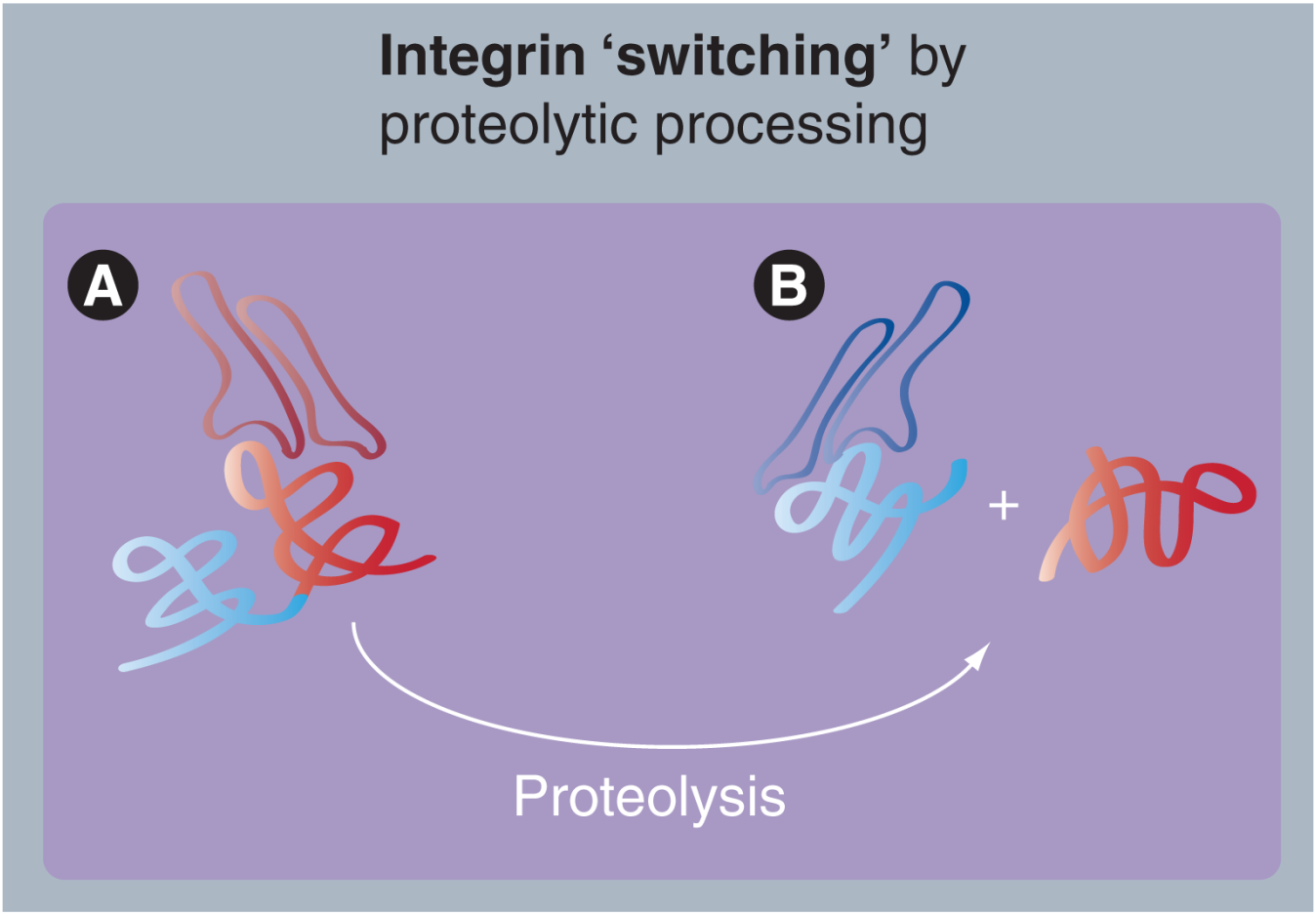

Figure 1. Extracellular matrix proteins are often degraded as a part of their natural processing

In certain cases, these proteolytic processing steps have the potential to expose cryptic, or buried, protein-binding sites, such as in the documented case of laminin during epidermal repair. Prior to proteolysis of laminin, integrin-$\alpha_3\beta_1$ is bound **(A)**, but proteolysis leads to exposure of binding sites that result in $\alpha_4\beta_6$ preferential binding **(B)**.

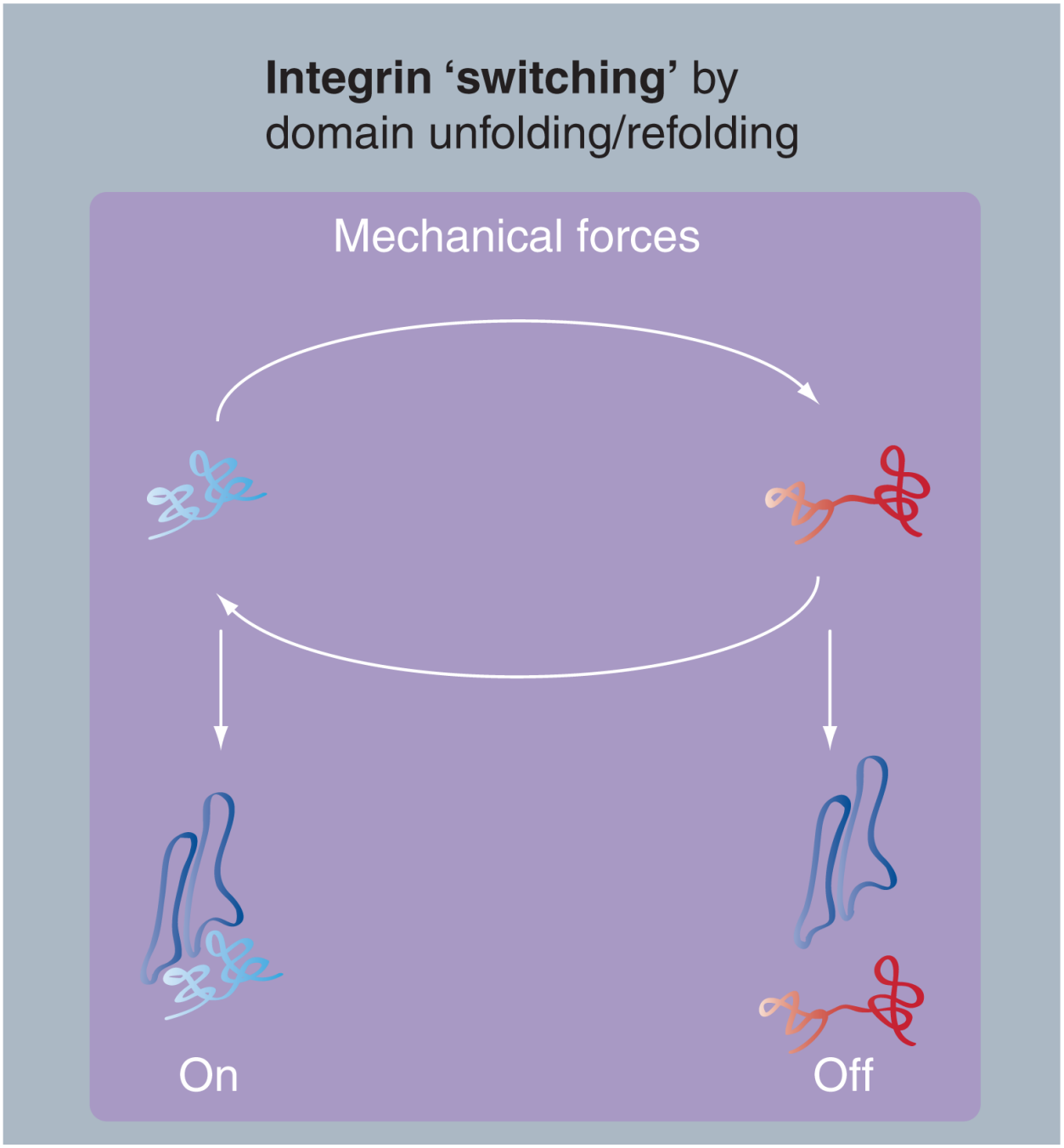

Figure 2. Many extracellular matrix proteins are sensitive to mechanical force-mediated unfolding

These proteins and protein fibers are capable of resisting cell-derived forces, yet they have the capacity to display partial unfolding, which has the potential for exposing cryptic, or buried, protein-binding sites, such as in the documented case of fibronectin during fibrillogenesis. Partial unfolding of molecules can also lead to an integrin switch such that prior to unfolding an integrin is bound (switch is 'on') and after unfolding the integrin is no longer bound (switch is 'off').