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Synergistic control of cell adhesion by integrins and syndecans

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Preface

The ability of cells to adhere to each other and to their surrounding extracellular matrices is essential for a multicellular existence. These interactions provide physical support for cells, regulate cell positioning, and enable microenvironmental sensing. Two adhesion receptor families, the integrins and the syndecans, mediate adhesion, but their relative, functional contributions to cell-extracellular interactions remain obscure. As recent advances have highlighted connections between the signalling networks controlled by these receptors, we have surveyed the evidence for their synergistic association in the control of adhesive function and their regulation of cell behaviour in response to the external environment.

Introduction

Cells in tissues are structurally and functionally integrated with their surrounding extracellular matrix (ECM) in a highly organised process that involves thousands of dynamic connections. The intracellular domains of adhesion receptors tether the contractile cytoskeleton to the plasma membrane and compartmentalise cytoplasmic signalling events. At the extracellular face, the same receptors direct and organise the deposition of the ECM itself. The membrane-proximal functions of adhesion receptors trigger distal processes within cells, which include alteration in the direction of cell movement and the regulation of cell fate, and determine long-range effects outside cells such as the construction of ECM networks and consequent shaping of higher-order tissue structure. Elucidating the molecular events that mediate the functional integration of multicellular tissues with spatially-oriented ECM and that control adhesion-regulated signalling is therefore of central importance if we are to understand the tissue-organising principles of metazoan life.

In keeping with the essential role of the ECM, aberrations in ECM organisation and cell-ECM interactions contribute widely to disease. Many of the major human diseases are caused by defects in cell-ECM coordination, are exacerbated by aberrant use of normal cell adhesive processes, or are potentially correctable by altering tissue structure or cell movement. For example: progressive extracellular remodelling in chronic atherosclerotic, fibrotic and neurodegenerative diseases ultimately leads to a loss of tissue integrity; altered adhesion is a defining characteristic of tumour malignancy; and the pathogenesis of inflammatory and thrombotic diseases relies on aberrant cell aggregation and/or migration. It is notable that the incidence of many of these conditions is growing in parallel with increasing longevity in the population. The development of strategies to correct adhesive dysfunction therefore has enormous promise as a route to improving the treatment of many clinical conditions and thereby enhancing quality of life.

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The cell surface receptors that mediate cell-ECM adhesion are primarily members of two gene families — the integrins and the syndecans. Intriguingly, nearly all ECM molecules contain binding sites for both types of receptor, and as reviewed below there is substantial evidence that a full cell adhesion response requires dual engagement. Integrins and syndecans are required for generating a physical link to the cytoskeleton, for force transduction, for spatial control of signalling complex assembly (see Text Box), and for the regulation of cytoskeletal dynamics. Over the past two decades, analyses *in vitro* have demonstrated a clear synergistic link between the two families. However, until recently, both the molecular basis of this synergy and the functional relevance of the association *in vivo* have been unclear.

This article draws together information from a series of recent studies that have shed light on both of these topics, beginning with the molecular signals that arise from synergistic engagement of integrins and syndecans, and continuing with the *in vivo* consequences of receptor cooperation.

Integrins and Syndecans

The integrins are a family of transmembrane glycoproteins (Fig. 1) that consist of non-covalent heterodimers. In mammals, 18 α and 8 β integrin genes encode polypeptides that combine to form 24 α,β receptors[1]. Integrins interact with various ligands including ECM glycoproteins and cell-surface proteins[2], whereas their cytoplasmic domains interact with components of the actin cytoskeleton. These bidirectional linkages exert spatial control on signalling and thereby control cellular differentiation and fate. Genetic analyses of mutant integrins have demonstrated roles in tissue structure and cell migration[3, 4]. These receptors also participate in adhesive events during many pathophysiological processes, including haemostasis and thrombosis, inflammation, wound healing and neoplasia.

The syndecans are a family of membrane-intercalated proteoglycans, comprising a protein core with covalently attached glycosaminoglycan sugar chains (Fig. 1). There are four members of the syndecan family in mammals, three of which (syndecans-1-3) have a restricted tissue distribution, and the fourth of which (syndecan-4) is expressed ubiquitously. The syndecans act as receptors for both ECM glycoproteins and growth factors, the large flexible glycosaminoglycan chains making them ideal receptors for ligands that are dilute or distant from the membrane. The cytoplasmic domains of syndecans comprise a pair of conserved regions, present in all isoforms, and a variable region that is unique to each syndecan. The variable region of syndecan-4 encompasses a binding site for PKCα, and the presence of this motif, combined with the ubiquitous expression of syndecan-4, has made syndecan-4 the primary focus of investigation into syndecan signalling.

It has been appreciated for two decades that focal adhesion (FA) formation during cell spreading on fibronectin depends upon engagement of an integrin and a cell-surface proteoglycan (specifically, $\alpha_5\beta_1$- integrin and syndecan-4 [5, 6]) (Fig. 2). RNAi knockdown and mutagenesis studies have subsequently revealed cooperation of $\alpha_v\beta_3$-integrin and $\alpha_v\beta_5$-integrin with syndecan-1 during adhesion to vitronectin[7,8], and $\alpha_2\beta_1$-integrin and $\alpha_6\beta_4$-integrin with syndecans during adhesion to laminin[9,10]. This range of integrin-syndecan receptor pairs suggests widespread cooperation between the families, and raises the possibility that, depending on cellular context, different receptor combinations can differentially modulate cellular responses to different extracellular stimuli. A key challenge for the field is to define the molecular basis for the functional connection between integrin and syndecan signalling.

The contribution of integrins to cell adhesion has largely been demonstrated through the use of inhibitory antibodies and over-expression studies. However, despite an extraordinary

effort by the field, the immediate connection between adhesion receptor occupancy and cytoplasmic signalling has remained elusive. Indeed, a convincing link between integrin and the cytoskeleton has been established only recently through the resolution of the atomic structure of the β_3-integrin cytoplasmic tail complexed with the head domain of the cytoskeletal protein talin[11]. Integrin conformation is influenced by the associations of both the extracellular and cytoplasmic domains, and cytoplasmic signals that lead to integrin activation from inside the cell (inside-out signalling) seem to converge on talin, as demonstrated by RNAi knockdown[12] and FRET analyses[13]. Conversely, the initiation of cytoplasmic signals, downstream of integrin, in response to an extracellular matrix (outside-in signalling) is reported to be talin-independent, and relies upon the activation of tyrosine kinases by an unresolved pathway[14]. Cytoplasmic signalling downstream of syndecan-4 is much easier to explain, due to the presence of well-characterised PKCα and PDZ domain-binding sites within the cytoplasmic domain of syndecan-4[15, 16]. Further putative interactions of syndecan cytoplasmic domains with tyrosine kinase complexes[17] and membrane-binding proteins[18] have been described, but the small size of the cytoplasmic domains (Fig. 1) precludes simultaneous association with all possible binding partners and suggests that the interactions might vary according to circumstance, allowing syndecans to act as environmental sensors.

Adhesion to the ECM regulates numerous signalling pathways, including those that involve Rho family GTPases[19], tyrosine kinases[20] and MAP kinases[21]. However, the downstream linkages that connect these pathways to the complex network of adhesion signalling have not been determined. Thus, although progress has been made in defining the effectors used by integrins and syndecans, the key nodal points at which receptor signalling is coordinated are unknown. Each of the key adhesion-regulated pathways downstream of ECM engagement will be considered in turn.

Signalling through protein kinases

Protein kinase C

A protein-kinase-binding motif, present in the cytoplasmic domain of syndecan-4 alone, was identified early during the characterisation of the receptor[22]. Unlike other members of the syndecan family, syndecan-4 specifically recruits PKCα to FAs and activates it through a unique interaction between the variable region of the syndecan-4 cytoplasmic domain, the kinase domain of PKCα, and the inositol lipid PI-4,5-P_2 [15] (Fig. 3). Formation of this ternary complex is itself regulated by the phosphorylation of the syndecan-4 cytoplasmic domain at a different site by PKCδ, which affects both activation of PKCα and oligomerisation of the cytoplasmic tails[23]. In support of a positive role for PKC signalling in syndecan function, dominant-negative PKCα blocks syndecan-4-induced migration[24]. Intriguingly, FRET analysis reveals a close association between active PKCα and β_1-integrin that promotes migration of cells on ECM substrates such as fibronectin, collagen and laminin[25]. These observations suggest a connection from syndecan-4 to integrin, via PKCα, that primes the integrin for migration, and indeed recent evidence suggests that the activation of $\alpha_{IIb}\beta_3$-integrin depends on both talin and active PKCα[26].

Increasingly, evidence suggests a role for PKC signalling in regulating the surface expression of integrins. PKC-induced migration of breast carcinoma cells is inhibited by blocking the endocytosis of β_1-integrin using a dominant-negative mutant of dynamin[25] and a direct interaction between syndecan-4 and dynamin-2 has been identified by yeast two-hybrid and immunoprecipitation experiments[18]. Although syndecan-1 and β_5-integrin have been found to co-precipitate[8], it appears that the relationship between β_1-integrin and syndecan-4 is one of regulation, rather than constitutive association, as dominant-negative dynamin has no effect on the endocytosis of syndecan-4 in endothelial cells[27]. Instead, the

surface expression of syndecan isoforms has been reported to depend on association with the second PDZ domain of the scaffolding protein syntenin[28] (Fig. 3). Mutating either syntenin or the C-terminus of syndecan prevents recycling of syndecans, causing the accumulation of syndecan and integrin in endosomes and compromising cell spreading. Together, these experiments suggest that the cooperation between integrin and syndecan is responsible for the delicate balance in integrin cycling that is necessary for rapid cell migration. Determining the extent to which integrin and syndecan molecules interact and regulate one another is now a priority for the field and, in particular, developing the ability to visualise and track syndecan-4 reliably will accelerate our understanding of receptor cooperation.

Integrins mediate attachment to various extracellular ligands[2], and there are indications that the signals activated as a consequence of integrin and syndecan engagement vary depending on the integrin involved. Syndecan-4-dependent PKCα regulation is essential for FA formation and migration in cells that adhere to fibronectin via $\alpha_5\beta_1$-integrin, but not $\alpha_4\beta_1$-integrin, which is syndecan-independent[24]. Similarly, the PKCα-phosphorylated form of myristoylated alanine-rich C-kinase substrate (MARCKS) redistributes from the membrane to the cytosol during the period of adhesion-induced PKC activity, and is essential for $\alpha_5\beta_1$-integrin-mediated spreading of myoblasts on fibronectin, but unnecessary for $\alpha_7\beta_1$-integrin-mediated spreading on laminin[29]. The requirement for PKC activity for adhesion to fibronectin (Fig. 2f) is a reflection of the synergy between $\alpha_5\beta_1$ and syndecan-4, and is consistent with the unique ability of syndecan-4 to regulate PKCα. So, although PKCα appears an essential mediator of signals downstream of syndecan-4, it may not be necessary for all integrin-mediated adhesion.

Tyrosine kinases

Unlike growth factor receptors, integrins lack intrinsic kinase activity, and therefore direct signalling cascades through the recruitment and activation of non-receptor kinases. ECM engagement of syndecan-4, which is itself phosphorylated by Src family kinases[30], has been linked to the activation of two key adhesion-dependent tyrosine kinases, focal adhesion kinase (FAK) and Src. A protein complex that includes Src and its close relative, Fyn, has been reported to bind to the membrane-proximal region of the syndecan-3 cytoplasmic tail and become activated in response to clustering of syndecan-3[17]. Although connections between syndecan-4 and Src are limited to reports of colocalisation, the fact that the Src-binding motif is conserved in all syndecans makes it likely that regulation of Src is relevant to the whole family (Fig. 3). Interestingly, it has been reported that both Src and Fyn are activated by direct association with the cytoplasmic tail of β_3-integrin, but not by the tails of β_1-integrin or β_2-integrin [14], and collectively these data may provide another example of cooperation between syndecans and a specific subset of integrins.

Evidence of syndecan-4 contributing to FAK regulation is more robust. FAK is activated by autophosphorylation of tyrosine-397 as cells spread on whole fibronectin, but not on a fibronectin fragment that lacks the syndecan-binding domain[31]. Levels of phospho-FAK are reduced by disruption of syndecan-4 and are not restored by activation of PKC with phorbol acetate[32]. Like PKCα, FAK has been linked to dynamin-mediated endocytosis. Microtubule-directed disassembly of FA is blocked by inhibition of FAK or dynamin, and recruitment of dynamin to FA depends on the association between dynamin and activated FAK[33]. As a consequence we might envisage a role for syndecan-4 in regulating dynamin-mediated endocytosis by a non-linear pathway involving both PKCα and FAK, and a similar level of crosstalk between serine/threonine and tyrosine kinases would go some way to explaining the contributions of syndecans to adhesion formation.

Signalling through Rho family GTPases

Rac

The Rho family of GTPases are central to cytoskeletal organisation and cycle between active (GTP-bound) and inactive (GDP-bound) forms, due to their intrinsic GTPase activity[19, 34]. The best-characterised members are Rac1, which drives membrane protrusion and the formation of nascent focal complexes, and RhoA, which drives FA maturation and actin filament bundling. Adhesion to fibronectin initiates the transient activation of Rac and downstream effectors[35], a process that is blocked by mutation of the cytoplasmic tail of β_1-integrin [36]. Recent investigation into Rac1 regulation revealed the extent of cooperation between $\alpha_5\beta_1$-integrin and syndecan-4 (Fig. 4). Engagement of both receptors was necessary for the activation of Rac1 in response to fibronectin, and localised activation of Rac1, at the leading edge of a migrating cell, was the direct consequence of syndecan-4 signalling[37]. Rac1 activity is also necessary for the endocytosis of both syndecan-4 and FGF2 [27], establishing a feedback loop between syndecan-4 and Rac1, as well as a link to growth factor signalling that might affect adhesion-dependent Rac1 signalling. Inhibition of the EGF receptor disrupts adhesion-dependent Rac1 regulation[38], suggesting crosstalk between integrins, syndecans and growth factor receptors.

Clustering of the cytoplasmic domains of syndecan-4, but not of syndecan-1, accelerates cell migration in scratch wound assays on fibronectin and is abrogated by deletion of the C-terminal PDZ-ligand motif[39]. The migration of fibroblasts on fibronectin fragments is compromised in the absence of syndecan ligand[40], and knock-down of syndecan-1 similarly compromises migration on vitronectin[7]. Each of these experiments is consistent with Rac regulation by syndecans, yet, surprisingly, disruption of syndecan-4 expression actually increases steady-state Rac activity[37, 41]. Rather than acting as a positive Rac regulator, expression of unengaged syndecan-4 suppresses Rac activity and only drives the activation of Rac, in a PKCα-dependent manner, at points of ECM engagement[37] (Fig. 4). Investigations into the role of Rac1 in cell migration have shown that Rac activity influences the persistence, rather than the speed, of migration[42, 43]. Over-activation of Rac1 limits the persistence of migration by increasing random protrusion. Thus, syndecan-4 limits Rac1 activity in the absence of ligand engagement and results in persistent migration by restricting Rac1 activity to the leading edge[37]. This finding could explain the crucial contribution of syndecan-4 to wound healing, discussed below.

Rho

ECM engagement of syndecan-4 has a similar effect on RhoA signalling. ECM engagement by syndecan-4 causes a wave of RhoA regulation[37, 44], and treatment of cells with the Rho inhibitor C3 exotoxin blocks FA formation in response to syndecan-4[45]. Syndecan-4-dependent regulation of both Rac1 and RhoA requires the activation of PKCα[37, 44], whereas independent activation of RhoA by lysophosphatidic acid (LPA) bypasses the requirements for syndecan-4 ligand or active PKC in FA formation[45]. These experiments demonstrate that GTPase regulation lies downstream of PKCα and provides a crucial link from the cytoplasmic domain of an adhesion receptor to GTPase signalling. As with PKC signalling, the relative contributions of integrin and syndecan-4 to GTPase signalling depend on the integrin involved. Overexpression of β_1-integrin, but not β_3-integrin, causes RhoA activation, whereas overexpression of neither integrin has a strong effect on GTP-loading of Rac[46]. Rather than activating Rac, antibody-clustering of β_1-integrin causes redistribution of Rac to the membrane by reorganisation of lipid microdomains: a response that could be enhanced by serum stimulation or expression of active Rac[47]. These alternative mechanisms of Rac regulation suggest that syndecan-4 and integrin might cooperate by separately driving GTP-loading and membrane recruitment, respectively, with both events being

necessary for the activation of downstream effectors. Such a model would highlight the importance of synergy between receptors and point to Rac as a convergence point for signals from integrins and syndecan-4.

The range of signalling pathways regulated by a variety of integrin-syndecan pairs points towards two key conclusions: first, as already outlined, the circumstances of syndecan-4 engagement, by the ECM, and the complement of other receptors involved will result in varied cellular responses to ECM; second, the localised regulation of signalling molecules, both spatially and temporally, by ECM receptors is more important than the holistic activation of a molecule throughout a cell. The ability to regulate signals differentially at the leading and trailing edges is particularly important for cell migration and will determine both the polarity and the velocity of migration. As we move on to consider the effects of integrin-syndecan function *in vivo*, it becomes increasingly clear that the regulation of cell migration is central to many integrin-syndecan-mediated biological processes.

Significance of integrin-syndecan synergy *in vivo*

Having considered the molecular basis of integrin and syndecan function, it is apparent that cooperative signalling between the two receptors is fundamental to cellular responses to ECM. In the following sections, the potential role of synergy between syndecans and integrins *in vivo* will be reviewed. Classical approaches to determining biological function (such as genetic analysis of development in whole organisms or analysis of inappropriate receptor expression during the pathogenesis of disease) have not yet been used to test the significance of integrin-syndecan co-signalling. However, there are many similarities between the physiological defects caused by interference with integrin or syndecan signals *in vivo* that could potentially be explained by the molecular synergy between the molecules. With this in mind, the cellular processes in which synergy appears to occur will be reviewed. Consistent with the role of syndecan-integrin synergy recently elucidated at the molecular and cellular level, it turns out that a common feature of these processes is the regulation of directional migration.

Wound healing

The process of wound healing is complex and involves precise regulation of clotting and coagulation, cell infiltration, angiogenesis and re-epithelialisation. It is well established that expression of many integrins is modulated during epithelial wound healing[48-50] with receptor levels often being regulated by growth factors or exposure to the ECM[51, 52]. Transgenic mice deficient in expression of β_3-integrin exhibit accelerated and dysregulated re-epithelialisation[51], suggesting that integrin function is pivotal to the process of wound healing. In uninjured mouse and neonatal human skin, syndecan-4 is found only in the epidermis; however, following injury, expression is upregulated on endothelial cells and fibroblasts in granulation tissue[53]. One of the defects detected in syndecan-4 knockout mice is impaired wound healing. As fibroblasts cultured from syndecan-4-/- mice displayed reduced migration in a scratch wound assay *in vitro*[54], it seems most likely that the reduced level of wound healing in syndecan-4-/- mice is, at least in part, a function of suppressed migration (although, as described below, syndecan-4 signalling may also regulate angiogenic processes in the wound bed). Moreover, a recent study has found that the GTPase Rac1, which can be regulated by $\alpha_5\beta_1$-integrin and syndecan-4 co-signalling[37], is required for efficient keratinocyte migration and wound healing *in vivo*[55], suggesting a possible means by which integrin-syndecan synergy could regulate wound healing.

A role for syndecan-1 in the wound healing response has long been established. In normal mouse tissue, syndecan-1 expression is restricted to the epithelium[56]. However, syndecan-1 expression is upregulated during tissue repair on dermal endothelial cells and granulation

tissue fibroblasts[57]. This switch mirrors the change in syndecan-1 expression during embryonic epithelial cell morphogenesis, when expression of syndecan-1 is reduced in the epithelium and elevated in associated mesenchymal cells[58]. After protease- or growth factor-mediated shedding, the syndecan-1 ectodomain accumulates in wound fluid and granulation tissue[59, 60]. Like syndecan-4 knockout mice, syndecan-1-deficient mice are healthy and fertile; however, they display a defect in both corneal and skin re-epithelialisation. This defect is a result of impaired keratinocyte proliferation, migration and integrin localisation[61]. Interestingly, targeted ablation of the syndecan-1 gene in mice results in elevated transcription of α_3 and α_9 integrin subunits[61], suggesting another mode by which syndecan and integrin function can be linked. Transgenic mice over-expressing syndecan-1 also exhibit impaired wound healing; this is a consequence of increased levels of shed syndecan-1 ectodomain at the wound site, acting as a dominant-negative regulator of syndecan-1 function[62]. Both syndecans-4 and -1, therefore, appear to be involved in wound healing, with syndecan-4 being primarily associated with fibroblast migration, wound contraction and angiogenesis, and syndecan-1 principally being involved in keratinocyte function and re-epithelialisation[54, 61].

Angiogenesis

Angiogenesis plays a central role in embryonic development, wound healing and tumour progression. *In vivo* studies have demonstrated that many integrins are involved in the precise regulation of both developmental and pathological angiogenesis. Of these, $\alpha_5\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, the integrins with which syndecans have primarily been shown to synergise, can be considered principal regulators of angiogenesis[63, 64].

Expression of the $\alpha_5\beta_1$ heterodimer is elevated in endothelial cells during angiogenesis and α_5-deficient mice exhibit embryonic lethal developmental defects including substantially disrupted vasculogenesis[65]. Developmental angiogenesis is unlikely to rely on the synergistic signalling between $\alpha_5\beta_1$ and syndecan-4 as there are no significant angiogenic defects in syndecan-4 null mouse embryos[54]. However, an angiogenic role for syndecans in adult animals is supported by the fact that syndecan-4-deficient mice exhibit reduced angiogenesis in the granulation tissue of the wound bed[54]. Therefore it is possible that syndecan-4, through cooperation with $\alpha_5\beta_1$, may modulate elements of post-natal and/or pathological angiogenesis. A separate study of syndecan-4 knockout mice has revealed that they also have reduced numbers of foetal vessels in the placental labyrinth, although the authors speculated that this may be a result of an impaired anti-coagulation mechanism as opposed to a direct effect on neovascularisation[66].

A role for syndecans in developmental vasculogenesis has been described only in *Danio rerio* (zebrafish). Syndecan-2 is expressed on cells proximal to the major trunk vessels and syndecan-directed gene targeting leads to decreased angiogenic sprouting during vascular development. Reconstitution of syndecan-2 expression rescues the angiogenic defect and depends on the integrity of the syndecan cytoplasmic domain and therefore, presumably, downstream signalling. Moreover, syndecan-2-mediated angiogenesis in zebrafish also requires vascular endothelial growth factor (VEGF) signalling[67]. A role for integrins in zebrafish angiogenesis has yet to be firmly established, but as so many integrins are associated with both physiological and pathophysiological angiogenesis in other organisms, it seems likely that *D. rerio* will be no exception.

Over the past decade it has become apparent that $\alpha_v\beta_3$ integrin function is central to the regulation of pathological angiogenesis (Fig. 5). Integrin $\alpha_V\beta_3$ is expressed *de novo* in angiogenic vascular tissue[68] and inhibition of $\alpha_V\beta_3$, like inhibition of $\alpha_5\beta_1$, induces caspase-dependent apoptosis of angiogenic vessels and reduces tumour growth[69-72]. However, surprisingly, $\alpha_V\beta_3$-deficient mice exhibit enhanced tumour angiogenesis as a

result of elevated VEGFR2 expression and signalling[73, 74]. Until recently, the integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ were considered to form distinct functional pairs with the proangiogenic growth factors bFGF and VEGF, respectively[64]. However, a recent study indicated that the ability of $\alpha_V\beta_3$ to mediate bidirectional signalling regulates the capacity of a tumour to secrete VEGF. Thus, tumours expressing inactive $\alpha_V\beta_3$ secreted reduced levels of VEGF and exhibited suppressed angiogenesis and tumour growth[75]. Moreover, on endothelial cells, phosphorylation of the β_3 subunit cytoplasmic domain is required to induce VEGF-stimulated, VEGFR2-phosphorylation-dependent, pathological angiogenesis[76].

Consequently, some of the key questions that now need to be addressed are: precisely what is the nature of the $\alpha_v\beta_3$-VEGF-VEGFR axis? Are the biological effects in response to $\alpha_v\beta_3$ antagonism entirely independent of those resulting from $\alpha_v\beta_3$-deficiency? How does pathological angiogenesis differ from developmental vascularisation? Intriguingly, *in vitro* studies in a limited number of cell types have demonstrated that syndecan-1 has the capacity to regulate $\alpha_v\beta_3$ activation, ligand-binding affinity and signalling[7, 77] and the activity of $\alpha_v\beta_5$ [8]. Moreover, syndecans are known to facilitate interactions between various growth factors and their receptors[60, 78]. When we consider these data it is apparent that syndecan-1 has the potential to influence many of the $\alpha_v\beta_3$- and VEGF-mediated functions that regulate pathological angiogenesis (Fig. 5). So integrin-syndecan synergy may hold the key to answering these important questions.

A role for syndecan-1 in the regulation of angiogenesis *in vivo* is supported by the fact that syndecan-1-deficient mice have enhanced susceptibility to injury-induced angiogenesis of the ocular vasculature[79] and during inflammatory angiogenesis, syndecan-1 is upregulated following stimulation of EphB4-positive endothelial cells with its pro-angiogenic ligand, ephrinB2[80]. Intriguingly, stimulation with ephrinB2 also significantly increases transcription of $\alpha_5\beta_1$-integrin, VEGF-C and hepatocyte growth factor (HGF) in endothelial cells[80].

The importance of integrins, especially of $\alpha_5\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, in tumour angiogenesis is now well established[64]. However, to date, there has been comparatively little research into the role of syndecans in neovascularisation of tumours, which is surprising given their synergistic relationship with these integrins. However, syndecan-1 expression on both tumour and stromal tissue appears to be differentially regulated in a variety of solid cancers[78] and elevated syndecan-1 is detected in the plasma of myeloma patients and the level of syndecan-1 detected in blood or bone marrow correlates with the density of microvessels and patient survival[81]. It is conceivable that expression of syndecan-1 could play either a pro- or anti-angiogenic role; presumably this would depend on the cellular context, syndecan shedding, and the extracellular environment (including availability of growth factors, ECM and guidance cues and expression of their receptors) (Fig. 6). The role of syndecan-1-mediated signalling in angiogenesis and α_v integrin regulation is unknown, but it is possible that spatially regulated ligation of integrins, syndecans and their coreceptors could elicit intracellular signals that ‘fine-tune’ angiogenic processes.

The signalling molecules associated with integrin-syndecan synergy at the molecular and cellular levels can also regulate angiogenesis, suggesting a possible role for synergistic signalling rather than simply functional synergy. Antisense oligonucleotide-mediated inhibition of PKCα *in vivo* suppresses neovascularisation in a model of cardiac ischaemia[82], whereas inhibition of PKCα *in vitro* decreases angiogenic sprouting, endothelial migration, and vascular tube formation and permeability[82, 83]. Transgenic over-expression of FAK in the endothelia of mice promotes angiogenesis in granulation tissue of wounds and in response to ischaemia[84], whereas endothelium-specific FAK deficiency results in defective developmental angiogenesis[85]. Furthermore, *in vivo* delivery of FAK siRNA decreases microvessel density and tumour mass in a mouse model of ovarian cancer[86]. Locally

increased expression of FAK promotes retinal neovascularisation in mice, whereas this phenomenon is reduced by the expression of a dominant-negative construct of FAK[87]. Also, inhibition of the cyclooxygenase-2 enzyme *in vivo* reduces αvβ3-dependent activation of Rac1, resulting in suppressed growth factor-induced angiogenesis[88]. Rac1 is required *in vitro* for endothelial branching morphogenesis, and its downstream effector, PAK, mediates endothelial cell motility[89]. In addition, Rac1 activation has recently been shown to increase the expression of VEGF and to promote angiogenesis[90]. So, modulation of signalling events downstream of syndecan and integrin engagement has a profound effect on angiogenesis and highlights the role that synergistic signalling could play in the regulation of this process.

Axonal guidance and neurite outgrowth

Axonal guidance is characterised by directional migration and response to external cues (including ECM molecules, growth factors and chemoattractive/repulsive factors). In vertebrates, syndecan-3 (N-syndecan) is expressed in neurons migrating along nerve bundles[91] and neuronal migration is perturbed in syndecan-3-null mice, possibly as a consequence of disrupted Src kinase activity[92]. Numerous β_1-integrin heterodimers and ECM molecules also have been demonstrated to modulate neuronal migration and function in mice[93, 94]. Functional synergy between integrins and syndecans in neuronal development and axonal guidance would appear to be evolutionarily conserved as integrins and syndecans also have a role in neural patterning in invertebrates.

The Slit/Robo system is a key regulatory pathway in the mediation of chemorepulsion of developing axons at the midline. Slit is a secreted ligand for Robo transmembrane receptors and acts as a short range chemorepellent to control the direction of axonal projections. In the developing *Drosophila* embryo, the single syndecan gene (*sdc*) is expressed in tissues adjacent to those that express Slit, and is coexpressed with Robo in longitudinal axons[95]. Axonal expression of syndecan, although having no influence on Slit or Robo expression, is essential for neuronal responsiveness to Slit. Thus, *sdc* mutants are phenotypically similar to *robo* mutants, producing neural defects in which axon bundles cross the midline. Syndecan also interacts both genetically and physically with Robo and Slit[95], thereby providing a means to regulate Slit localisation[96] (FIG. 6). In *C. elegans*, the only syndecan orthologue (SDN-1) regulates axonal guidance and neural outgrowth, at least in part, by modulating the Slit/Robo pathway[97]. A role for integrins in Slit-mediated axonal guidance has also been described, whereby integrin-mediated adhesion of *Drosophila* neurons modulates axonal responsiveness to Slit-mediated chemorepulsion[98].

Interestingly, the Slit/Robo pathway, which has been characterised primarily in the mediation of axonal guidance, has also been implicated in tumour angiogenesis and vascular guidance. Slit2 is expressed on many human tumours, and its receptor, Robo1, is expressed on the surface of vascular endothelial cells. Slit2 acts as a chemoattractant for Robo1-expressing endothelial cells and induces endothelial tube formation *in vitro*, and tumour growth and angiogenesis *in vivo* can be inhibited by antibody-mediated blockade of Robo1 function[99]. Owing to the involvement of integrins and syndecans in the modulation of Slit/Robo function during axonal guidance it is possible that regulation of the Slit/Robo system may provide another means by which integrin-syndecan synergy can control angiogenesis *in vivo*.

The various signalling molecules implicated in integrin-syndecan synergistic signalling also appear to have a role in neural patterning and guidance. For instance, modulation of PKC and FAK expression or activity, both *in vivo* and *ex vivo*, influences neuronal migration, neurite outgrowth and axonal branching[100-103]. Active Src is detected in rat axons during neuronal regeneration and disrupted SRC-1 expression in *C. elegans* induces growth cone migration and directionality defects[104, 105]. Furthermore, expression of Fyn is increased in

rat neurons following neural trauma[106] and Fyn over-expression in *Xenopus* primary sensory neurons results in aberrant axonal targeting[107]. The Rac orthologues expressed in *Drosophila* (Rac1, Rac2 and Mtl) and *C. elegans* (CED-10, MIG-2 and RAC-2), and their downstream PAK effectors, are essential for axon growth, pathfinding and branching[108-111]. Indeed, membrane targeting of PAK in rat cells *in vitro* is sufficient to promote neurite outgrowth[112]. A recent study has demonstrated the complexity of Rho GTPase signalling *in vivo*, and highlighted the requirement for multiple Rac- and Rho-dependent signalling pathways, both convergent and divergent, to precisely regulate both axonal growth and targeting in *Drosophila*[113]. Given the complexity of this system, it is likely that the discrete spatial and temporal regulation of these pathways will be mediated by cross-talk between numerous cell surface receptors acting as environmental sensors.

The complexity of integrin-syndecan synergy *in vivo*

While this review concentrates primarily on the synergistic relationship between integrins and syndecans, it is clear that regulation of cellular functions by these receptors *in vivo* is considerably more complicated and involves cross-talk with many other receptor systems. Consequently, it is unlikely that integrin-syndecan synergy is mediated by a single universal mechanism, but rather through a number of different mechanisms (Fig. 6) that can each regulate a subset of functions depending on cellular context and the dynamic regulation, both spatial and temporal, of interactions with ECM, growth factors, chemokines, directional cues and receptor tyrosine kinases/phosphatases. Therefore, we propose four mechanisms by which integrins and syndecans could functionally synergise and interact with a variety of different ligands and receptors to elicit specific intracellular signals *in vivo* (Fig 6), and suggest that the transition between these different modes of interaction and signalling could be regulated by dynamic changes in the extracellular environment. However, it is apparent that an even greater level of complexity exists in the regulation of such synergistic signalling as the localisation, through direct association, of growth factors to the ECM, the integrin-mediated activation of specific growth factors, integrin affinity-modulation, endocytosis of receptors and the regulation of syndecan signalling through extracellular domain shedding, could all further modulate cross-talk between integrins and syndecans.

Perspectives

The breadth of signalling pathways considered in this review reveals a substantial literature supporting a functional synergy between integrins and syndecans that affects many of the adhesion-dependent processes observed *in vivo*. Perhaps the most surprising discovery arising from these studies is that disruption of syndecan expression is a non-lethal event, suggesting that the cooperation of receptors is most apparent when stationary cells are challenged to migrate by a change in environment rather than during developmental morphogenesis. Whether the difference between developmental and pathological cell migration is due to varied compensation between syndecans during development or a shift towards reduced cell migration in adult animals is unclear, but we speculate that some higher functions of eukaryotes, such as repair, inflammation and angiogenesis, required the evolution of a second tier of adhesion and signalling molecules that include the syndecans and a subset of integrins (such the β2 and αV sub-families)[114]. Major areas of future investigation are likely to include elucidation of signalling networks that mediate synergistic signalling, with emphasis on the spatial and temporal regulation of these events. Also, a more detailed analysis of the contributions of integrins and syndecans to repair processes *in vivo*, in particular in wound healing, is called for. It is notable that the majority of *in vitro* studies have focused on syndecan-4 signalling, whereas most *in vivo* studies have dealt with syndecan-1, leaving large sections of syndecan biology unresolved. Given the crosstalk and feedback that exists between signalling pathways, there is unlikely to be a single solution to

the challenge of enhancing repair processes *in vivo*, but the therapeutic implications of understanding receptor synergy make such investigations of utmost importance.

Glossary

Synergistic/ cooperative signalling	At least two molecules/receptors facilitate the transduction of a common signalling pathway (signalling convergence)
Functional synergy	At least two molecules/receptors transduce separate intracellular signals that regulate independent cellular processes, yet these processes synergise to modulate the same cellular event *in vivo*

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Text Box 1

Adhesion contacts

In most cells *in vitro*, adhesion signalling complexes are distributed focally rather than diffusely, and are manifested as asymmetric patches, flecks and stripes. These contact points are found all over the ventral surface of cells and are usually associated with the contractile polymers of the cytoskeleton. Detailed morphological and functional analyses in fibroblasts have defined three major forms of adhesion contact: focal complexes (FC), focal adhesions (FA) and fibrillar adhesions (FB)[115]. These contacts reflect different stages of interactions of cells with the ECM, and each is formed and disrupted in a cyclical manner as cells translocate. Initially, FC form at the posterior edge of ruffling membrane, where they anchor the short filopodial struts and lamellipodial meshes of actomyosin that mediate membrane protrusion. When protrusion ceases, or the lamellipodium retracts, FC transform into larger FA, which provide a more robust anchorage via transcellular, contractile actomyosin-containing stress fibres. In turn, FA evolve into centrally-located FB, which are the major sites of FN matrix deposition[116]. In keeping with their variable size, shape and location, there is some evidence for heterogeneity in the composition of adhesion contacts: FC are reported to lack zyxin[117], while FB lack phosphotyrosine and α_V integrins[118], but contain tensin. Adhesion contact-like structures have been observed *in vivo* in smooth muscle cell plaques and myotendinous junctions[119], and recently in embryonic 3D ECM[120], thereby validating the use of cell cultures for analysis. However, as cells are normally surrounded by an extracellular matrix in vivo, rather than being immobilised on a two-dimensional surface, it is likely that the relative proportions of different adhesion complexes will vary between cell types. In this context, analysis of embryonic fibroblasts revealed abundant fibrillar adhesions[120].

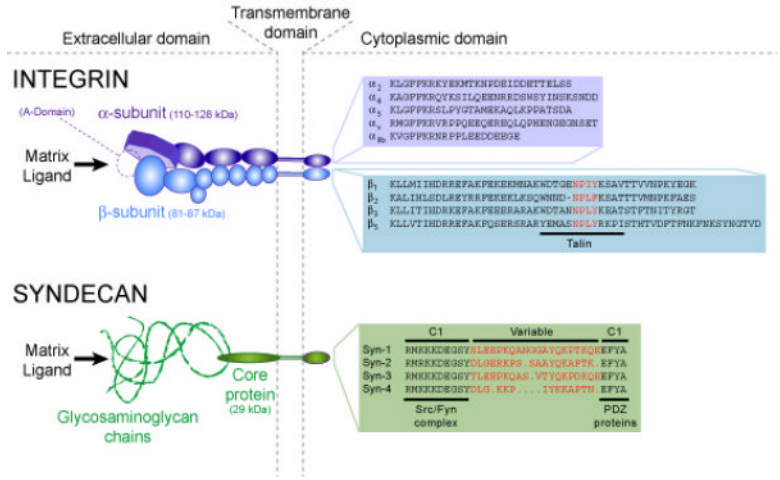

Figure 1. Domain structures of integrin and syndecan-4, transmembrane matrix receptors

Integrins consist of α,β heterodimers, comprising large extracellular and short cytoplasmic domains[121, 122].

The extracellular domain structure of each subunit is conserved between isoforms, with the exception of a subset of α-subunits (α_1, α_2, α_{10}, α_{11}, α_X, α_M, $\alpha_{L,}$ α_D and α_E) that include an inserted "A-domain" within the ligand binding pocket[2]. The cytoplasmic interactions of integrins are mostly mediated by the β-subunit tail, most notably by recruitment of the cytoskeletal protein talin[11]. Syndecans exist as homodimers and bind to the matrix through glycosaminoglycan chains substituted at 3-5 positions on the extracellular domain[60]. The short cytoplasmic domains can be subdivided into two conserved regions, C1 and C2, that bind a Src/Fyn tyrosine kinase complex and PDZ-domain-containing proteins, respectively, and a central variable region. The variable region confers specific properties on each syndecan, and most notably is a PKCα binding site in syndecan-4.

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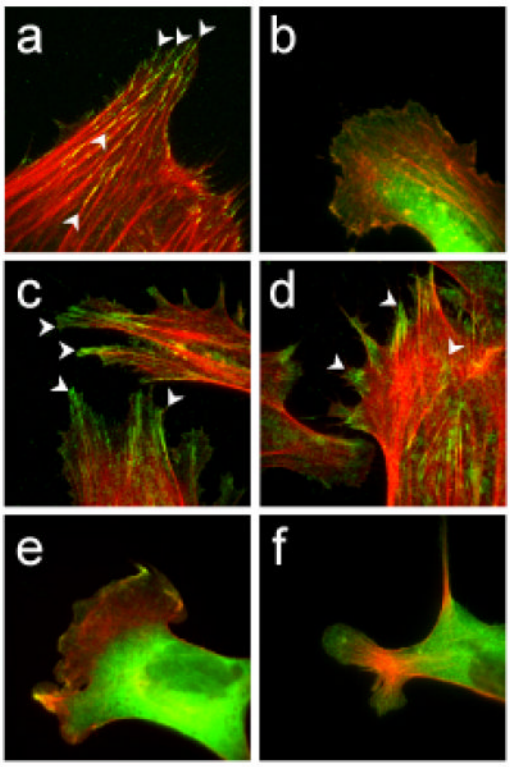

Figure 2. Adhesion formation is dependent on engagement of syndecan-4

Unlike cells plated onto fibronectin (a), cells plated onto an integrin ligand spread but fail to form FA (b) unless stimulated with an antibody against syndecan-4 (c) or a syndecan-binding fragment of fibronectin (d). Adhesion contact formation, in response to a syndecan-4 ligand, can be blocked by disrupting expression of syndecan-4 (e) or PKCα (f), highlighting the specific role of syndecan-4 in adhesion contact formation and the immediate relationship with classical adhesion signalling pathways. Images portray fixed fibroblasts stained for the adhesion marker vinculin (green) and actin (red), and arrows indicate focal adhesions.

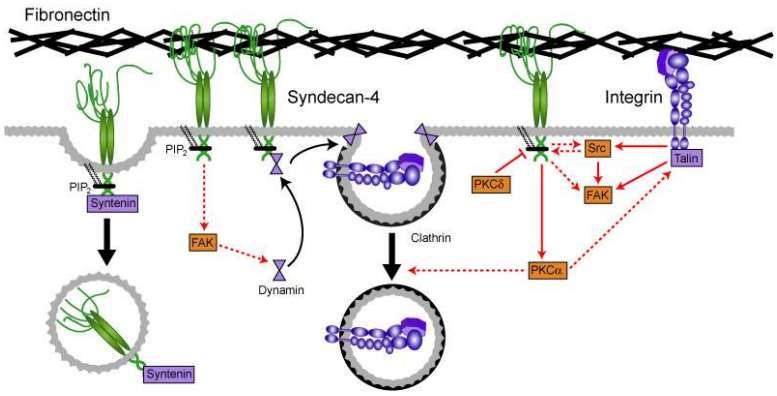

Figure 3. Protein kinase signalling depends on synergy between integrin and syndecan-4 and regulates endocytosis

Syndecan-4 regulates PKCα and tyrosine kinases, FAK and Src, in synergy with $\alpha_5\beta_1$ integrin. Activation of PKCα depends on direct association (solid arrows) with the syndecan-4 cytoplasmic domain and PIP_2, and can be prevented by the phosphorylation of syndecan-4 by PKCδ[15]. Both PKCα and FAK play key roles in cell migration due to the regulation of clathrin-dependent endocytosis of $\alpha_5\beta_1$-integrin, which is mediated by another syndecan-4-binding protein, dynamin[18]. The contributions of PKCα and FAK to integrin endocytosis are long-range effects (dotted arrows), and do not depend on direct association between $\alpha_5\beta_1$-integrin and syndecan-4. Endocytosis of the syndecan itself depends on association between the C terminus of the syndecan cytoplasmic domain and one of the paired PDZ domains of the small scaffolding protein syntenin[28].

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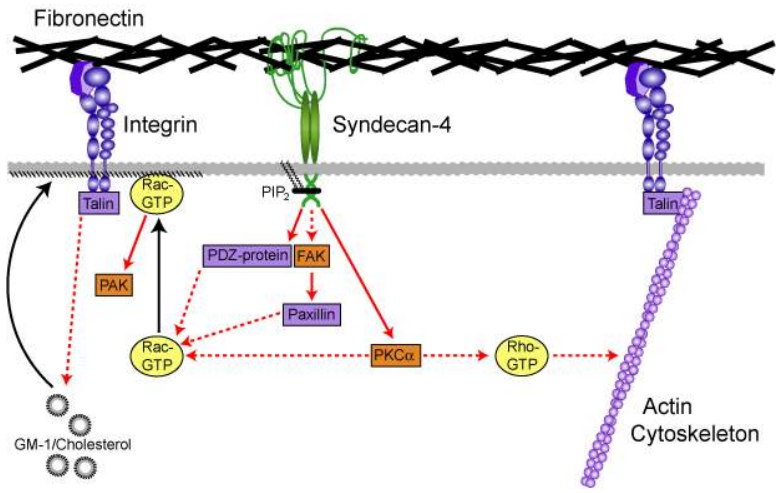

Figure 4. Adhesion-dependent GTPase signalling depends on synergy between integrin and syndecan-4

Efficient activation of the cytoskeletal regulators Rac and Rho, in response to fibronectin, depends on engagement of syndecan-4 by the ECM. Direct association (solid arrows) between PKCα and the cytoplasmic domain of syndecan-4 is necessary for GTP-loading of both Rac and Rho, and the activation of Rac also depends on the PDZ-binding motif of syndecan-4[37]. ECM engagement of integrins causes redistribution of cholesterol into detergent insoluble membrane microdomains, and is necessary for the redistribution of GTP-Rac to the membrane where it associates with downstream effectors, such as PAK[47]. It is the localised activation of Rac and Rho that initiates signalling cascades (dotted arrows) that result in the organisation of the actin cytoskeleton.

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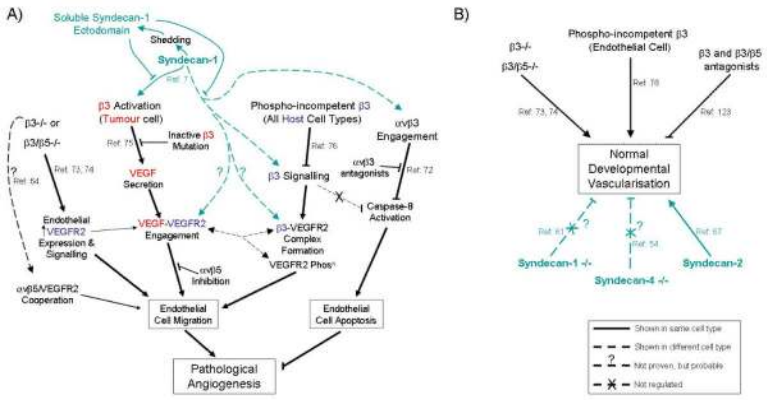

Figure 5. Interplay between β3 integrin, VEGFR and syndecan function in vascularisation

Perturbing $\alpha_v\beta_3$ function *in vivo* can have remarkably different effects on pathological angiogenesis (A) depending on the nature of the perturbation and the cell type expressing the integrin (tumour (Blue) vs. host (Red)). Antagonism of $\alpha_v\beta_3$, or expression of mutant β_3 subunits with compromised signalling capabilities, inhibits angiogenesis (seemingly through the modulation of different pathways), whilst β_3-integrin-deficient mice exhibit enhanced pathological angiogenesis. Further characterisation of these phenomena has demonstrated an important link between $\alpha_v\beta_3$ function and VEGF/VEGFR signalling. *In vitro* studies suggest that syndecan-1 has the capacity to differentially regulate this $\alpha_v\beta_3$ and VEGF/VEGFR signalling network.

There would also appear to be fundamental differences between $\alpha_v\beta_3$-dependent regulation of pathological angiogenesis (A) and developmental neovascularisation (B); inhibition of β_3 ligand-binding can disrupt developmental vascularisation whereas perturbation of β_3 expression or signalling has no effect. Syndecan-1 (and syndecan-4) deficient mice, like β_3-integrin null mice, appear developmentally normal, suggesting that they do not exhibit significant developmental angiogenic defects.

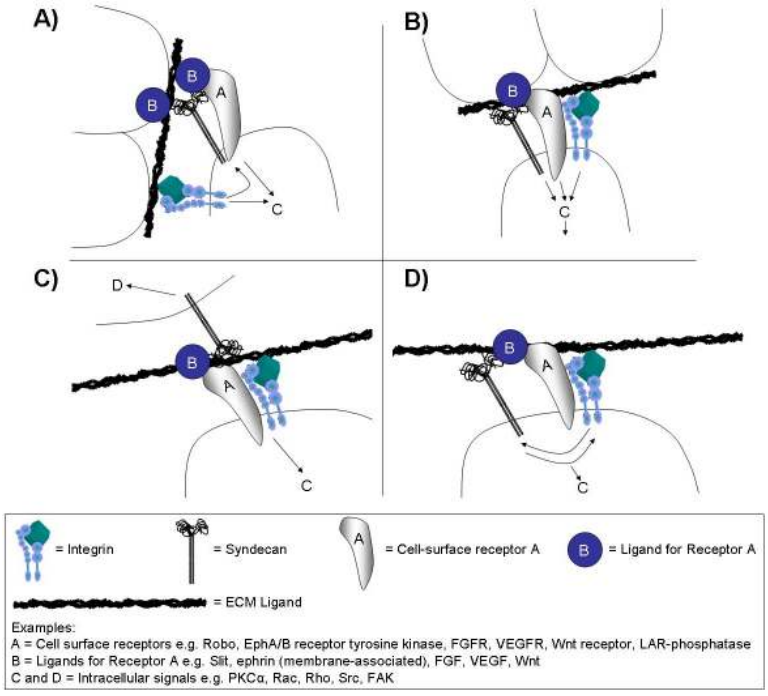

Figure 6. Models of the complexity of integrin-syndecan synergy *in vivo*

In each model, depending on the cellular context, molecules A, B, C and D may correspond to different proteins (see Key)

A) Ternary complex formation. Syndecan associates with cell surface receptor and its ligand (either soluble or membrane-bound), and generates intracellular signals. ECM engagement of integrins, and possibly syndecans, is required to potentiate the syndecan-mediated signals.

B) Multimeric complex formation. Cell surface receptors for chemoattractive, repulsive or growth factors are central to the formation of complexes with their ligands, syndecans, integrins and ECM molecules. Cooperative intracellular signals are generated from each receptor, initiating a coordinated cellular response.

C) Syndecan-mediated ligand-presentation in *trans*. Syndecan on an adjacent cell binds to and localises a soluble ligand, to allow interaction with its receptor (possibly in complex with an integrin). Such interactions could initiate both syndecan and integrin signalling events. Integrin and syndecan activity in this model may or may not require association with ECM molecules

D) Syndecan-mediated ligand-presentation in *cis*. Syndecans, viewed as pioneering molecules, bind to and localise chemoattractive/repulsive/growth factors, to allow interaction with their receptors and integrins on the same cell. Collaborative intracellular signals may be elicited from both syndecans and integrins in such a model, but may also require interaction with ECM molecules