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Syndecan-4: Dispensable or indispensable?

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Studies examining the role of the cell-surface heparan sulfate proteoglycan syndecan-4 have yielded a plethora of information regarding its role in both cell-matrix and growth-factor mediated signaling events. Many of the initial conclusions drawn from such research placed syndecan-4 in a keystone position within various signaling pathways though the generation of syndecan-4 null mice have surprised many in the field by indicating otherwise. These contradictory results place researchers in the frustrating and yet exhilarating position of still asking the question, "What role does syndecan-4 play in life?"
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Introduction

When Merton Bernfield described the first syndecan family member [1], he initiated the formation of a complex and often contradictory field in cell biology. Biologists who study the syndecans may likely feel akin to the Indian folktale of 6 blind men and the elephant (poetized by John Godfrey Saxe [2]). Each laboratory is able to conclusively show an interaction or activation/inhibition that occurs optimally in the presence of a syndecan and results in a dramatic change in cell behavior-but linking these observations together to make a coherent "animal" is much more difficult. To add to this confusion, the generation of knock-out mice for 3 of the 4 family members (syndecan-1, -3 and -4) have not easily rectified this problem. Syndecan-3 deficient mice exhibit slight defects in the hippocampus but are otherwise healthy [3,4] and neither syndecan-1 nor syndecan-4 null mice display overt morphological defects upon initial observation [5,6].

Given the enormous volume of research others and we have dedicated to this field, the lack of a dramatic effect upon gene deletion was surprising. The results led many to the thought exemplified by the title—are these molecules, in our case syndecan-4 in particular, dispensable as the *in vivo* data would initially suggest, or indispensable, as attested to by years of biochemical and molecular study. We believe, not without a little bias, that although syndecan-4 may not be necessary for life it is very important for survival. Only time will tell if our hypothesis holds and if it is applicable to the other syndecan family members. In the meantime we are grateful to Merton Bernfield for his legacy-for discovering an "elephant" large enough to allow all of us to actively pursue our observations for many years to come.

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HSPG to syndecan-4 structure

The interaction of cells with their surrounding environment can contribute to modifications in cell proliferation, migration, morphogenesis and survival. The factors that trigger changes in cell behavior may be soluble (such as growth factors or chemokines) or insoluble (components of the extracellular matrix). The members of a superfamily that allow cells to interact with both types of factors are the heparan sulfate proteoglycans (HSPGs). These localize to either the basement membrane (perlecan and agrin) or the cell surface (syndecan and glypican families) [7] and consist of glycosaminoglycan (GAG) chains covalently bound to a protein core. The heparan sulfate chains are composed of alternating disaccharide residues (glucuronic acid or iduronic acid with glucosamine) that are post-translationally modified by acetylation, epimerization and sulfation in sub-domains that occur along the length of the carbohydrate chain [8].

The two subfamilies of cell-surface HSPGs are structurally quite dissimilar. The six members of the glypican family are glycosylphosphatidylinositol-linked to the cell membrane to which the GAG attachment sites are closely adjacent. Fourteen conserved cysteine residues that promote the formation of a structurally conserved compact and globular shape make up the N-terminal distal portion of the core protein [9,10].

The syndecan family (syndecans-1, -2, -3 and -4) is characterized by a core protein that is believed to be highly extended due to a large number of proline residues [11]. Although the extracellular domain of each family member is divergent except at sites of GAG attachment, all four proteins share a highly conserved transmembrane domain and two constant regions separated by a unique variable region within the cytoplasmic domain. At the terminus of the cytoplasmic domain is an invariant amino acid sequence (EFYA) that comprises a binding site for PDZ (post synaptic density-95, discs-large-1 and zonulin-1) domain-containing proteins. The first syndecan-binding protein to be identified, syntenin, contains a tandem repeat of PDZ domains and both domains act synergistically for optimal syndecan binding [12,13]. Studies indicate that syntenin is associated with cellular membranes, localizing to sites of nascent cell-cell contact as well as early sites of cell-substrate adhesion and endocytic vesicles [14,15]. Its prominent distribution in many subcellular compartments suggests that it may serve to promote the correct recycling or localization of receptor and cytoskeletal-associated proteins [14,15]. CASK/LIN-2, another PDZ domain-containing protein, is a member of the membrane-associated guanylate kinase family that has been shown to bind both syndecans and the actin/spectrin binding protein 4.1 indicating that it may act as a scaffolding protein that localizes proteins to the proper cell membrane domain [16]. Indeed, expression of CASK during the postnatal development of the rat brain shifts from an axonal distribution (where it colocalizes with syndecan-3) to a somatodendritic distribution that corresponds with syndecan-2 expression [17,18]. Synectin was isolated as a syndecan-4 binding protein and found to be widely distributed in tissues and adherent cell lines [19]. Immunoprecipitation experiments demonstrated an indirect association of protein kinase C-α (PKC-α) with synectin, presumably via syndecan-4 acting as a linker, suggesting that a signaling complex containing all three proteins may exist *in vitro*. Interestingly, activation of PKC by phorbol myristate acetate (PMA) induces expression of synectin in U937 suspension cells as well as an adherent phenotype, and transfection of synectin in ECV304 cells results in impaired migration in *in vitro* wound healing assays [19]. The most recent protein to be isolated, synbindin, does not contain any classical PDZ domains though a stretch of 60 amino acids near the N-terminus is homologous to several PDZ domain-containing proteins [20]. Binding of synbindin to syndecan-2 restricts its localization to dendritic spines where it may play a role in membrane trafficking in postsynaptic sites [20]. The extensive overlap of these proteins for a binding site on syndecan family members suggests that a higher level of regulation must exist to prevent inappropriate signaling events from occurring. Indeed, the various syndecans as well as their potentially interacting PDZ-containing partners seem to be limited by the cell-specific coordinated expression or sub-cellular localization of both [15,18,20].

Structural specificity of each syndecan family member is conferred by the extracellular domain and the unique variable region located within the short cytoplasmic domain. The extracellular domain of the syndecan-4 core protein contains a cell-binding region (corresponding to amino acids 56-109 of the mouse protein) that mediates fibroblast attachment and cannot be competed for by other family members [21,22]. Intracellularly, syndecan-4 has been shown to specifically bind two cytoplasmic proteins. Syndecan-4 binds PKC-α through the intermediary phosphatidylinositol-4,5-bisphosphate at the variable region [23–26] and the cytoplasmic protein syndesmos through both the variable and membrane-proximal constant regions [27]. Although syndesmos binding also incorporates one of the constant regions shared by all four syndecans, it does not ligate syndecans 1–3 indicating the contributory importance of the variable region for binding.

Syndecan-4 expression

Syndecan expression patterns are highly regulated in tissues and during development. Although most cells express more than one form of syndecan, stereotypically syndecan-1 is the predominant form of epithelial cells, syndecan-2 of fibroblasts and syndecan-3 of neural tissue. Syndecan-4 is unique in that it is the most ubiquitious of all the syndecans although it is expressed at lower levels [9,28–31].

Expression of syndecan-4 has been found in all stages of embryonic development and in most adult tissues [32,33] though it is not necessary for viability [6]. Beta-galactosidase expression driven by the syndecan-4 promoter in syndecan-4 null mouse embryos indicates that syndecan-4 is expressed in vertebrae, ribs, skull base, hair follicles, whiskers, salivary glands, kidney, lung, heart and urinary bladder [32]. Adult tissue expresses high levels of syndecan-4 in liver, kidney and lung while the heart and brain show moderate expression levels [33,34].

Syndecan-4 has been shown to act as an immediate-early gene in smooth muscle cells following changes in mechanical stress [35]. Cells exposed to cyclic strain for increasing lengths of time display a rapid upregulation of syndecan-4 mRNA. In addition, a change in its subcellular distribution and enhancement of its shedding from the cell surface is also observed. This modification is correlated with an increase in cell motility suggesting that syndecan-4 localized to the plasma membrane acts as a negative regulator of smooth muscle cell migration.

Alterations in syndecan-4 expression are associated with woundhealing. Studies have documented increases in its cell-surface expression in response to exposure to the proline-rich antimicrobial peptide PR-39 [36,37] which induces changes in the rate of cell motility [38,39]. The syndecan-4 extracellular domain is shed from the cell surface and this activity is upregulated in acute dermal wound fluid by a metalloprotease which is sensitive to a tissue inhibitor of metalloprotease-3 (TIMP-3) following stimulation by PKC, plasmin, thrombin or epidermal growth factor [40,41]. The shed syndecan-4 ectodomain has been shown to serve as an effector in maintaining the proteolytic balance in the inflammatory environment by binding

proteases and protecting them from protease inhibitors [42]. Muscle satellite cells, which repair damaged skeletal muscle tissue, express syndecan-4 [43]. Inhibition of GAG synthesis attenuates satellite cell activation, proliferation and differentiation suggesting that syndecan-4 may help regulate signaling events associated with muscle cell regeneration [43]. A potential role of syndecan-4 in healing has also been demonstrated following myocardial infarction. Syndecan-4 expression is increased in response to hypoxia in cardiac myocytes [44,45] and is localized to the repair region of damaged cardiac tissue [46].

Collectively, these studies suggest that syndecan-4 is important in combating physiological stresses and/or maintaining homeostasis. Our data from recently generated syndecan-4-deficient mice confirm this [6]. Mice null for the syndecan-4 core protein are viable, fertile and show no overt morphological defects. However, upon subjecting the mice to various physiological stresses a phenotypic deficiency is uncovered. Syndecan-4 null mice exhibit a delay in the rate of healing of excisional wounds and a defect in angiogenesis of the granulation tissue characterized by an alteration in the size of the infiltrating blood vessels. Dermal fibroblasts isolated from the syndecan-4 knockout mice migrate slower than their wildtype counterparts in *in vitro* wound healing studies suggesting that cell motility is adversely affected by the loss of syndecan-4.

Renal function studies demonstrated increased mortality in syndecan-4 null mice following κ-carrageenan induced obstructive nephropathy [47], see accompanying article by Ishiguro et al. Examination of the syndecan-4 deficient mice following treatment indicated a substantial increase in blood urea nitrogen levels, dilation of renal tubules and degeneration of the inner medulla. Lipopolysaccharide-induced septic shock also increased the mortality of syndecan-4 null mice [48], see accompanying article by Ishiguro et al. Both systolic blood pressure and left ventricular fractional shortening were greatly reduced and inhibition of IL-1 β production by transforming growth factor-β (TGF-β) was impaired, indicating a role for syndecan-4 in promoting survival following lipopolysaccharide-induced sepsis.

Syndecan-4 and focal adhesions

Syndecan-4 is the only syndecan family member that localizes to focal adhesion sites in fibronectin-adherent cells [49,50]. Its specific role in cell adhesion was unclear until our subsequent research demonstrated that fibroblasts adherent to the cell-binding domain of fibronectin (which contains only the RGD integrin binding site) will generate focal adhesions and actin stress fibers following incubation with antibodies to the extracellular domain of syndecan-4 [51]. Furthermore, the syndecan-4 dependent formation of focal adhesions and stress fibers acts via a Rho-dependent mechanism [51]. Interestingly, lack of syndecan-4 signaling (due to adhesion only to the cell-binding domain) stimulates cell entry into a caspase-mediated apoptotic pathway [52] suggesting that ligation of syndecan-4 to fibronectin contributes to cell survival.

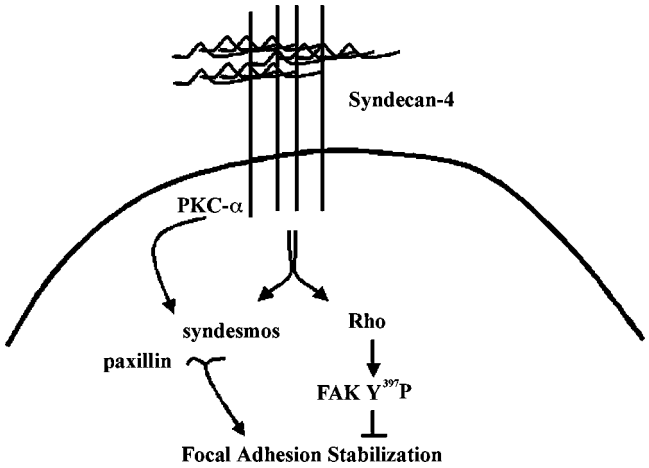

Figure 1. Syndecan-4 signaling modulates focal adhesion stability. (Left side) Clustering of syndecan-4 activates PKC-α and also promotes syndesmos localization to the plasma membrane. PKC-α activation enhances the interaction between syndesmos and paxillin thereby stabilizing focal adhesions. (Right side) Syndecan-4 signaling increases the phosphorylation of FAK at tyrosine-397 through a Rho-dependent mechanism. Activation of FAK inhibits focal adhesion stability by facilitating turnover. How these two pathways are temporally regulated is currently unknown.

Syndecan-4-mediated stress fiber and focal adhesion formation could potentially be due to changes elicited either by the heparan sulfate chains or the protein core. GAG chains from syndecan-4 and glypican-1 exhibit some minor structural differences but both bind to the Hep II domain of fibronectin similarly [53]. Studies which have generated syndecan-glypican chimeras to assess the relative contributions of either the GAG chains or the protein core have demonstrated that signals elicited by syndecan ligation cannot be mimicked by glypican [54,55] implying that signaling events are transduced through the protein core. In support of this, our studies utilizing both parental CHO-K1 cells and the derived CHO-745 cells, that lack xylosyltransferase which is necessary for transferring the initial xylose residue needed to initiate GAG chain addition, show increased focal adhesion and stress fiber formation following transfection of the syndecan-4 core protein [56]. Transfection of syndecan-4 constructs containing a partial or full deletion of the cytoplasmic domain into CHO-K1 cells results in a decrease in stress fiber organization and focal adhesion formation [57]. These data imply that a direct interaction between the cytoplasmic domain of syndecan-4 and other cytoplasmic signaling components must occur. Indeed, we have found that syndecan-4 binds selectively to the cytoplasmic protein syndesmos and over-expression of syndesmos enhances cell spreading [27]. Recently we have shown that syndesmos also binds the focal adhesion adaptor protein paxillin [58] indicating a mechanism by which syndecan-4 may be linked to focal adhesion-associated machinery (Figure 1).

Syndecan-4 and cell migration

Fibroblasts isolated from syndecan-4 null mice will develop focal adhesions and actin stress fibers when plated on a fibronectin substrate ([6,59], see accompanying article by Ishiguro et al.). This result indicates that a compensatory mechanism (presumably another syndecan family member) exists to generate focal adhesions and stress fibers in the absence of syndecan-4. Activation of the specific syndecan-4 mediated pathway in the syndecan-4 null fibroblasts does not stimulate focal adhesion and stress fiber formation [59]. This indicates that syndecan-4 still plays a role in focal adhesion formation, although it may serve a specialized function in this process.

Syndecan-4 serves not only to promote the formation of focal adhesions but also may facilitate their turnover. We have found that syndecan-4 null fibroblasts migrate more slowly in *in vitro* wound healing assays compared with their wildtype counterparts [6]. Recently lack of syndecan-4 has been shown to correspond with a reduction in the phosphorylation level of focal adhesion kinase (FAK) tyrosine-397 [60]. Fibroblasts generated from FAK null embryos have demonstrated that FAK expression is required for focal adhesion turnover [61] and that phosphorylation of tyrosine-397, in particular, is necessary for optimal cell migration [62]. Syndecan-4 mediated phosphorylation of FAK is dependent on the activity of the small GTPase Rho. The levels of GTP-bound Rho are significantly diminished in syndecan-4 null fibroblasts suggesting that syndecan-4 ligation either activates Rho or maintains it in an active conformation [60].

Given these data, the story of syndecan-4 mediated migration is not just a simple "on" (migratory phenotype) versus "off" (stationary phenotype) scenario. Not only does the loss of the syndecan-4 gene diminish cell migration, overexpression of both the full-length or cytoplasmically deleted syndecan-4 core protein in CHO-K1 cells also impairs migration [57]. These results suggest that under basal conditions an optimal level of cell surface syndecan-4 is required for efficient cell migration. Thus too much syndecan-4 generates an enhanced adhesive phenotype that prevents migration while loss of syndecan-4 results in the failure of cytoplasmic signaling mechanisms to be activated thus impairing focal adhesion turnover (Figure 2). Other studies point to syndecan-4 acting to enhance or inhibit regulated cell migration. Blockage of syndecan-4 ligation by tenascin-C increases glioblastoma and breast carcinoma cell migration on fibronectin [63]. Transfection of syndecan-4 full-length or of a chimera containing the syndecan-4 cytoplasmic domain enhances migration and tubule formation in ECV304 endothelial cells [55]. In neutrophils, lymphocytes and monocytes, where syndecan-4 acts as an antithrombin receptor [39,64], ligation of antithrombin III to syndecan-4 inhibits chemokine mediated chemotaxis while enhancing antithrombin III directed cell migration [64,65]. The selective cellular response to migration following ligation of syndecan-4 (as in the case of the peripheral blood leukocytes) implies that syndecan-4 initiated intracellular signaling events do not act solely in a linear manner but have the capability to influence other receptor-mediated signaling pathways.

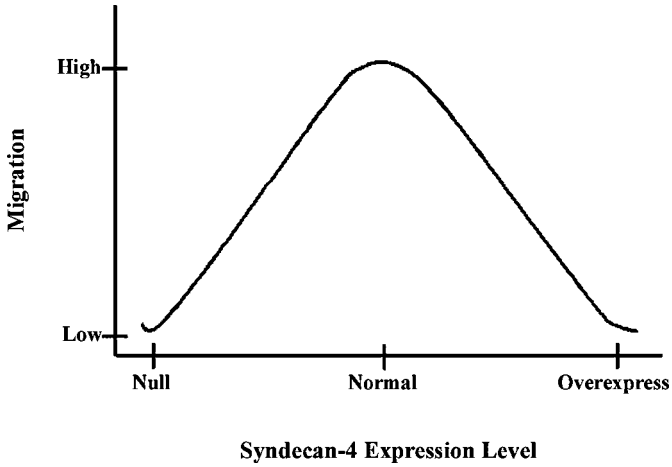

Figure 2. Theoretical model of effect of syndecan-4 on cell migration. Optimal cell migration occurs in the presence of a "normal" level of cell-surface syndecan-4. Too little (Null) or too much (Over-express) diminishes the ability of the cell to efficiently migrate as described in the text.

Syndecan-4 and PKC

Cell spreading and focal adhesion formation not only relies on syndecan-4 mediated Rho GTPase activity but also on the activation of PKC [66]. Although studies demonstrated that both PKC and syndecan-4 localize to cell-matrix adhesion sites [49,67], neither study linked the two molecules into a single signaling pathway. Definitive proof that PKC regulated syndecan-4 localization to focal adhesions was demonstrated when syndecan-4 was observed to redistribute to the focal adhesions of quiescent fibroblasts following treatment with phorbol myristate acetate [50]. Subsequently PKC and syndecan-4 have been shown to be intimately associated. Syndecan-4 binds PKC-α, through the binding intermediary phophatidyl inositol-4,5-bis phosphate at the variable region in the cytoplasmic domain [23,25]. Ligation to syndecan-4 potentiates PKC-α activity and reduces the requirement for calcium [23,24,26]. Interestingly, monomeric syndecan-4 is not capable of potentiating PKC-α activity. Peptides made of the syndecan-4 variable region will self-oligomerize and the degree of oligomerization parallels the ability of the peptides to activate PKC-α [25]. Peptides made to the full cytoplasmic domain of syndecan-4 do not form higher-level oligomers unless incubated in the presence of phophatidyl inositol-4,5-bis phosphate [25].

The capability of syndecan-4 to potentiate PKC-α activity is not without regulation. PKC-dependent activation of a tyrosine kinase increases the shedding of syndecan-4 from the cell surface [41]. This matrix metalloprotease dependent process cleaves the proteoglycan at the juxtamembrane region, generating a soluble effector that maintains all extracellular binding attributes [40,41]. Increased loss of syndecan-4 is associated

with cellular responses to stress (i.e., hyperosmolarity, heat shock and ceramide treatment) [41] indicating a potential role of syndecan-4 in helping to return to a balanced homeostatic state following physiological disruption.

Horowitz and Simons [68] demonstrated that phosphorylation of serine-183 on the syndecan-4 cytoplasmic domain reduced the ability of syndecan-4 to oligomerize and to potentiate PKC-α activity without diminishing its ability to bind to the kinase. Analysis of the location of serine-183 in relation to the phophatidyl inositol-4,5-bis phosphate binding site suggested that phosphorylation of this serine residue interferes with the binding affinity of syndecan-4 for phophatidyl inositol-4,5-bis phosphate [68]. Further examination indicated that another PKC isoform, PKC-δ, is responsible for phosphorylating serine-183 [69].

Given that all of these data indicate a close and complex relationship between syndecan-4 and PKC, many researchers are now addressing what possible roles they play within the cell. The slowly emerging answers are as convoluted as the pathway itself. The association of PKC activation with both focal adhesion formation and the localization of syndecan-4 to more established adhesion sites, and not nascent contacts, indicates that these molecules may act to maintain cell-matrix adhesions. In support of this, we have shown that the syndecan-4 binding protein syndesmos also binds the focal adhesion adaptor protein paxillin upon PKC activation [58]. Cells isolated from paxillin null embryos exhibit delayed cell spreading and abnormal focal adhesion formation [70,71] whereas syndesmos enhances cell spreading, focal adhesion and actin stress fiber formation when overexpressed [27]. Thus a likely scenario could be that clustering of syndecan-4 leads to localization of syndesmos to the cell periphery and the activation of PKC which would subsequently enhance the association of syndesmos with paxillin facilitating cell spreading and focal adhesion formation. This pathway is separate from the likely role of syndecan-4 in the turnover of focal adhesions, as PKC activation did not increase the level of phosphorylation of FAK tyrosine-397 [60] (Figure 1).

The mechanism by which this pathway is initiated may be through the FGF-2 receptor. Incubation of cells with FGF-2 results in the activation of a serine/threonine phosphatase type 1/2A which dephosphorylates syndecan-4 at serine-183 [68,72]. Dephosphorylation allows for the subsequent activation of PKC-α by syndecan-4/phophatidyl inositol-4,5-bis phosphate as described above [68,69,72]. Endothelial cells incubated with FGF-2 show an increase in cell migration, proliferation and tubule formation [72] that can be inhibited upon transfection of the cells with either a constitutively active PKC-δ, a dominant negative PKC-α or a syndecan construct mutated at the phophatidyl inositol-4,5-bis phosphate binding site [69,72].

Syndecan-4 and growth factors

Both extracellular and cell-surface heparan sulfate proteoglycans have been shown to bind heparin-binding growth factors (such as the fibroblast growth factor (FGF) superfamily, hepatocyte growth factor (HGF) and heparin-binding isoforms of vascular endothelial growth factor (VEGF)). In addition to protecting growth factors from proteolysis and thermal denaturation [73,74], proteoglycan ligation can help direct the binding of growth factors to their primary receptors [75–80] and, therefore, regulate potential downstream physiological responses [65,79,81–85]. Adding to the complexity, HSPGs that normally enhance growth factor signaling when membrane-bound may act in an inhibitory manner when soluble [86,87].

Many studies have been devoted to elucidating the role of syndecan-4 in FGF-2-mediated signaling. Syndecan-4 binds FGF-2 directly and increases the affinity of FGF-2 for FGF-receptor-1 when co-immobilized with the receptor in cell-free binding assays [79,88]. Richardson et al. [89] demonstrated that FGF-2 mediated proliferation is dependent on cell density in stromal fibroblasts and this correlates with the level of syndecan-4, and not of the FGF-receptor, expressed on the cell surface. ECV304 endothelial cells transfected with various glypican-1 and syndecan-4 constructs increase the number of low-affinity FGF-2 binding sites on the cell surface but only respond physiologically to FGF-2 (enhanced growth and migration) when the transfection is of cDNA for full-length syndecan-4 or a construct containing the syndecan-4 cytoplasmic domain [55].

The ability of syndecan-4 to cluster, in the absence of fibronectin ligation, may also be dependent on FGF-2 binding. Whereas syndecan-4 is present in non-raft fractions of the cell membrane under quiescent conditions, it translocates to lipid rafts upon FGF-2 induced clustering [90]. These lipid rafts do not contain caveolae, as designated by the absence of co-localization with the marker caveolin-1, but the authors noted a parallel relocalization of syndecan-4 and caveolin-1 [90]. The purpose of the coordinated movement is currently unclear. However, heparin-binding growth factor receptors such as those for FGF, platelet-derived growth factor, and vascular endothelial growth factor are found in caveolae [91–93] so syndecan-4 may serve to transport the ligand to its primary receptor. Alternatively, localization to membrane subdomains may allow syndecan-4 to interact with additional signaling components. PKC-α translocates to caveolar compartments upon activation whereby it can stimulate downstream signaling pathways [94,95]. Mutation of either the phophatidyl inositol-4,5-bis phosphate binding site or PDZ domain on syndecan-4 results in an attenuation of FGF-mediated signaling events due to a decrease in the syndecan-4 dependent activation of PKC-α [72]. Therefore, the localization of syndecan-4 to areas adjacent to caveolae may facilitate its ability to bind and activate PKC-α upon dephosphorylation of serine-183.

Studies have not yet explored the FGF-2 signaling pathway in syndecan-4 null mice but one study has evaluated FGF-2 mediated nitric oxide release in cardiac myocytes isolated from transgenic mice carrying rat syndecan-4 cDNA [35]. The presence of the syndecan-4 transgene enhanced nitric oxide production

and vasodilation following FGF-2 stimulation compared with controls [35]. Interestingly, myocytes do not normally generate syndecan-4 so their responsiveness would be dependent on the presence of proteoglycans shed from the surface of neighboring endothelial cells. Shedding of syndecan-4 is accelerated during the process of wound repair [40] and, in this regard, would serve as a paracrine modulator of FGF-2 regulated signaling.

Conclusion

Many recent studies have helped in the elucidation of the role of syndecan-4 in focal adhesion formation, cell migration and growth factor signaling. The answer to its "indispensability" is still unknown. This enigmatic molecule seems to serve as a mechanism to facilitate physiological responses to various stresses under acute conditions as well as to enhance or "make more efficient" more routine signaling pathways. Given that, its presence may not lead to the activation of the same downstream effectors and its absence under static conditions is not detrimental. The characterization of the recently generated syndecan-4 null mice will help further the understanding of this complex molecule.

Acknowledgments

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