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Syndecan-2 regulation of morphology in breast carcinoma cells is dependent on RhoGTPases ☆

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ABSTRACT

Background: While syndecan-2 is usually considered a mesenchymal transmembrane proteoglycan, it can be up-regulated in some tumour cells, such as the malignant breast carcinoma cell line, MDA-MB231. Depletion of this syndecan by siRNA, but not other syndecans, has a marked effect on cell morphology, increasing spreading, microfilament bundle and focal adhesion formation, with reduced cell migration.
Methods: A combination of siRNA transfection, immunofluorescence microscopy, phosphoprotein analysis and migration assays was used to determine how syndecan-2 may influence the cytoskeleton.
Results: The altered adhesion upon syndecan-2 depletion was dependent on the RhoGTPases. p190ARhoGAP relocated to the margins of spreading cells, where it codistributed with syndecan-4 and active β_1-integrin. This was accompanied by increased RhoGAP tyrosine phosphorylation, indicative of activity and RhoGTPase suppression. Consistent with this, GTP-RhoA was strongly present at the edges of control cells, but lost after syndecan-2 reduction by siRNA treatments. Further, RhoA, but not RhoC was shown to be essential for the anchored phenotype of these breast carcinoma cells that accompanied siRNA-mediated loss of syndecan-2.
Conclusions: Syndecan-2 has a key role in promoting the invasive activity of these cells, in part by regulating the RhoGTPases.
General significance: Syndecan-2, as a cell surface receptor is accessible for targeting to determine whether breast tumour progression is altered. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

The syndecan proteoglycans are an evolutionarily ancient family, with four members in mammals. They are widespread, and since all can interact with the actin cytoskeleton, have been implicated in cell adhesion, migration and extracellular matrix assembly [1,2]. On their ectodomains are multiple glycosaminoglycan chains, usually heparan sulphate. This endows syndecans with an ability to interact with a large number of proteins that have heparin- or heparan sulphate-binding properties. These include many growth factors, such as fibroblast growth factors, morphogens, cytokines, chemokines, enzymes such as metalloproteinases and lipases, as well as extracellular matrix glycoproteins and collagens. With such a diverse array of binding molecules, it is apparent that numerous inputs through syndecans may lead to a conservative cytoplasmic output.

Nevertheless, specificity among syndecans has been recorded on many occasions. Syndecan-4, for example, can promote focal adhesions and its loss in the knock-out mouse leads to tissue repair and migration deficits that are not overcome by other members of the family [3,4]. In addition, syndecan-1 expression in breast cancer is associated with poor prognosis, especially where it is expressed in the tumour stroma [5,6]. The same is not true for syndecan-4 [6]. Therefore, while having common features, such as heparan sulphate chains and linkage to the cytoskeleton, there is specificity in function across the syndecans, even where multiple members of the family are expressed on the surface of a single cell type. This specificity is not well understood.

In our recent studies of the triple negative breast carcinoma cell line, MDA-MB231, lacking oestrogen receptor α, progesterone receptor and the Her2/Neu receptor, we noted the presence of syndecans-1, -2 and -4 [unpublished results]. Experiments with exogenous heparan sulphate or heparin treatment, and specific depletion of syndecans by siRNA revealed specific and important properties for syndecan-2. When depleted, these highly invasive cells spread, formed cell–cell and cell–matrix junctions, microfilament bundles and were markedly reduced in their ability to invade and degrade native type I collagen [unpublished results]. It was further shown that the microfilament bundles appeared to form in a conventional signalling pathway involving Rho kinases and elevated phosphorylation of myosin light chain [7,8]. Syndecan-2, however, is poorly understood in terms of its signalling capacities. Its cytoplasmic domain can interact with PDZ domain proteins that are probably involved in trafficking to or from the cell surface [9]. It can also interact with ezrin, but these properties are common to all

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syndecans [10]. Preliminary data suggest that the effect of syndecan-2 depletion in MDA-MB231 cells was specific to this syndecan and not reproduced by reductions in either syndecan-1 or -4 [unpublished results]. As with other syndecans, association with integrins and integrin-mediated adhesion has also been recorded in various cell types, but this is not fully understood in molecular terms.

Given the striking change in behaviour of MDA-MB231 cells when depleted of syndecan-2, this proteoglycan deserves further scrutiny. While we showed an association with, and regulation of, caveolin-2 in these carcinoma cells, it remains unclear how syndecan-2 effectively suppresses the spreading and adhesion functions that are released once the proteoglycan has been depleted. Here, we determine the features of the spreading and adhesion formation, with emphasis on the RhoGTPases that are well known to influence many aspects of adhesion and locomotion.

2. Materials and methods

2.1. Cell culture

The human breast carcinoma MDA-MB231 line was maintained in Dulbecco's modified Eagle's media (Invitrogen) containing 10% foetal bovine serum (FBS) at 37 °C and 5% CO_2. Cultures were tested routinely for mycoplasma. Cells were transfected with siRNA targeting syndecan-2 (Santa Cruz Biotechnology), syndecan-4 (siGENOME SMARTpool, Thermo Scientific), p190RhoGAP A and B (siGENOME SMARTpool, Thermo Scientific), RhoA (Qiagen), RhoC (Qiagen) or non-targeting siRNA (siGENOME SMARTpool, Thermo Scientific) using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

2.2. Antibodies

The following antibodies were used: syndecan-2 (cat. H00006383-B04P; Abnova); syndecan-4 (cat. LS-C150078; LSBio), p190ARhoGAP (cat. 610150), p190BRhoGAP (cat. 611613) and p120RasGAP (cat. 610040) from BD Bioscience; RhoA-GTP (cat. 26904; Neweast Biosciences), RhoC (cat. 3430) phospho-Src (Tyr 416; cat. 2101) and phospho-(Ser) PKC substrate (P-S^3-101) (cat. 2261) from Cell Signaling; active β_1-integrin (clone HUTS-4, cat. MAB2079Z), β_1-integrin (cat. AB1592) and paxillin (clone 5H11, cat. no. 05–417) from Millipore; β-tubulin (clone TUB2.1, cat. no. T4026) from Sigma-Aldrich; Alexa Fluor-conjugated and phalloidin and Alexa Fluor-conjugated secondary antibodies used in immunofluorescence staining were obtained from Molecular Probes, Invitrogen; and peroxidase-conjugated secondary antibodies used in western blotting analysis were from Dako.

2.3. Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde/PBS and permeabilised with 0.1% Triton X-100/PBS for 10 min. This was followed by incubation in 0.1 M NH_4Cl for 20 min to quench free aldehydes and blocked with 5% heat-denatured bovine serum albumin for 30 min. Cells were incubated with indicated primary antibodies overnight at 4 °C, followed by incubation with appropriate Alexa Fluor-conjugated antibodies and/or Alexa-conjugated phalloidin for 1 h at room temperature. Coverslips were mounted with Prolong Gold mounting media (Invitrogen) and viewed on a Zeiss Axioplan-2 microscope (Carl Zeiss) using an Aprochromat 63× objective and analysed with MetaMorph software (version 6.2r6).

2.4. Western blotting, immunoprecipitation and Rho-GTPase pulldown assay

Cells were directly lysed using sample buffer before subjected to SDS–polyacrylamide gel electrophoresis and followed by electrophoretic transfer and western blotting. For p190RhoGAP immunoprecipitations, transfected cells were lysed with RIPA buffer containing 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, phosphatase inhibitors (Sigma) and protease inhibitors (Roche). The lysates were centrifuged for 5 min then precleared with protein G-agarose beads for 30 min at 4 °C before incubation with 5 μg p190ARhoGAP antibody for 1 h at 4 °C. This was followed by further incubation with protein G-agarose beads (EZview Red, Sigma) for 1 h at 4 °C before elution with sample buffer. All the samples were analysed by western blotting for phospho-tyrosine and p120RasGAP. The blots were stripped for subsequent p190ARhoGAP detection. In certain experiments, transfected cells were further treated with 10 μM of Src kinase inhibitor I (SrcI1) for 2 h before lysis. GST-Rhotekin agarose beads were prepared and Rho-GTP pulldown assays were performed as previously described [11].

2.5. Flow cytometry analysis

Transfected cells were incubated with dissociation buffer (Invitrogen), then suspended in ice-cold sterile 1% BSA/PBS and incubated with syndecan-2 antibody (1:75) or syndecan-4 antibody (1:100) for 1 h on ice. For active β_1-integrin detection, the cells were fixed with 1% paraformaldehyde/PBS for 7 min and followed by PBS washing before antibody (1:100) incubation for 1 h on ice. Incubation of appropriate Alexa Fluor-conjugated secondary antibodies for 30 min was carried out on ice. The stained cells were analysed on a FACSCalibur flow cytometer and data processed by using CellQuest Pro v6.0 software (Becton Dickinson).

2.6. Cell migration assays

The transwell inserts (12-well insert; pore size 8 μm, BD Falcon) were coated with 10 μg/ml type I collagen (PureCol™, Nutacon) and incubated for 1 h at 37 °C. Approximately 15×10^3 transfected cells were plated on the inserts in serum free medium and complete medium was placed in lower chamber as a chemoattractant. After 24 h, the cells that migrated through the filter were fixed with 4% paraformaldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted.

2.7. Statistical analyses

Error bars are presented as standard error of mean. Two-tailed paired *t*-test was used to compare between groups. $p < 0.05$ was considered significant. All statistical analysis and graphs were plotted using GraphPad Prism 6.

3. Results

3.1. Syndecan-2 regulates the distribution and activity of p190ARhoGAP to promote cell migration

The molecular mechanism by which syndecan-2 regulates cytoskeletal rearrangements is yet to be defined. However, p190RhoGAP is often involved in the regulation of contractility and is implicated in cell migration. Previous studies by Bass et al. [12] identified syndecan-4 as a regulator of p190ARhoGAP localisation in normal fibroblasts. To test whether this RhoGAP is also relevant to syndecan-2 regulated MDA-MB231 cell adhesion, we knocked down syndecan-2 levels by siRNA, which was confirmed by FACS (Fig. 1A). Control siRNA treated cells were rounded and motile in morphology while syndecan-2 depleted cells showed enhanced spreading, microfilament bundle and focal adhesion assembly (Fig. 1B). In transwell migration assays, cell migration was commensurately reduced in syndecan-2 depleted cells (Fig. 1C). In addition, p190ARhoGAP (p190A) was redistributed to the cell periphery in syndecan-2 depleted cells while no effect was noted in the localisation of p190BRhoGAP (p190B) (Fig. 1D), suggesting that syndecan-2 prevents recruitment of p190A to cell periphery where it normally exerts its activity [13,14].

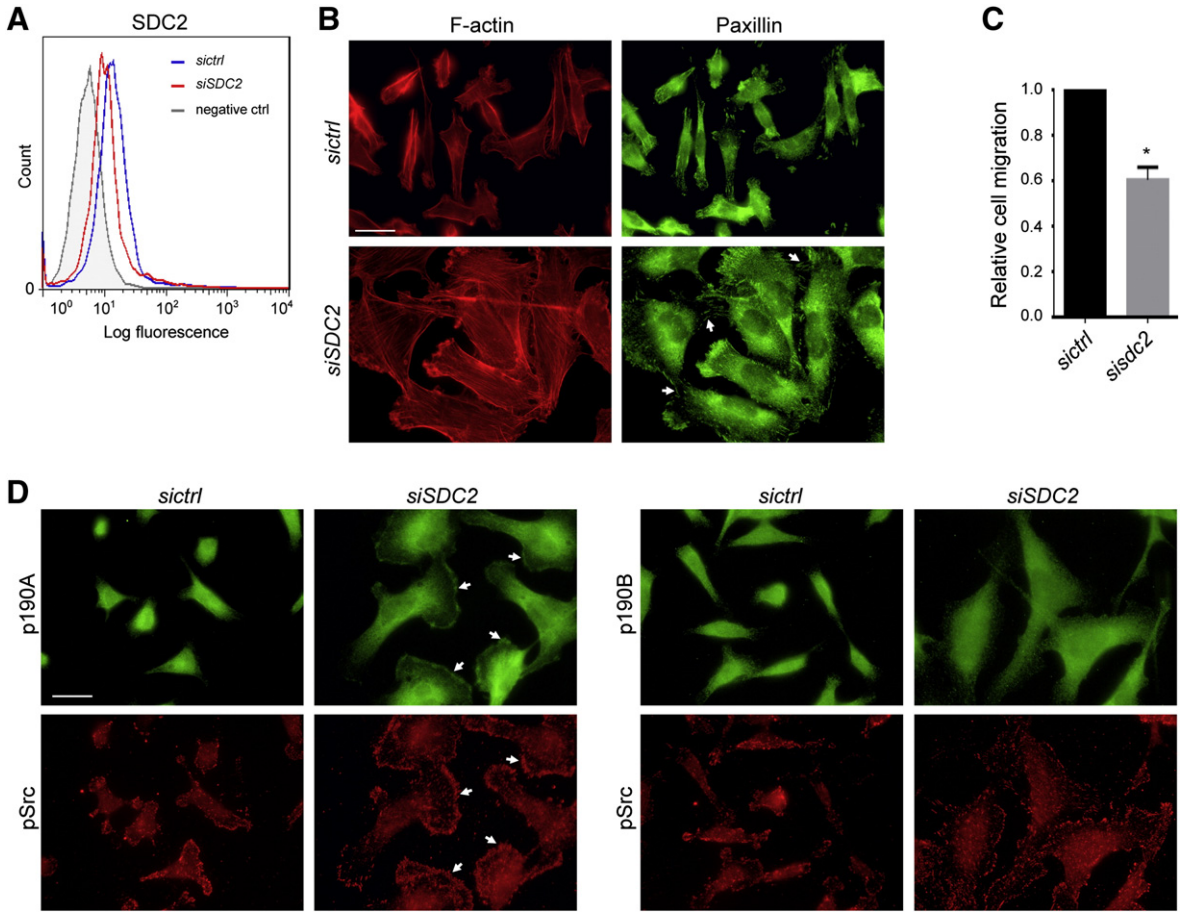


Fig. 1. Reduction in syndecan-2 in MDA-MB231 cells promotes adhesion, spreading and relocalisation of p190ARhoGAP. A. Syndecan-2 levels on the cell surface were reduced by siRNA treatment, as shown by FACS analysis. B, C. Cell spreading, F-actin containing microfilament bundles and paxillin-containing focal adhesions were promoted by siRNA for syndecan-2. In addition, cell migration was reduced. Quantitation in C, $n = 4$, $p < 0.05$. D. While p190ARhoGAP was relocated to the cell margins after syndecan-2 depletion, p190BRhoGAP distribution was unaffected. sictrl: control siRNA. Bar = 25 μm.

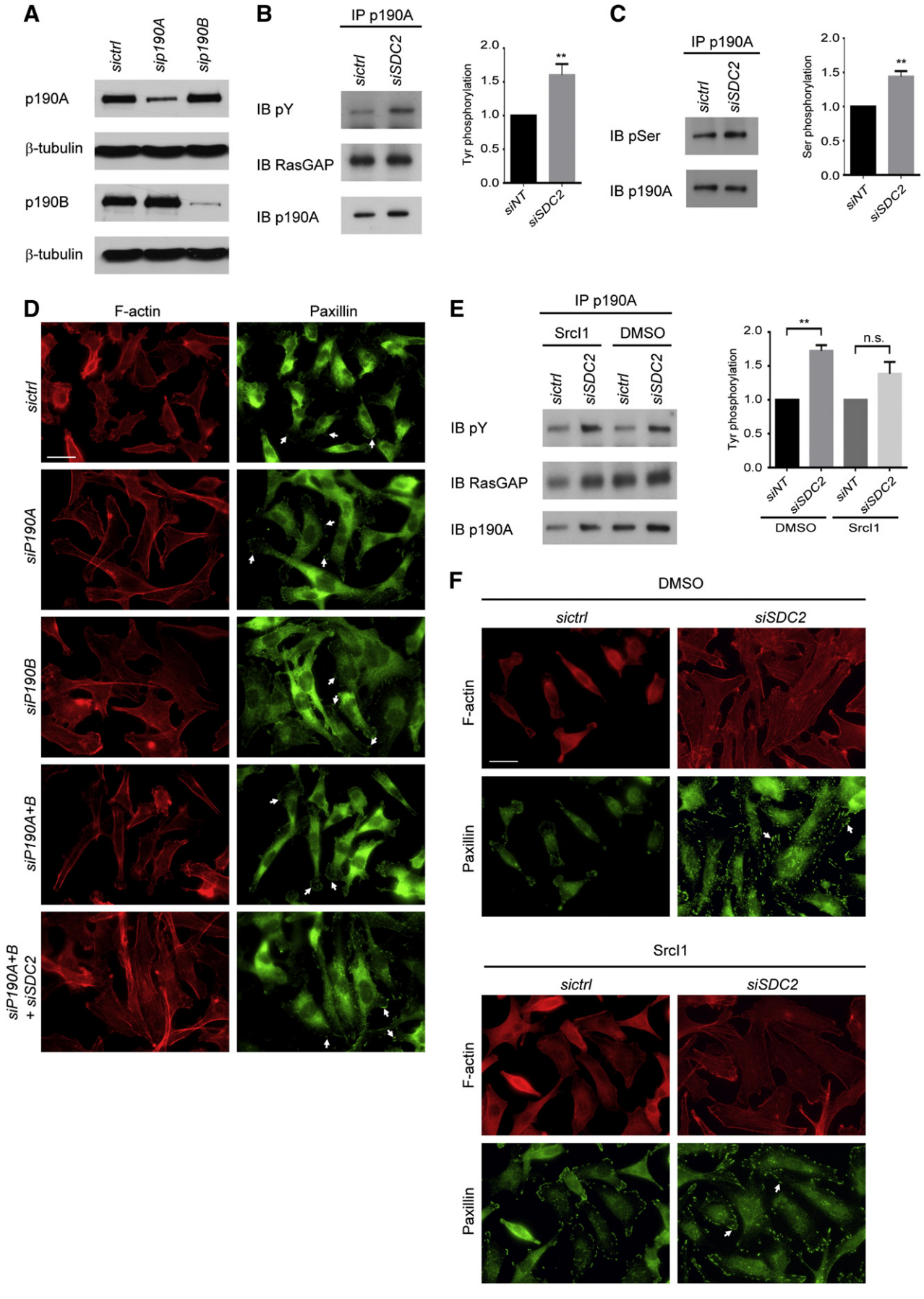
3.2. Syndecan-2 controls Src-dependent p190A activity

Given that syndecan-2 may regulate the localisation of p190A, we therefore investigated the effects of its depletion on MDA-MB231 cell morphology. Both p190A and p190B were knocked-down efficiently (Fig. 2A). Cells acquired an elongated morphology upon p190A siRNA treatment, but had no obvious changes in size and number of focal adhesions (Fig. 2B). When both p190RhoGAP isoforms and syndecan-2 were depleted simultaneously, the cell morphology resembled p190A depletion alone, suggesting that the GAP may be controlled downstream of syndecan-2 (Fig. 2B).

To establish whether syndecan-2 regulates the activity of p190RhoGAP, the protein was immunoprecipitated from syndecan-2 depleted cells, followed by detection of phosphotyrosine, since tyrosine phosphorylation is a measure of GAP activity [15–17]. Immunoprecipitation followed by western blotting showed that p190ARhoGAP-tyrosine phosphorylation was induced in syndecan-2 depleted cells (Fig. 2C). In addition, p120RasGAP co-immunoprecipitated with p190A. This is consistent with seminal findings where p190RhoGAP activity correlated with its interaction with p120RasGAP [18]. Therefore, it appears that syndecan-2 may function to modulate the activity of p190A in malignant cells. Activity of p190B, on the other hand, was not altered after depletion of syndecan-2 by siRNA treatment, consistent with its unchanged localisation (data not shown). In addition, p190A in MDA-MB231 cells can also be serine phosphorylated as detected by a phospho-serine PKC substrate antibody. In immunoprecipitates of p190A an upregulation in serine phosphorylation was observed in syndecan-2 depleted cells (Fig. 2D), which is consistent with a previous report indicating that p190RhoGAP may be phosphorylated by PKCα [19] and its membrane localisation is dependent upon it [12].

Src is a major tyrosine kinase that can phosphorylate p190ARhoGAP [16]. Fibroblasts transformed by Src enhanced phosphorylation of this GAP and led to cytoskeletal rearrangement associated with RhoA suppression [20]. We observed that redistribution of p190A to cell periphery in syndecan-2 depleted cells concomitantly colocalised with Tyr416 phosphorylated Src (Fig. 1D). To explore this further, we treated MDA-MB231 cells with Src kinase inhibitor I (SrcI1) and examined tyrosine phosphorylation levels after syndecan-2 depletion. Treatment with SrcI1 inhibited phosphorylation levels of p190A induced by syndecan-2 depletion, indicating that in MDA-MB231 cells, syndecan-2 suppresses Src-dependent phosphorylation of p190A (Fig. 2E). In addition, inhibition of Src increased cell spreading in control siRNA treated cells and interestingly, microfilament bundles formed in syndecan-2 depleted cells were partially abolished by Src inhibition. Numbers of focal adhesions

Fig. 2. p190ARhoGAP activity is a regulator of MDA-MB231 morphology. A. Specific depletion of the two RhoGAP isoforms could be achieved by siRNA treatment. B. Depletion of p190ARhoGAP or both RhoGAPs led to an elongated morphology, while suppression of p190BRhoGAP led to some cell spreading. However, knockdown of both RhoGAPs prevented the spreading and cytoskeletal changes induced by syndecan-2 siRNA. Bar=25μm. C, D. Tyrosine and PKC-mediated serine phosphorylation of p190ARhoGAP were increased by syndecan-2 siRNA. E. The increased tyrosine phosphorylation of p190ARhoGAP was prevented by Src inhibitor 1 (SrcI1), but p120RasGAP remained in the RhoGAP immunoprecipitations. In C, D and E $n=3$, $p<0.01$ from densitometic analysis of blots, calibrated to total p190ARhoGAP. (F) Src inhibitor 1 treatment (10μM for 2h) promoted cell spreading in sictrl cells and partially abolished microfilament bundles in syndecan-2 siRNA treated cells. The number of focal adhesions in syndecan depleted cells was substantially reduced after SrcI1 treatment.


A
sictrl
sip190A
sip190B
p190A
β-tubulin
p190B
β-tubulin
B
IP p190A
sictrl
siSDC2
IB pY
IB RasGAP
IB p190A
Tyr phosphorylation
2.0
1.5
1.0
0.5
0.0
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siNT
siSDC2
C
IP p190A
sictrl
siSDC2
IB pSer
IB p190A
Ser phosphorylation
2.0
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siNT
siSDC2
D
F-actin
Paxillin
sictrl
siP190A
siP190B
siP190A+B
siP190A+B + siSDC2
E
IP p190A
Srcl1
DMSO
sictrl
siSDC2
sictrl
siSDC2
IB pY
IB RasGAP
IB p190A
Tyr phosphorylation
2.0
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siNT
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DMSO
Srcl1
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DMSO
sictrl
siSDC2
F-actin
Paxillin
Srcl1
sictrl
siSDC2
F-actin
Paxillin

were also reduced in syndecan-2 depleted cells upon Src inhibition (Fig. 2F).

3.3. RhoA and RhoC have distinct functions in syndecan-2-regulated morphology and cell behaviour

Rho-GTPases are important in regulation of many cellular processes including cell migration, trafficking, cell polarity and cytokinesis [21]. As a GAP protein, p190RhoGAP can control Rho protein activity. Therefore, we next sought to investigate the involvement of Rho-GTPases, in particular RhoA and RhoC, in regulating cell morphology in syndecan-2 depleted cells. RhoA and RhoC knockdown efficiency was confirmed by western blotting (Fig. 3A). Consistent with previous findings, RhoA-depleted cells showed elongated morphology, whereas RhoC depleted cells had increased spread cell area (Fig. 3B). Furthermore, increased microfilament bundle assembly was also observed in RhoC depleted cells (Fig. 3B). It therefore appears that RhoA and RhoC are antagonistic in terms of regulating cell morphology. However, no obvious changes in size and number of focal adhesion upon RhoA or RhoC depletion were seen (Fig. 3B). Since RhoC depleted cells yielded a similar cell morphology to syndecan-2 depleted cells, we double depleted syndecan-2 with either RhoA or RhoC to determine the downstream target of syndecan-2. Simultaneous depletion of syndecan-2 with RhoA or RhoC yielded cell phenotypes resembling RhoA or RhoC depletion alone (Fig. 3C). Together, these results indicated that syndecan-2 depleted cells required RhoA to enhance microfilament bundle and focal adhesion formation, while RhoC promotes a motile and rounded morphology in MDA-MB231 cells downstream of syndecan-2.

RhoA is often associated with inhibition of cancer cell invasion. RhoC, on the other hand, plays a critical role in promoting cancer cell invasion [22]. We have previously shown that cell invasion into native type I collagen gels was markedly impaired in syndecan-2 depleted MDA-MB231 cells [unpublished results]. We hypothesised that cells treated with syndecan-2 siRNA would control the balance of RhoA and RhoC levels whereby RhoA activity is increased while RhoC activity is attenuated. To address this, we performed GTPase pulldown assays after syndecan-2 depletion. However, neither GTP-RhoA nor GTP-RhoC levels were affected by syndecan-2 depletion (Fig. 3D). This indicates that cytoskeletal and cell behaviour changes upon syndecan-depletion are not accompanied by changes in the global activity of either RhoA or -C. Next, we examined the distribution of GTP-RhoA by immunofluorescence staining specific for the activated form of RhoA (GTP-RhoA). In syndecan-2 depleted cells, GTP-RhoA staining was minimal at the leading edge compared to the control cells, but concentrated intracellularly, though not obviously associated with the microfilament bundles (Fig. 3E). This is consistent with recruitment of p190A to cell periphery with concomitant, local suppression of RhoA activity in cells spreading in response to syndecan-2 siRNA treatment. In addition, we observed that treatment with EHT-1864, a Rac inhibitor, promoted cell spreading (Fig. 3F). This suggests that the protrusive but more rounded phenotype of untreated MDA-MB231 cells is Rac-dependent, while the extensively spread phenotype with focal adhesions is not, or may even be inhibited by Rac.

3.4. Increased cell surface active β_1-integrin levels in syndecan-2 depleted cells

Engagement of integrin with syndecan-2 in cancer cells has been established. Syndecan-2 cooperates with $\alpha_2\beta_1$ integrin in mediating cell adhesion and migration in colon cancer cells [23]. Activation of β_1-integrin leads to tyrosine phosphorylation of p190RhoGAP [24] and RhoA activity is subsequently blocked by c-Src-dependent activation of p190RhoGAP upon integrin ligation [16]. In conjunction with all these previous studies, we also found increased tyrosine phosphorylation and redistribution of p190A in the absence of syndecan-2; therefore, the cell surface levels β_1-integrin were examined. FACS analysis indicated that active β_1-integrin levels in syndecan-2 depleted cells were enhanced (Fig. 4A). Concomitantly, active β_1-integrin, detected by a conformation-sensitive antibody, was detected at cell periphery in the absence of syndecan-2 (Fig. 4B). Moreover, β_1-integrin was co-distributed with p190A at cell periphery (Fig. 4C). As a whole, our findings suggested that recruitment of p190A to cell periphery in syndecan-2 depleted MDA-MB231 cells correlates with accumulation of active β_1-integrin at the cell surface.

3.5. Syndecan-2 suppresses syndecan-4 function in promoting p190A redistribution

Several studies have suggested that syndecan-2 can influence the syndecan-4 signalling pathway since they are similar in structure and may have overlapping roles [25,26]. However, the compensatory mechanism is effectively unknown. We have preliminary observations that syndecan-2 suppresses the roles of syndecan-4 in focal adhesion formation in MDA-MB231 cells [unpublished results]. Bass et al. [12] provided a link between p190A, syndecan-4 and integrin in fibroblasts, where localisation of p190A was regulated by syndecan-4, while its activity was modulated by $\alpha_5\beta_1$ integrin. Therefore, we addressed the question of how p190A relocalised to plasma membrane in the absence of syndecan-2. Cell surface levels of syndecan-4 were, in fact, elevated in the absence of syndecan-2, suggesting a compensatory upregulation had occurred (Fig. 5A). We next investigated whether syndecan-4 played a role in facilitating p190A distribution in MDA-MB231 cells. Syndecan-4 depletion was confirmed by western blotting, but surface levels of syndecan-2 were unchanged (Fig. 5B). Indeed, syndecan-4 depletion did not cause redistribution of p190A, which is consistent with the previous report [12] (Fig. 5C). However, double depletion of syndecan-2 and syndecan-4 revealed weak p190A staining at cell periphery arguing that syndecan-4 was required for p190A relocalisation (Fig. 5C). Furthermore, syndecan-4 and p190A were colocalised at cell periphery in syndecan-2 depleted cells (Fig. 5D). To investigate this further, we tested if syndecan-4 also affected the activity of p190A, as measured by its tyrosine phosphorylation. Unlike in fibroblasts, we observed that tyrosine phosphorylation of p190A was increased in syndecan-4 depleted cells and double depletion of syndecan-2 and syndecan-4 yielded higher tyrosine phosphorylation levels compared to either syndecan alone (Fig. 5E). Collectively, our data suggest that syndecan-4 is responsible for the relocalisation of p190A when syndecan-2 is unavailable.

4. Discussion

Syndecan-2 is usually considered a mesenchymal receptor, but in breast cancer we have shown it to be upregulated in the epithelial compartment, both in primary tumour and lymph node metastasis [unpublished results]. Moreover, it is also considered to be an important tumour progression entity in colon carcinoma and melanoma [23,27,28]. Increasingly, syndecans are now under investigation for roles in tumour growth and progression. Roles for p190RhoGAP in cancer cells have, however, been examined rarely. Two isoforms of p190RhoGAP, p190A and p190B exist in mammals. Both of them have distinct roles in mammary gland development [29]. p190A but not p190B is needed for mammary architecture development and also mammary epithelial cell differentiation. In the present study, we have provided a functional link between syndecan-2 and p190A in spatio-temporal control of cytoskeletal rearrangement and cell migration in MDA-MB231 cells. Syndecan-2 depletion had a marked impact on actin cytoskeletal organisation, where cell spreading, microfilament bundle and focal adhesion assembly were enhanced. Furthermore, cell migration was blocked in transwell migration. These data suggest that syndecan-2 is a major player in maintaining a dynamic arrangement of the actin cytoskeleton for cell motility. Intriguingly, activity and localisation of p190RhoGap, specifically the A isoform are controlled

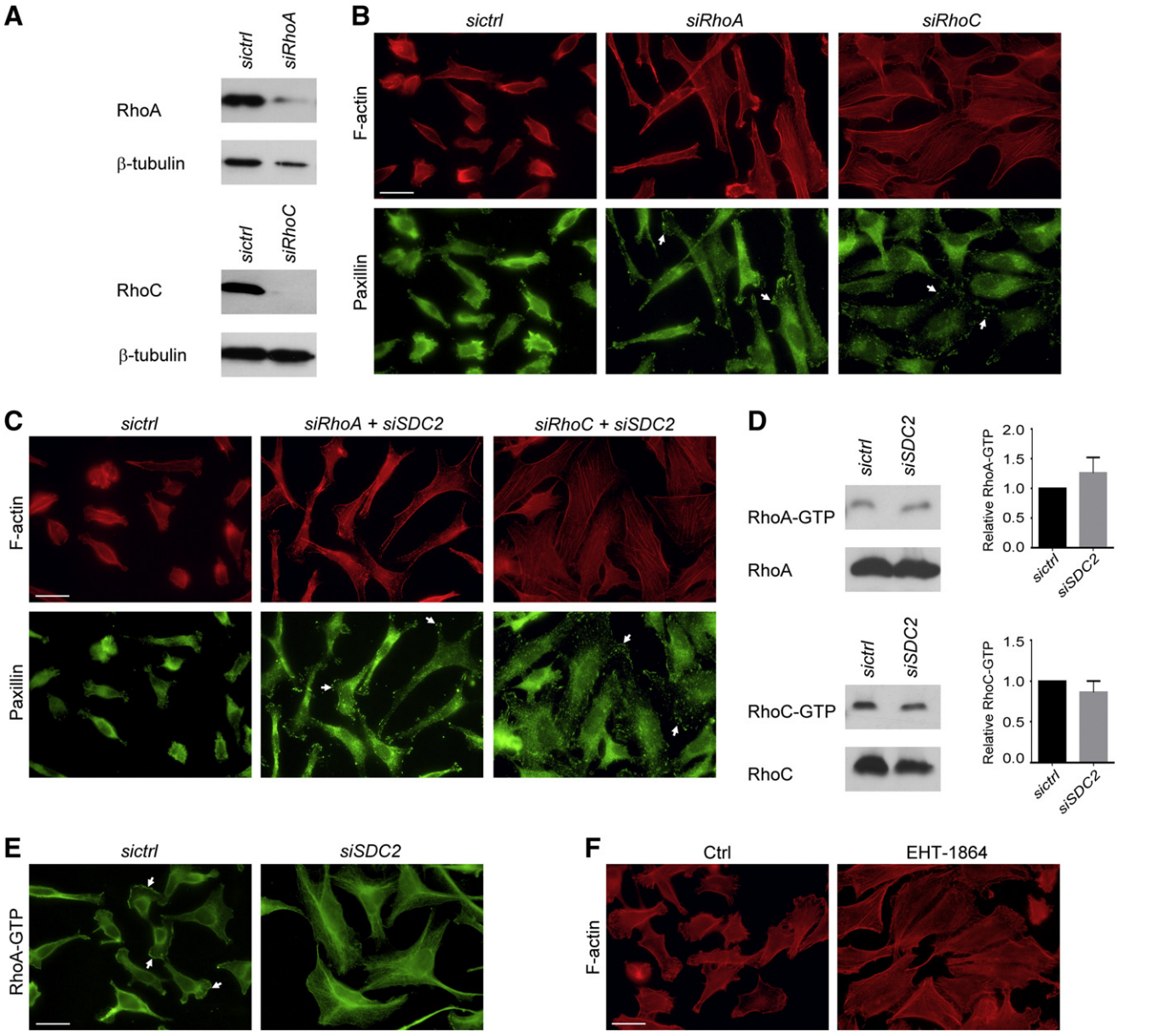

Fig. 3. RhoA and RhoC have opposing roles in the regulation of MDA-MB231 cell morphology. A. Specific knockdown of RhoA and RhoC could be achieved by siRNA treatment. B, C. While RhoA depletion by siRNA yielded an elongated but not well spread morphology, spreading, microfilament bundles and focal adhesions were promoted by knockdown of RhoC. Additional knockdown of syndecan-2 had minor effects, suggesting that the spreading response to syndecan-2 depletion requires RhoA. D, E. No changes in GTP loading of either RhoA or RhoC were noted in total cell lysates after syndecan-2 siRNA treatment. However, GTP-RhoA was lost from the protrusive edges of control cells when syndecan-2 was knocked down. F. Partial spreading but no microfilament bundles resulted from treating cells with the Rac inhibitor, EHT-1864 (20 μM for 24 h). Bars = 25 μm.

by the presence of syndecan-2. As a negative regulator of RhoGTPases, activity of p190RhoGAP is dependent on tyrosine phosphorylation by Src [17,30]; however the localisation of p190A is also important. Previous studies have demonstrated that p190A localised to the cell periphery of spreading cells [24,31]. We observed that p190A redistributed to the cell periphery in syndecan-2 depleted MDA-MB231 cells in which cell spreading was much enhanced. In addition, Src-dependent tyrosine phosphorylation levels were elevated in syndecan-2 depleted cells. Overexpression of syndecan-2 in sarcoma cells was reported to increase p190A tyrosine phosphorylation [32], which is the converse of our observations and suggests cell-type specificity. However, our data suggest that p190 and syndecan-2 are aligned in the same pathway. Therefore, the specific role of syndecan-2 in controlling a RhoGAP protein and thereby RhoGTPase-mediated signalling is proposed. This is consistent with actin cytoskeleton changes and reduced cell migration in syndecan-2 depleted cells.

Increased active β_1-integrin at the cell periphery accompanied syndecan-2 depletion, but the detailed molecular mechanism is not yet known. Syndecan-4 can regulate integrin recycling in non-transformed cells [33] and this may be altered by syndecan-2, which can both suppress syndecan-4 function and alter tyrosine phosphorylation status through the phosphatase CD148 (PTPRJ), with impact on integrin activity [34]. In contrast to depletion of syndecan-2, two previous studies showed that syndecan-1 reduction by siRNA led to enhanced MDA-MB231 cell adhesion to, and migration on, fibronectin substrates [35,36]. It was inferred that this involved increased levels of β1 integrin at the cell surface, coupled with increased RhoA and C and cadherin-11 protein levels.

Cell spreading and microfilament bundle formation are linked to members of the RhoGTPase family [21,37]. In particular RhoA has been shown many times to promote these structures, while GTP-Rac promotes spreading and ruffling behaviour [21]. Kurokawa et al. [38] showed that

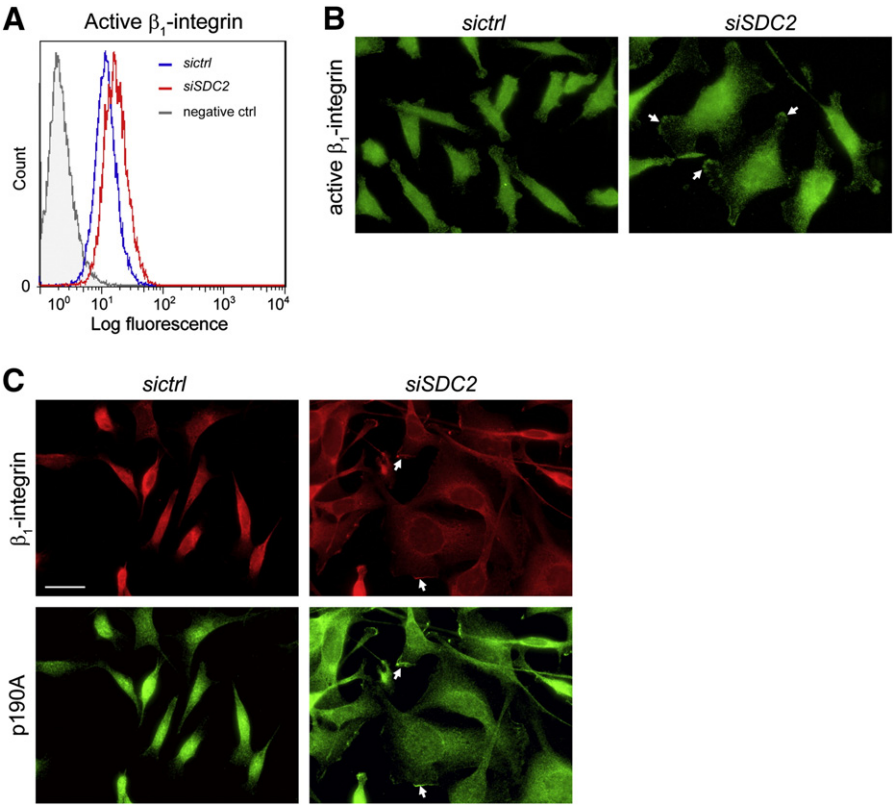

Fig. 4. Active β_1-integrin is increased and localised in response to syndecan-2 depletion. A. FACS analysis shows increased levels of active β_1-integrin on the surface of syndecan-2 siRNA treated cells, using a conformation-specific antibody. B, C. Loss of syndecan-2 promotes spreading and localisation of β_1-integrin to protrusive cell margins. Bars = 25 μm.

fibroblast spreading was accompanied by a transient increase in RhoGAP activity, which in turn leads to decreased GTP-RhoA levels. Later in the spreading process, GTP-RhoA levels rose again, facilitating stress fibres and focal adhesions. We have investigated whether these processes are involved in the morphological changes that accompany syndecan-2 reduction. While global levels of GTP-RhoA or GTP-RhoC did not change, we nevertheless noted translocation of p190ARhoGAP to the cell edge along with its increased tyrosine phosphorylation, an indicator of activity. Notably, it has been shown that p190A knockout mice did not show global changes Rho-GTP at tissue level [39]. Therefore, it is not surprising that we did not observe global changes in RhoA activity although tyrosine phosphorylation p190RhoGAP was upregulated in syndecan-2 depleted cells. It is known that Src family tyrosine kinases are keys to p190RhoGAP tyrosine phosphorylation, and that was confirmed here for the breast carcinoma cells. Of interest, however, is the contribution of syndecan-4. This proteoglycan, which is also widely distributed, including in MDA-MB231 cells, can trigger protein kinase Cα activity, and Bass et al. [12] have proposed that p190A is a target. Moreover, PKC phosphorylation was proposed to direct the RhoGAP to the membrane. Our data also suggest that PKC-mediated phosphorylation of p190A is increased as it is translocated to the cell membrane, and that syndecan-4 may be involved. All this suggests that syndecan-2 in these tumour cells can suppress the signalling activity of syndecan-4. The microfilament bundles that form in MDA-MB231 cells in response to syndecan-2 siRNA treatment are probably syndecan-4 dependent [unpublished results] as they are in other cells [1,2,7,40]. What remains to be understood is how syndecan-2 controls syndecan-4. They are quite closely related in terms of structure and primary sequence, although the central variable regions of their cytoplasmic domains are distinct. That of syndecan-4 can bind PKCα and also the actin-associated protein α-actinin [40,41]. These properties are believed to be not shared by syndecan-2. On the other hand, syndecan-2 cytoplasmic domain can be phosphorylated by PKCγ, an essential process for left–right asymmetry in Xenopus development [42].

Around 10 years ago, we proposed that syndecans may be regulators of cell surface microdomains [1]. Much work since then has shown relationships between syndecans and endocytic activities, as well as lipid rafts (or detergent-resistant membranes, DRMs). In this way, it is possible to visualise how multiple syndecans that may be present on a single cell type can have specific functions, even though ligand binding through their heparan sulphate chains may be a common feature. Consistent with this hypothesis, preliminary data show the syndecan-2 can interact with caveolin-2 [unpublished results]. Moreover, exogenous heparan sulphate treatment of MDA-MB231 cells can displace caveolin-2 from DRM pools into a Triton-soluble pool [unpublished results]. This suggests a role for syndecan-2 in association with caveolar domains.

RhoC was identified as a hallmark of the transformed state by Clark et al. [43] some years ago. In further exploring the regulation of the cytoskeleton in MDA-MB231 cells, we used siRNA treatments to knock down RhoA or RhoC. The phenotypes that emerged from these treatments were very distinct. Loss of RhoA caused cells to become attenuated, not well spread and lacking microfilament bundles. The same phenotype was observed if both RhoA and syndecan-2 were depleted. This suggests, consistent with other examples [22,44] that the cytoskeletal organization, with stress fibres and focal adhesions, that here results from syndecan-2 depletion, is dependent on RhoA. However, this process was not reliant on RhoC. Indeed, siRNA treatment to decrease RhoC levels led directly to spreading and microfilament bundles, showing clearly that these two related Rho isoforms have quite different properties in tumour cells. This effect was seen regardless of whether syndecan-2 was present or not, suggesting that RhoC may be downstream of syndecan-2 and that a key property of the proteoglycan is to suppress the syndecan-4/PKCα/RhoA axis that has been documented previously [7]. The relationship between syndecan-2 and RhoC should be a focus of future study. Moreover, these studies have concentrated on one, well characterised, cell line. It would be important to determine how widely applicable these results are to other breast carcinoma lines.

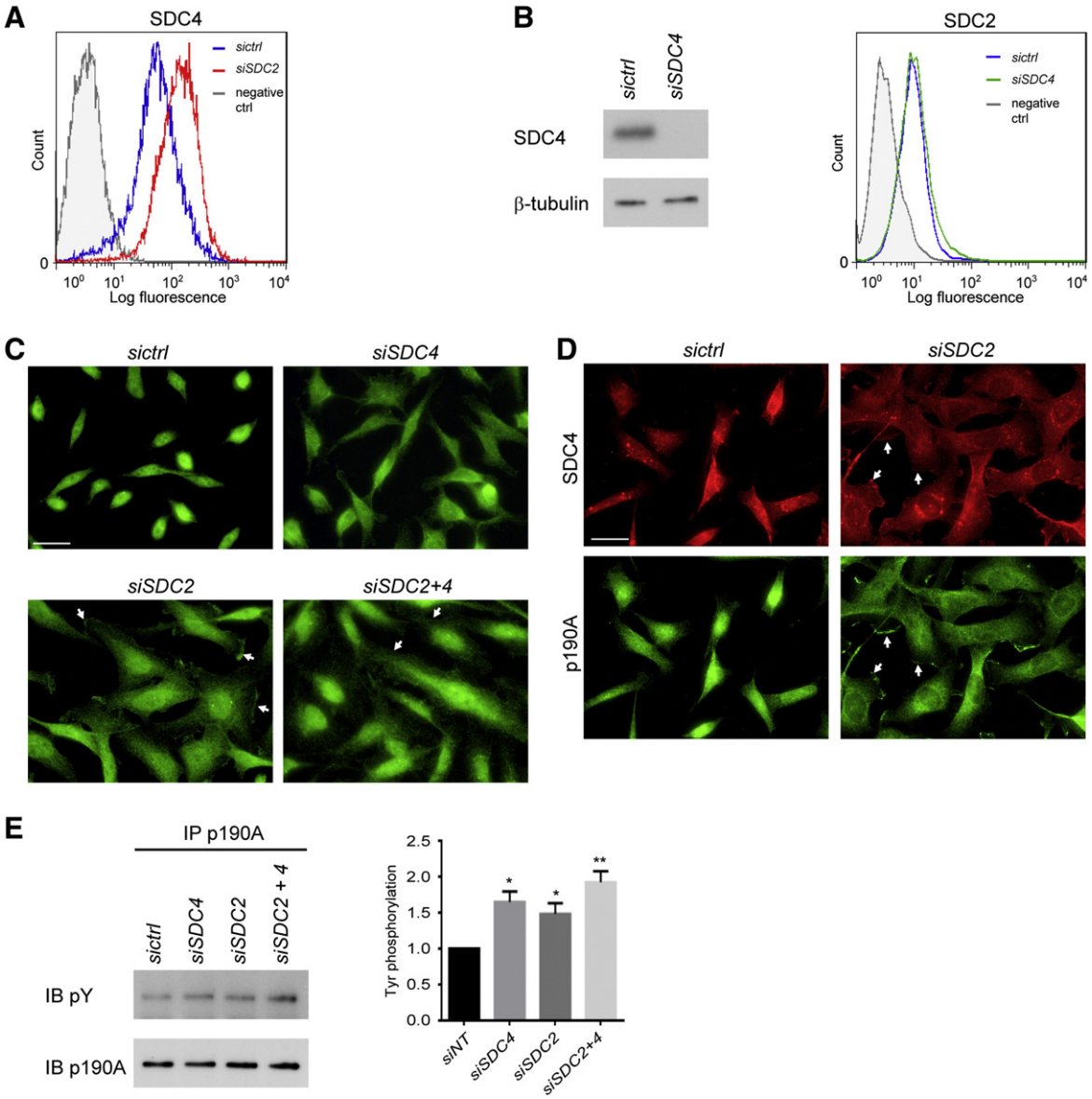

Fig. 5. Syndecan-4 regulates the movement of p190RhoGAP to cell margins in MDA-MB231 cells. A. Levels of cell surface syndecan-4 are increased when syndecan-2 is depleted by siRNA treatment. B. Syndecan-4 is efficiently reduced in response to specific siRNA treatment. FACS analysis shows that syndecan-2 levels on the cell surface are not affected by loss of syndecan-4. C. Syndecan-4 depletion does not promote spreading or relocalisation of p190ARhoGAP to the cell edges, in contrast to syndecan-2 reduction. D. Syndecan-4 and p190ARhoGAP are colocalised at cell edges after syndecan-2 reduction by siRNA treatment. E. Tyrosine phosphorylation of p190ARhoGAP is increased by loss of either or both of the syndecans. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$.

Breast cancer remains the major cause of cancer-related death in women worldwide, particularly in Europe and the US, although this form of cancer is less common in Asia. Triple negative or basal types can be aggressive and particularly difficult to treat. Our work suggests that at least one cell surface receptor, syndecan-2, has an important role in maintaining an invasive phenotype through its regulation of the actin cytoskeleton. It should be determined in the future whether this represents a viable route to controlling tumour progression.

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