Original Paper

Syndecan-4 Promotes Epithelial Tumor Cells Spreading and Regulates the Turnover of PKC α Activity under Mechanical Stimulation on the Elastomeric Substrates

Chun-Ping Huang^a Chao-Min Cheng^b Hong-Lin Su^a Yi-Wen Lin^{c,d}

^aDepartment of Life Sciences, National Chung Hsing University, Taichung, ^bInstitute of Nanoengineering and Microsystems, National Tsing Hua University, Hsinchu, ^cGraduate Institute of Acupuncture Science, China Medical University, Taichung, ^dResearch Center for Chinese Medicine & Acupuncture, China Medical University, Taichung, Taiwan

Key Words

Mechanotransducer • Syndecan-4 • Polydimethylsiloxane • Tumor cell development

Abstract

Background: Heparan sulfate proteoglycans (HSPGs) at the cell surface play an important role in cell adhesion, spreading, formation of focal adhesion complexes (FACs), and sensing mechanical stress. Syndecans are members of the HSPGs family and are highly expressed in various tumor cells. Syndecan-4 (SDC4) is a unique member of syndecans that activates protein kinase C alpha (PKC α). However, syndecan-4 in tumor cells development is not clear when receiving mechanical stress. **Aims:** Here we investigate the role of syndecan-4 in tumor cells spreading and its downstream kinases under mechanical stimulation. **Methods:** Epithelial tumor cells were seeded onto elastomeric polydimethylsiloxane (PDMS) membranes coated with poly-L-lysine (PL), fibronectin (Fn), or anti-SDC4 antibody and stretched with a modified pressure-driven cell-stretching (PreCS) device. **Results:** When cells received mechanical stimulation, engagement of syndecan-4 promoted the phosphorylation of focal adhesion kinase (FAK) at tyrosine 397 and PKC α at serine 657. Furthermore, we analyzed the cell contractility marker—myosin light chain 2 (MLC2) in 30 min time courses. The levels of phosphorylated MLC2 at serine19 were augmented through ligations of syndecan-4 but not integrin binding motif (RGD) at 10 min mechanical stimulation and were suppressed at 30 min and this phenomenon was associated with the activity of PKC α . **Conclusion:** Our data demonstrate that syndecan-4 is essential for transmitting the mechanotransduction signals via activation of PKC α and is important for tumor cells spreading, assembly of actin cytoskeleton and cell contractility.

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Dr. Yi-Wen Lin,
and Dr. Hong-Lin Su,

Graduate Institute of Acupuncture Science, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, R. O. C. (Taiwan) and Department of Life Sciences, National Chung Hsing University, 250, Kuo-Kuang Road, Taichung, 402, (Taiwan)
E-Mail yiwenlin@mail.cmu.edu.tw and E-Mail suhonglin@nchu.edu.tw

Introduction

The extracellular matrix (ECM) microenvironment plays a crucial role in tumor cell progression, invasion, and metastasis. For example, invadopodia formation allows tumor cells to penetrate the basement membrane and interstitial stroma [1]. Attachment to ECM allows suspension cells to form focal adhesion complexes (FACs) and activate focal adhesion kinase (FAK) to turn on multiple intracellular signaling pathways. Overexpression of FAK has been reported in various cancers [2]. FAK activation is induced by integrin–ECM engagement [3], growth factor collaboration [4], and mechanical stretch [5]. However, until now, the molecular mechanisms that link mechanical stimuli to FAK activation in tumor cells have been poorly understood.

Mechanical stress is converted into biochemical information by activating mechanotransducers [5]. Traditionally, it is believed that integrins are important mechanotransducers that connect the ECM with the actin cytoskeleton inside the cell. These integrin–ECM mechanotransduction signals including activation of Src [6], FAK [7], and ERK1/2 [8] lead to the expression of metalloproteinases (MMPs) and proteolysis of ECM to make tumor cells invasion and metastasis [9]. Cell surface heparan sulfate proteoglycans (HSPGs) play an important role in cell spreading [10] and sensing shear stress [11]. Furthermore, the disruption of HSPGs by heparinase treatment reduces mechanotransduction-sensing and differentiation [12, 13]. It is believed that HSPGs not only function as co-receptors, but also act as mechanotransducers in cell development and migration [14].

Syndecans are members of the HSPGs family [15] that belong to the group of adhesion receptors; together with integrins, they initiate synergistic signaling, control cell adhesive functions, and regulate cell behavior [12]. Syndecans are highly expressed in various tumor cells and promote tumor growth, invasion, metastasis, and angiogenesis [16]. Among the four subtypes of syndecans (syndecan-1 to -4), syndecan-4 (SDC4) has the unique ability to activate calcium-dependent protein kinase C α (PKC α) to bind to phosphatidylinositol 4,5-bisphosphate (PIP₂) at the V-region [17], forming a ternary complex. Syndecan-4 also regulates the activation of FAK [18] and RhoA [19] and serves as a mechanotransducer [20–22]. In particular, SDC4 binds to the heparin-binding domain of fibronectin [23] and activates RhoA through PKC α [24]. This evidence supports the idea that ligation of SDC4 induces the activation of FAK and RhoA through PKC α .

Mechanical stress triggers the activation of the Rho and its downstream effectors, Rho-associated kinase (ROCK) and mDia, through focal contacts [25]. ROCK inhibits the activity of MLC phosphatase and promotes the phosphorylation of myosin light chain (MLC), thus increasing focal adhesion formation [26] and cell contractility [27]. Indeed, RhoA-mediated contractility causes ECM remodeling of tumor stroma and feedback to induce the tumor cell proliferation [28]. The increase in ECM stiffness by mechanical stress critically promotes tumor progression and metastasis [9, 29]. However, the role of SDC4 in mechanical stress-mediated cytoskeleton remodeling in tumor cells is, as yet, unclear.

In this study, we hypothesize that syndecan-4 acts as a mechanotransducer to sense the microenvironmental mechanical force and activates the FAK and PKC α signaling pathways in tumor development. We examined the mechanical signal transmitting pathway of SDC4 in the regulation of PKC α and FAK activity through mechanical stretch stimulation. We seeded HeLa cells, human cervical cancer cells, and mouse melanoma cells (B16-F10) onto elastomeric polydimethylsiloxane (PDMS) membranes [30] coated with non-ECM (poly-L-lysine) and ECM molecules (fibronectin), or anti-SDC4 antibody. We stretched cells using a modified pressure-driven cell-stretching (PreCS) device [21] and observed the cellular morphology and phosphorylation of kinases triggered by SDC4 in the unstretched adhesion condition and under mechanical stimulation. Our results suggest that SDC4 may act as a mechanotransducer in the progression of cancer.

Materials and Methods

PDMS membrane and silanization

PDMS (Sylgard 184) was purchased from Dow Corning (Taipei, Taiwan). A ration of 6.0 g base to 0.4 g of curing agent (15:1 ratio) was mixed in a 90 mm Petri dish (Biomate, Taipei, Taiwan) and set aside for 7 days at room temperature. The elastic modulus of PDMS with this ratio has a Young's modulus of approximately 173 kPa [31]. The membrane was chemically silanized to produce biological molecules conjugated to the surface due to the hydrophobic character of PDMS. PDMS membranes were treated for 5 minutes with air plasma and UV sterilization, followed by 24 hours of 2% 3-aminopropyltriethoxysilane (AMEO; Sigma, St. Louis, MO, USA) in ethanol. The membranes were subsequently heat-treated in an -80°C oven for 8 hours, and rinsed with ethanol and phosphate buffer saline (PBS) [21].

Immobilized ECM molecules and antibody coating

The silanization PDMS membranes were coated with 15 mL solution of poly-L-lysine (0.1%; Sigma, St. Louis, MO, USA), fibronectin (10 μ g/mL; Sigma, St. Louis, MO, USA), RGD (10 μ g/mL; Sigma, St. Louis, MO, USA) or anti-SDC4 5G9 antibodies (10 μ g/mL; Santa Cruz Biotechnology, Texas, USA) in PBS for 24 hours at 4°C. Finally, the PDMS membranes were treated with a 1% BSA (Sigma, St. Louis, MO, USA) in ddH₂O solution for 30 minutes to block nonspecific binding.

Cell culture

HeLa cells and B16-F10 were cultured in 75 cm² flask (Falcon, Tewksbury, MA, USA) and maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) with 100 units penicillin per mL, and 100 μ g streptomycin per mL (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C, 5% CO₂, and humidified atmosphere. For spreading and stretch assays, 1 X 10⁶ cells per Petri dish were trypsinized with 0.25% Trypsin-EDTA (Gibco, Carlsbad, CA, USA) and seeded onto PDMS membranes pre-coated with poly-L-lysine (PL), fibronectin (Fn), or anti-SDC4 antibody in DMEM medium containing 10% FBS overnight. After cell adhesion and spreading, the DMEM medium was removed and cells were washed with PBS thrice, and subsequently serum starved for 18 hours to reduce interference [18, 32].

Mechanical stretch experiment

The pressure-driven cell-stretching device [21] was modified to enlarge the diameter to 90 mm in order to fill the space with a PDMS membrane. This modified device was handmade and purchased from BIOCHIEFDOM (Taipei, Taiwan). The pressure of mechanical stretch experiment was controlled at 5 lb/in² with a pressure regulating valve (San-Yuan, Taichung, Taiwan). After cell starvation, the membranes were cut around the edges of Petri dishes to loosen them and cell-laden membranes were laid on our modified pressure-driven cell-stretching (PreCS) device for 5, 10, and 30 minute periods in serum-free DMEM medium containing 1 mM sodium vanadate (Sigma, St. Louis, MO, USA).

Immunofluorescent staining and spreading area analysis

The unstretched or stretched PDMS membranes were washed with cold PBS containing 0.5 mM sodium vanadate thrice, cut into 1 X 1 cm² squares, and subsequently fixed with 4% paraformaldehyde for 15 minutes at room temperature, before being permeabilized and blocked with PBS containing 1% bovine serum albumin, 0.1% Triton X-100, and 0.02% sodium azide for 2 hours at 4°C. Samples were further incubated with primary antibodies at 4°C overnight. The primary antibodies were: anti-syndecan-4 monoclonal antibody (1:100, Santa Cruz Biotechnology, Texas, USA), anti-phosphospecific FAK at tyrosine 397 polyclonal antibody (1:200, Novus Biologicals, Littleton, CO, USA), anti-phosphospecific PKC α at serine 657 polyclonal antibody (1:200, Upstate, Billerica, MA, USA), anti-phosphospecific myosin light chain 2 at serine 19 monoclonal antibody (1:200, Cell Signaling Technology, Boston, MA, USA). The secondary antibodies were incubated with phalloidin conjugated with rhodamine (1:1000, Molecular Probes, Carlsbad, CA, USA), Goat anti-mouse IgG DyLight488 (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) and Goat anti-rabbit IgG DyLight594 (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at 4°C. The stained HeLa cells were mounted with glycerol, sealed under a coverslip, and then photographed using a fluorescent microscope system (Olympus, BX-51, Japan). Stained B16-F10 cells were

mounted with DAPI-contained mounting medium (Sigma, St. Louis, MO, USA) and photographed by confocal microscope detection system (Leica, TCS SP2, Germany). The area of cell spreading was examined using NIH ImageJ software (Bethesda, MD USA), and the fluorescent image of fluorescent actin (80-120 cells from three independent experiments) was hand-measured.

Immunoblotting

HeLa cells were harvested by scrapers using 200 μ L of lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 0.02% NaN₃) and a protease inhibitor cocktail (Amresco, Solon, USA). The extracted proteins (20 μ g per sample assessed by BCA protein assay) were subjected to 8 or 10% SDS-Tris glycine gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in tris-buffered saline (TBS) buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.05% Tween 20), incubated with primary antibodies: anti- α -tubulin monoclonal antibody (1:2500, Santa Cruz Biotechnology, Texas, USA), anti-phosphospecific ERK1/2 at threonine 203 / tyrosine 205 polyclonal antibody (1:1000, Novus Biologicals, Littleton, CO, USA), anti-pFAKy397 polyclonal antibody (1:1000, Novus Biologicals, Littleton, CO, USA), anti-pPKC α s657 polyclonal antibody (1:1000, Upstate, Billerica, MA, USA), and anti-pMLC2s19 monoclonal antibody (1:1000, Cell Signaling Technology, Boston, MA, USA) in TBS with 1% bovine serum albumin at 4°C overnight. Peroxidase-conjugated anti-rabbit antibody and anti-mouse antibody (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA) were used as a secondary antibody. The bands were visualized using an enhanced chemiluminescent substrate kit (Thermo Scientific, Waltham, MA, USA) with LAS-3000 Fujifilm (Fuji Photo Film Co. Ltd). Where applicable, the image intensities of specific bands were quantified with NIH ImageJ software (Bethesda, MD, USA).

Sandwich enzyme-linked immunosorbent assay (ELISA)

B16-F10 cells were harvested by scrapers using 500 μ L of ELISA lysis buffer (FIVEphoton Biochemicals, San Diego, USA) containing inhibitor cocktail (Amresco, Solon, USA) and 1 mM PMSF. The extracted proteins (20 μ g per sample assessed by BCA protein assay) were used to detect pFAKy397 (R&D Systems, Minnesota, USA), pMLC2s18 (Assay Biotechnology, CA, USA) and pPKC α t638 (Assay Biotechnology, CA, USA) following manufacturer's protocol. The absorbance of each sample was read by spectrophotometry (BioTek, VT, USA).

Statistical analysis

All statistic data are presented as the mean $\pm$ standard error. Statistical significance was tested using the ANOVA test, followed by a post hoc Tukey's test ($p < 0.05$ was considered statistically significant).

Results

HeLa cells expanded on the fibronectin or anti-syndecan-4 antibody-coated elastomeric PDMS membranes

To determine the role of syndecan-4 in tumor cells adhesion and spreading on elastomeric matrix, we cultured HeLa cells on elastomeric PDMS membranes. HeLa cells are adenocarcinoma epithelial cells transformed by the human papillomaviruses 18 (HPV-18) and are used widely in cancer and cell biology research areas [33]. SDC4 mRNA is abundant in epithelial cells and expressed in HeLa cells [34]. After adhesion and starvation, cells were fixed and visualized by immunofluorescent staining for SDC4 (green) and filamentous actin (F-actin, red). On PI (Fig. 1A), a synthetic, positively charged amino acid polymer, the morphology of HeLa cells appeared condensed and rounded. On fibronectin, the glycoprotein of ECM and a ligand for both integrins and SDC4 [23], cells exhibited extended morphology and well-formed FACs (Fig. 1A). Furthermore, we analyzed the area of cell spreading with ImageJ software and the data showed the area of seeding on Fn ($386.0 \pm 30.8 \mu\text{m}^2$, $p < 0.05$) or SDC4 ($478.1 \pm 29.6 \mu\text{m}^2$, $p < 0.05$) -coated membrane was significantly higher than that for the PI ($156.4 \pm 5.5 \mu\text{m}^2$, $n = 80$ -120 cells) group (Fig. 1B). These results demonstrate that the engagement of SDC4 could promote the spreading of HeLa cells and the formation of FACs on the elastomeric polymer matrix.

Fig. 1. HeLa cells expanded on fibronectin- or anti-SDC4 antibody-coated elastomeric PDMS membrane under starvation conditions. (A) HeLa cells were seeded onto poly-L-lysine (PI), fibronectin (Fn), or anti-SDC4 antibody pre-coated PDMS membrane overnight and starved for 18 hours. After starvation, cells were subsequently fixed and visualized by immunofluorescent staining for SDC4 (green) and filamentous actin (F-actin) (red). Arrows indicate co-localization of SDC4 and F-actin in focal adhesions. (B) The area of cell spreading was measured by hand-drawing with NIH ImageJ software and depended on morphological analysis. The area of seeding on fibronectin or anti-SDC4 antibody-coated membrane was greater than that of the PI group ($n = 80$ – 120 cells, three independent experiments). Data represent mean $\pm$ SE, and $*p < 0.05$, as compared to that of the PI group (scale bar = $25 \mu\text{m}$).

Fig. 2. Mechanical stretch induced the phosphorylation of focal adhesion kinase at tyrosine 397 on the elastomeric PDMS membranes. (A) HeLa cells were seeded onto PI, Fn, or anti-SDC4 antibody pre-coated PDMS membrane and stained as described previously. (B) The membrane was washed with PBS thrice and laid on a modified pressure-driven cell-stretching (PreCS) device for 5 minutes at 5 lb/in^2 in DMEM medium containing 1 mM sodium vanadate. After mechanical stretch, cells were subsequently fixed and visualized by immunofluorescent staining for SDC4 (green) and phosphospecific antibodies to FAKy397 (pFAKy397) (red). Arrows indicate the co-localization of SDC4 and pFAKy397 in FAKs around the edges of cells (scale bar = $25 \mu\text{m}$).

Mechanical stretch induced the phosphorylation of focal adhesion kinase at tyrosine 397 through the engagement of syndecan-4

We hypothesized that SDC4 senses microenvironmental mechanical stimuli and turns on the FAK signaling pathway in cancer development. FAK plays a key role in

Fig. 3. Mechanical stretch induced the phosphorylation of downstream SDC4 kinases: pPKC α , pFAK and pERK 1/2. (A) HeLa cells were harvested from PDMS membrane for protein extracts before and after mechanical stretch (5 minutes, 5 lb/in²) and analyzed with antibodies to α -tubulin, phosphospecific antibodies to ERK1/2 (pERK1/2), FAK at tyrosine 397 (pFAKy397) and PKC α at serine 657 (pPKC α s657) by immunoblotting. (B) Densitometry analysis of phosphorylation level was quantified with NIH ImageJ software. Relative intensity was calculated based on defining the PI lane (Unstretched) as 100%. After mechanical stretch, the levels of pERK1/2 and pFAKy397 were increased in all groups. Noticeably, the levels of pPKC α s657 were increased in the Fn and SDC4 group, but not in the PI group. Each bar represents the average of four independent experiments (* $p < 0.05$, as compared to each of the unstretched adhesion group).

mechanotransduction and phosphorylation at tyrosine 397, and is crucial in response to mechanical force [35]. Our results indicate that FAK was phosphorylated at tyrosine 397 (pFAKy397) and co-localized with SDC4 in the unstretched condition (Fig. 2A). After mechanical stretch (5 minutes, 5 lb/in²), pFAKy397 was activated around the edges of cells in both the Fn and SDC4 groups (Fig. 2B). These data suggest that cells sensed mechanical stimulation by the ligation of SDC4 and induced the activation of FAK.

Mechanical stretch induced the phosphorylation of syndecan-4 downstream kinases: PKC α , FAK, and ERK 1/2

The ligation of SDC4 and matrix induces the activation of PKC α in cell adhesion and spreading, but its role in signal transmitting under mechanotransduction is poorly understood. To elucidate the mechanism of SDC4, HeLa cells were harvested from PDMS membranes for protein extracts before and after mechanical stretch (5 minutes, 5 lb/in²). In the unstretched condition, the mechanotransduction-related signaling kinases showed basal levels of phosphorylation (Fig. 3A). After mechanical stretch, the phosphorylation levels of ERK1/2 were all increased compared to each of member of the unstretched control (PI group: $148.8 \pm 12.2\%$; Fn group: $158.2 \pm 7.9\%$; SDC4 group: $154.9 \pm 9.7\%$, $p < 0.05$). Noticeably, the phosphorylation levels of PKC α at serine 657 were increased in the Fn ($136.0 \pm 8.6\%$, $p < 0.05$) and SDC4 ($141.3 \pm 3.1\%$, $p < 0.05$) group, but not in the PI ($108.0 \pm 5.7\%$) group. The phosphorylation levels of FAKy397 were increased after mechanical stretch (PI group: $146.0 \pm 3.2\%$; fibronectin group: $132.6 \pm 3.2\%$; SDC4 group: $130.5 \pm 2.6\%$, $p < 0.05$) (Fig. 3B). These data suggest that the SDC4 molecule received mechanical stimulation to promote the phosphorylation of PKC α at serine 657, thus activating the downstream mechanotransduction-related kinases such as FAK and ERK1/2.

Mechanical stretch induced the phosphorylation of MLC 2 at serine19 through the engagement of SDC4

Shear stress induces transient inactivation of Rho and promotes the formation of focal contacts [36]. Rho can be activated by PKC α to maintain the phosphorylation of MLC2 and promote actin cross-linking and contractility. To dissect the role of SDC4 in cell contractility

Fig. 4. Mechanical stretch induced the phosphorylation of myosin light chain 2 at serine19 on the Fn or anti-SDC4 antibody-coated PDMS membrane. (A) HeLa cells were seeded on the Fn, anti-SDC4 antibody or RGD pre-coated PDMS membrane as described previously, and visualized by immunofluorescent staining for phospho-MLC2 serine19 (pMLC2s19) (green) and pPKC α 657 (red). (B) After mechanical stretch (10 minutes, 5 lb/in²), pPKC α 657 and pMLC2s19 were activated in the Fn and SDC4 group, but not in the RGD group. Arrows indicate the co-localization of pPKC α 657 and pMLC2s19 (scale bar = 25 μ m).

Fig. 5. Mechanical stretch promoted the phosphorylation of myosin light chain 2 and dynamic changes were associated with the activity of PKC α . (A) Cells were treated with mechanical stretch at different time courses (0, 10, 30 minutes, 5 lb/in²) and harvested. Phosphospecific antibodies to MLC2 at serine 19 (pMLCs19) and PKC α at serine 657 (pPKC α 657) were used to recognize phosphospecific proteins by immunoblotting. (B) Densitometry analysis of pMLCs19 was quantified with NIH ImageJ software as bar chart. Relative intensity was calculated based on defining the Fn lane (0') as 100%. Mechanical stretch increased the phosphorylation of MLCs19 in the Fn group, but not in the RGD group at 10 minutes. The pMLCs19 was suppressed in the SDC4 and RGD group at 30 minutes (Fig. 5B#). (C) Mechanical stretch induced the phosphorylation of PKC α 657 at the 10 minute time point and decreased at the 30 minute time point in the Fn and SDC4 groups, but not in the RGD group. In particular, the phosphorylation of PKC α in the SDC4 group was suppressed at the 30 minute time point, which is consistent with the phosphorylated levels of pMLC2s19 (Fig. 5C#). Each bar represents the average of three independent experiments. (* p < 0.05, as compared to each of the unstretched group; # p < 0.05, as compared to that of the Fn stretch-treated group).

Fig. 6. The engagement of SDC4 delivered mechanical signaling and activated PKC α in B16-F10 mouse melanoma cells. B16-F10 cells were seeded onto elastomeric PDMS membranes pre-coated with Fn, anti-SDC4, or RGD as described previously and treated with mechanical stretch in time intervals (0, 5, 10, 30 minutes, 5 lb/in²), and further stained for phospho-MLC2 serine19 (pMLC2s19) (green), pPKC α s657 (red), and nucleus (DAPI, blue). The ligation of fibronectin (A), SDC4 (B) or RGD (C) showed basal levels of pMLC2s19 in the unstretched condition (0'). After mechanical stretch, the pMLC2s19 was increased in all groups (5'-10'). The pPKC α s657 was activated and merged with pMLC2s19 in the SDC4 (5') and Fn (10') groups, but not in the RGD group. Arrows indicate the co-localization of pPKC α s657 and pMLC2s19 (scale bar = 20 μ m).

under mechanical stimulation, HeLa cells were seeded onto Fn, anti-SDC4 antibody or RGD pre-coated PDMS membranes, and were visualized by immunofluorescent staining for phospho-MLC2 at serine19 (pMLC2s19) (green) and pPKC α s657 (red). When cells were plated on RGD, the integrin-binding motif of fibronectin, the cells were also expanded, suggesting integrin-mediated cell adhesion (Fig. 4A). The engagement of Fn, SDC4 or RGD showed basal activation of pMLC2s19 (Fig. 4A). After mechanical stretch (10 minutes, 5 lb/in²), the pPKC α s657 and pMLC2s19 were activated in the Fn and SDC4 group, but not in the RGD group (Fig. 4B). These data suggest that engagement of the SDC4 molecule promoted the phosphorylation of MLC2s19 under mechanical stimulation.

Fig. 7. Mechanical stretch induced FAK activation at the initial stage and PKC α was important to maintain the phosphorylation of FAK and MLC2. B16-F10 cells were treated with mechanical stretch as described above and harvested with ELISA lysis buffer. pFAKy397, pMLCs18, and pPKCat638 were analyzed by sandwich ELISA. (A) The concentration of pFAKy397 was calculated according to standard curve, and each set of samples assayed. Mechanical stimulation increased the concentration of pFAKy397 in the Fn, SDC4, and RGD groups at the 5-minute time point. (B) The phosphorylation levels of pMLC2s18 were elevated at the 5-minute time point in the Fn, SDC4, and RGD groups. Noticeably, pMLC2s18 was immediately dephosphorylated in the RGD group at the 10-minute time point. (C) The phosphorylation of PKCat638 was increased in the Fn and SDC4 groups at 5 to 10-min time point, but no elevation was observed in the RGD group. Relative intensity was calculated based on defining the absorbance of Fn (0') as 100%. Each bar represents the average of three independent experiments. (* $p < 0.05$, as compared to each of the unstretched group; # $p < 0.05$, as compared to each 5 min time-point stretch-treated group).

The phosphorylation of MLC2 was associated with the activity of PKC α under mechanical stimulation

In order to elucidate the dynamic activity of PKC α , we analyzed the phosphorylation of MLC2 in HeLa cells under mechanical stretch at different time intervals (0, 10, 30 minutes, 5 lb/in²). The engagement of fibronectin, SDC4, or RGD showed basal levels of pMLC2s19 in the unstretched condition (Fig. 5A). We observed an increase of pMLC2s19 in the SDC4 group when cells were unstretched, but no statistical difference (Fig. 5B). After 10 minutes of mechanical stretch, the pMLC2s19 was increased in the Fn group (Fig. 5B, $146.3 \pm 11.7\%$, $p < 0.05$) compared to our unstretched control. When receiving long-term mechanical stimulation (30 minutes), the phosphorylation levels of MLC2s19 were still higher in the Fn group ($153.5 \pm 5.2\%$, $p < 0.05$), but restored to basal levels in the SDC4 group (Fig. 5B) and the pMLC2s19 in the RGD group remained at basal levels (Fig. 5B). The pPKC α s657 showed at basal level when cells were unstretched in all groups (Fig. 5C). Mechanical stretch induced the phosphorylation of PKC α s657 in the Fn ($152.8 \pm 11.8\%$, $p < 0.05$) and SDC4 ($150.4 \pm 3.9\%$, $p < 0.05$) groups at the 10-minute time point and decreased at the 30-minute time point, but showed no change in the RGD group (Fig. 5C). These data suggest that the phosphorylation of MLC 2 was associated with the activity of PKC α through SDC4.

Fig. 8. Summary diagram of how SDC4 regulates the dynamic activity of kinases through PKC α under mechanical stimuli. Our data shows that SDC4 acts as a mechanotransducer to initiate the phosphorylation of PKC α , FAK, and ERK1/2 at an early stage (solid arrow). Furthermore, the phosphorylation of myosin light chain 2 is associated with the activity of PKC α . At later stages, low activity of PKC α caused dephosphorylation of FAK and myosin light chain 2 (dotted arrow).

SDC4 delivered mechanical signaling and activated PKC α in B16-F10 mouse melanoma cells

Our data suggest that SDC4 could transmit a mechanical signal into biochemical information in the adenocarcinoma epithelial cells. We used malignant mouse melanoma cells (B16-F10) to confirm the role of SDC4 in mechanical signal transmitting. B16-F10 is an epithelial-like tumor cell line that has highly metastatic ability and is resistant to anticancer drugs treatment [37, 38]. Cells were seeded onto elastomeric PDMS membranes pre-coated with Fn, anti-SDC4, or RGD, as described above, and treated with mechanical stimulation in time intervals (0, 5, 10, 30 minutes, 5 lb/in²), further harvested, and stained for pMLC2s19 (green) and pPKC α s657 (red). The data shows basal levels of pMLC2s19 in the unstretched condition (Fig. 6, 0'). After mechanical stretch, the pMLC2s19 was increased from the 5 to 10 minute time-point in all groups (Fig. 6, 5'-10'). Noticeably, we observed that pPKC α s657 was activated and merged with pMLC2s19 after receiving mechanical stretch in the SDC4 (Fig. 6B) and Fn (Fig. 6A), but this did not occur in the RGD group (Fig. 6C). These results correspond to phenomena we observed in HeLa cells.

FAK activation increased at the initial stage and PKC α was important for maintaining the activity of MLC2 under mechanical stimulation

We further analyzed the phosphorylation of FAK, PKC α and MLC2 for the duration of 30 minutes by sandwich ELISA. Mechanical stimulation increased the concentration of pFAKy397 in the Fn (56.0 ± 0.9 pg/ μ g, $p < 0.05$), SDC4 (60.1 ± 9.1 pg/ μ g, $p < 0.05$) and RGD (44.0 ± 6.6 pg/ μ g, $p < 0.05$) groups at the 5-minute time point (Fig. 7A). The phosphorylation levels of pMLC2s18 were raised at the 5-minute time point (Fig. 7B) in the Fn ($117.1 \pm 2.0\%$, $p < 0.05$), SDC4 ($117.2 \pm 0.4\%$, $p < 0.05$), and RGD ($115.9 \pm 1.5\%$, $p < 0.05$) groups. Noticeably, pMLC2s18 was immediately shut down in the RGD group at the 10-minute time point ($92.7 \pm 4.6\%$, $p < 0.05$). Otherwise, mechanical stretch through the engagement of fibronectin or SDC4 maintained the phosphorylation of MLC2 at the 10-minute time point (Fig. 7B). The phosphorylation of PKC α 638 was increased in the Fn and SDC4 groups at the 5- and 10-min time points, but no raise was observed in the RGD group (Fig. 7C). These ELISA results further confirmed that activity of PKC α was necessary to maintain the phosphorylation of MLC2 through the engagement of SDC4.

Discussion

In this study, we have shown that HeLa cells display condensed morphology on low stiffness substrates; however, immobilized ECM molecules such as fibronectin, RGD, or

specific ligation (anti- SDC4 antibody) could promote cell spreading and formation of focal adhesions. The finding demonstrates that the SDC4 molecule promotes cell adhesion and spreading and overcomes the low stiffness of ECM. Mechanical stress plays a critical role in both normal cellular physiology and in diseases such as cardiac hypertrophy and atherosclerosis [39]. However, only a few studies have focused on the effects of mechanical stress in cancer progression. Since the major mechanical stress effector, FAK, is overexpressed in numerous cancers, we hypothesized that mechanical stress can induce the activation of FAK through HSPGs, especially SDC4. Our results show that mechanical stimulation promotes the phosphorylation of FAK at tyrosine 397 site through the engagement of fibronectin and unique syndecan-4 molecules. The levels of pFAK^{Y397} on the PI-coated group were greater than those of the Fn- and anti-SDC4-coated groups. This suggests the involvement of other mechanotransducers [40-42] that were adsorbed by poly-L-lysine and enhanced the effects induced by mechanical stimulation.

Mechanical stress increases the expression of SDC4 mRNA within 30 minutes [43], and PKC α is translocated to the cell membrane within 2 minutes [44]. Until now, the relationship between SDC4 and PKC α under mechanical stimulation was unclear. Our data suggest that SDC4 activates the PKC α downstream signaling pathway to initiate the activation of FAK and ERK1/2.

Mechanical stress mediates actin cytoskeleton remodeling and increases the contractility of cells by promoting the assembly of stress fibers [28, 45]. These stress fibers consist of α -actinin cross-linked actin filaments stabilized by interaction with myosin IIA [46]. The phosphorylation of myosin II light chain is regulated by MLC kinase (MLCK) and ROCK and promotes actomyosin contractility. Mechanical stress has been shown to increase the activities of both MLCK [47] and ROCK [26]. Integrins [48] and SDC4 [49] signaling transiently suppress the activity of RhoA through the regulation of p190RhoGAP at early stages of cell adhesion and migration. The activity of RhoA is rapidly reduced after 5 minutes of shear stress and recovered after 30 minutes [36]. As the details regarding the process by which mechanical stimuli regulate RhoA activity are unclear, we investigated the phosphorylation of myosin II light chain at serine 18 and 19 when cells were placed under mechanical stretch for 30 minutes. Our data demonstrate that 10 minutes of mechanical stimuli changed the morphology of cells into an extended shape and pPKC α s657 and pMLC2s19 were activated by SDC4, but not by the integrins RGD motif. Immunoblotting and ELISA results further confirmed that the phosphorylation of MLC2 was associated with the activity of PKC α . The ligation of cell surface receptors and matrix determines specific mechanotransduction signaling pathways in cells.

SDC4 increased the phosphorylation of myosin II at the early 10 minute time point, a process that may be mediated by PKC α -dependent activation of RhoA [24]. After 30 minutes of mechanical stimuli, the phosphorylation of myosin II was reduced; down regulation may be induced by p190RhoGAP [49]. Our data suggest that SDC4 activates PKC α to turn on the downstream kinase before p190RhoGAP during mechanical stimuli. Otherwise, RGD binding motif-mediated suppression of myosin II phosphorylation may be induced by the FAK—Src—p190RhoGAP pathway [50]. Furthermore, serine 183 phosphorylation in SDC4 is critical for regulation of PKC α activity [51]. The conformational change near the C2 region in phosphorylated SDC4 [52] suppresses the activity of PKC α [53]. Our data show that SDC4 promotes the activity of PKC α during the early stages of mechanical stress and suppresses it at the later stages. It is likely that phosphorylation at serine 183 [7] at different time points plays a role in regulating the activity of PKC α .

Conclusion

In summary, we applied mechanical stimulation with a modified pressure-driven cell-stretching (PreCS) device to elucidate the role of SDC4 in tumor cells. By using immobilized ECM molecules pre-coated with elastomeric PDMS membrane, the signals transmitted by

engagement of fibronectin, RGD, or SDC4 were analyzed. Our experiments show that SDC4 supports cells spreading on the elastomeric matrix and acts as a mechanotransducer to initiate the phosphorylation of PKC α at threonine 638 and serine 657, FAK at tyrosine 397, and ERK1/2 (Fig. 8, solid arrow). Furthermore, we discovered that phosphorylation of MLC2 at serine 18 and 19 was associated with the activity of PKC α (Fig. 8, dotted arrow). These findings help us to understand the functions of SDC4 in sensing microenvironmental force during tumor cell progression.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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