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Tumor promoting effect of podoplanin-positive fibroblasts is mediated by enhanced RhoA activity

Suguru Ito [a,b], Genichiro Ishii [b,*], Ayuko Hoshino [a,b], Hiroko Hashimoto [b], Shinya Neri [b,c], Takeshi Kuwata [b], Morihiro Higashi [d], Kanji Nagai [c], Atsushi Ochiai [a,b,*]

[a] Laboratory of Cancer Biology, Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan
[b] Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan
[c] Thoracic Oncology Division, National Cancer Center Hospital East, Chiba, Japan
[d] Department of Pathology, Saitama Medical Center, Saitama Medical University, Saitama, Japan

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ABSTRACT

There is growing evidence that stromal fibroblasts can promote tumor progression via several mechanisms. We previously reported that podoplanin (PDPN) expressed on stromal fibroblasts is functionally protein responsible for the promotion of tumor formation in mouse subcutaneous tissue. The purpose of the present study was to reveal the molecular mechanism by which PDPN on stromal fibroblasts promotes tumor formation. The subcutaneous co-injection of the human lung adenocarcinoma cell line A549 and human fibroblasts (hFbs) overexpressing wild-type podoplanin (WT-PDPN) promoted subcutaneous tumor formation, compared with the co-injection of A549 and control hFbs (64% vs 21%). On the other hand, hFbs expressing PDPN mutant in which the cytoplasmic domain of PDPN was deleted (PDPN-Del.IC), resulted in a relatively lower level of tumor formation (33%). Since PDPN reportedly regulates RhoA activity through its cytoplasmic domain, we measured the activation state of RhoA in hFbs expressing WT-PDPN. RhoA activity was 2.7-fold higher in WT-PDPN expressing hFbs than in control hFbs. Furthermore, the subcutaneous co-injection of hFbs expressing constitutive active RhoA (G14VRhoA) and A549 cells enhanced tumor formation compared with the co-injection of the same cell line and control hFbs. These results indicate that enhanced RhoA activity in hFbs expressing PDPN may be one of the mechanisms resulting in the promotion of tumor formation, suggesting that biomechanical remodeling of the microenvironment by stromal fibroblasts may play important roles in tumor progression.

1. Introduction

Cancer tissue is composed of not only cancer cells but also several types of stromal cells including fibroblasts, immune cells and endothelial cells. These stromal cells, especially fibroblasts are thought to be functionally organized to influence the proliferation, survival or invasion of cancer cells [1–3]. The biological importance of stromal fibroblasts in tumor progression has been supported by clinicopathological evidences and experimental animal models [4]. Accumulating evidence suggests that stromal fibroblasts can promote tumor progression by several mechanisms such as by inducing angiogenesis, secreting growth factors, and remodeling the extracellular matrix [5–7]. Recently, the presence of physical tracks in the extracellular matrix created by stromal fibroblasts has been proposed as a mechanism of cancer cell invasion [8].

Podoplanin (PDPN) is a transmembrane protein that consists of 162 amino acids. PDPN is reportedly expressed in several cancer cells. The extracellular domain of PDPN binds to CLEC-2 [9–13], and CD44 [14]. On the other hand, the ERM binding domain is the only known functional domain in the intercellular region of PDPN. PDPN reportedly regulates RhoA activity through its ERM binding domain [15].

We previously reported that PDPN is expressed in cancer associated fibroblasts and that the presence of PDPN(+) human fibroblasts predicted a poor outcome of patients with lung adenocarcinoma [16,17]. Furthermore we also confirmed that PDPN(+) human fibroblasts enhance A549 (human lung adenocarcinoma cell line) tumor formation using a mouse xenograft model and that PDPN is a functional protein responsible for the promotion of tumor formation [18]. However, the molecular mechanism by which PDPN in fibroblasts promotes A549 tumor formation remains unknown.

In our previous reports, A549 did not express CLEC-2 or PDPN [18], suggesting that PDPN expressing fibroblasts do not interact

* Corresponding authors. Address: Pathology Division, Research Center for Innovative Oncology, National Cancer Center, Kashiwa, 6-5-1 Kashiwanoha, Kashiwa-City, Chiba 277-8577, Japan. Fax: +81 4 7134 6865.
E-mail addresses: gishii@east.ncc.go.jp (G. Ishii), aochiai@east.ncc.go.jp (A. Ochiai).

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directly with A549 cells through CLEC-2. Therefore, we explored whether the intracellular domain of PDPN expressed by human fibroblasts has a functional role in the promotion of A549 tumor formation. First, we compared tumor formation using human fibroblasts expressing mutant PDPN in which the intracellular domain of wild type PDPN had been deleted (PDPN-Del.IC), and hFbs expressing wild-type PDPN (PDPN-WT). We also examined the possibility that human fibroblasts expressing PDPN may promote tumor formation via a higher RhoA activity level.

2. Materials and methods

2.1. Cell culture

Human fibroblasts (hFbs) from vascular adventitia obtained from surgically resections at our hospital were cultured as described previously [19,20]. The human lung adenocarcinoma cell line A549 (RIKEN BioResource Center) was cultured in DMEM (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, Sigma), and 1% penicillin and streptomycin (Sigma); the cultures were incubated at 37 °C in an atmosphere containing 5% CO_2. The study was approved by the Institutional Review Board of the National Cancer Center.

2.2. Constructs

Constructs for wild-type podoplanin (PDPN-WT) and mutant podoplanin, lacking the cytoplasmic region (PDPN-Del.IC) (Fig. 1A) were generated by synthesizing each cDNA carrying *BamH1* and *Eco*RI restriction sites to facilitate subcloning into the CSII-CMV-RfA-IRES2-Venus vector (RIKEN BioResource Center). cDNAs for the PDPN-WT (residues 1–162) and PDPN-Del.IC (residues 1–153) were obtained by PCR amplification using the pcDNA3/human-WT-podoplanin vector (kindly provided by Dr. N. Fujita, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research). The oligonucleotides used for the amplification of all these constructs are described in Table S1 in Supplementary material. All PCR-derived constructs were sequenced using the Genetic Analyzer 3500 (Hitachi, Japan) to confirm that the nucleotide sequences were correct.

Human RhoA (G14VRhoA) lentivirus vector was generated by subcloning pcDNA3/G14VRhoA (Cell Biolabs Inc., CA) into the CSII-CMV-RfA-IRES2-Venus vector.

2.3. Transfection

The lentiviruses were produced using 293T cells transfected with PCAG-HIV, pCMV-VSV-G-RSV-Rev, and either a PDPN-WT, PDPN-Del.IC, or G14VRhoA vector (CSII-CMV-RfA-IRES2-Venus). Transfection was achieved using LipofectAMINE 2000 reagent (Invitrogen, CA) according to the manufacturer's instructions. Vector-containing medium was filtered through a 0.45 μm filter and 8 μg/mL of Polybrene (Sigma) was added for target cell transduction.

2.4. Flow cytometry

The cells were incubated with anti-podoplanin antibody (gp36, clone 18H5, Abcam, Cambridge, UK) and excess antibody was removed by washing with PBS (containing 1% FBS). Goat anti-mouse IgG F(ab′)$_2$-APC (Santa Cruz, CA) was added as a secondary antibody. The cells were then rinsed with PBS and a FACS scan was performed using FACSCalibur (BD Biosciences).

2.5. Animal studies

All the animal experiments were performed in accordance with a protocol approved by the local Animal Experiment Committee of the National Cancer Center (K03-011). Female SCID mice (Charles River Lab) between 8 and 12 weeks old were used. A549 cells and human fibroblasts were co-injected into the subcutaneous tissue of SCID mice. The tumor volume was calculated as the product of a scaling factor of 0.52 and the tumor length, width, and height.

2.6. Histological examination

All the engrafted tumor specimens were fixed in 10% formalin and embedded in paraffin. Serial 4-μm sections were stained with hematoxylin and eosin. For immunohistochemical staining, the sections were deparaffinized and heated in citric acid buffer solution at 95 °C for 20 min. The endogenous peroxidases were quenched with 0.3% H_2O_2 in PBS. The sections were incubated overnight at 4 °C using mouse anti-human MIB-1 antibody (Dako Cytomation, Denmark). The sections were then washed and incubated using the Envision+® system (Dako Cytomation) at room temperature. The color reaction was developed in 2% 3,3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% H_2O_2.

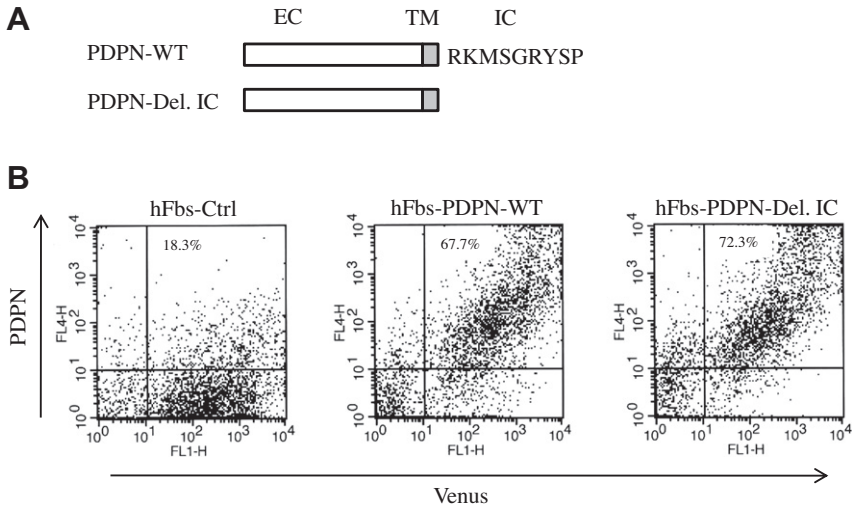

Fig. 1. (A) Structure of wild-type (WT) PDPN with extracellular (EC), transmembrane (TM), and intracellular (IC) domains and PDPN construct lacking the IC domain (Del.IC). (B) Expression of wild-type PDPN, mutant PDPN (Del.IC), or empty vector (Ctrl) in hFbs. The expression of PDPN on the cell surface was detected using flow cytometry.

The sections were counterstained with Meyer's hematoxylin, dehydrated and mounted. The proliferative activities of the tumor cells assessed using the MIB-1 labeling index were calculated as the percentage of at least 500 tumor cells.

2.7. Western Blot analysis

The cells were lysed in whole-cell extraction buffer (20 mM HEPES–NaOH, 0.5% NP-40, 15% glycerol) containing complete, a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). The proteins were separated on a 12% SDS–polyacrylamide gel and transferred to an Immobilon-P, PVDF membrane (MILLIPORE, Billerica, MA). The blots were incubated overnight at 4 °C with anti-human mouse monoclonal RhoA antibody (Cell Signaling, CA) or polyclonal goat actin antibody (Santa Cruz). After washing in TBS-T, the membranes were incubated with HRP-rabbit anti-mouse IgG or HRP-rabbit anti-goat IgG (Zymed, San Francisco, CA). ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) were used to develop the high-performance chemiluminescence film (GE Healthcare, Buckinghamshire, UK).

2.8. Cell growth in soft agar

The anchorage-independent growth of A549 cells with human fibroblasts was examined using a colony formation assay in soft agar (Difco™ Agar Noble, BD, Sparks, MD). A total of 5×10^3 cells/35 mm dish were suspended in culture medium containing 0.4% agar (1.5 mL) and immediately overlaid onto a 0.5% bottom agar in the culture medium (3 mL). The cells were then incubated at 37 °C. Two weeks later, the number of colonies with a diameter greater than 200 μm was counted.

2.9. Measurement of RhoA activity

Direct RhoA activation was measured using a G-LISA assay (Cytoskeleton Inc., CO) according to the manufacturer's instructions. Briefly, the RhoA G-LISA kit used 96-well plates coated with the Rho-binding domain of the RhoA effector Rhotekin. Rho-GDP was removed during washing steps and Rho-GTP was detected using a RhoA-specific antibody and chemiluminescence.

3. Results

3.1. PDPN mutant protein

We generated human fibroblasts expressing a wild-type PDPN (hFbs-PDPN-WT) and a mutant form of PDPN lacking the cytoplasmic region (hFbs-PDPN-del.IC) (Fig. 1A). Flow cytometry using antibodies recognizing the extracellular domain of PDPN revealed that the expression level of cell surface PDPN in cells expressing PDPN-del.IC was similar to that in cells expressing PDPN-WT (Fig. 1B).

3.2. PDPN cytoplasmic domain mediated enhanced tumor formation in mouse subcutaneous tissue

A549 cells (1×10^5) were subcutaneously injected with either hFbs-PDPN-WT or hFbs-PDPN-Del.IC (1×10^5 cells each) into SCID mice. The tumor formation rate of A549 cells co-injected with hFbs-Ctrl was 21% ($n = 24$), at 2 weeks (Fig. 2A). On the other hand, the tumor formation rate of A549 cells co-injected with hFbs-PDPN-WT and hFbs-PDPN-del.IC was 64% ($n = 22$) and 33% ($n = 24$), respectively, at 2 weeks. At 3 weeks after the injection, however, the tumor formation rates among these three groups were almost equal. The growth kinetics of these three groups of engrafted tumors was also similar once the tumors became detectable (data not shown).

The histological examination revealed that engrafted tumors displayed moderately to poorly differentiated adenocarcinoma, and no distinct differences in morphological feature were obvious among the three groups (Fig. 1B). No significant differences in the proliferative activity (as revealed by Ki-67 staining) of the engrafted tumor cells were seen (Fig. 2C). These results suggested that fibroblasts expressing PDPN did not affect the proliferation of the A549 cells, but promoted the tumor engraftment in SCID mice.

We next performed an *in vitro* colony assay. The number of the colonies with a diameter over 200 μm was significantly increased when the A549 cells were seeded with hFbs-Ctrl, hFbs-PDPN-WT, or hFbs-PDPN-Del.IC compared with A549 cells alone. However, no significant differences in the large colony numbers for A549 seeded with hFbs-Ctrl, hFbs-PDPN-WT, or hFbs-PDPN-Del.IC were seen (Fig. 2D).

3.3. hFbs-PDPN-WT exhibited higher RhoA activity

In MDCK cell (Madin–Darby canine kidney cell), PDPN cytoplasmic domain reportedly regulates RhoA activity via the ERM binding motif. We measured the RhoA activity of hFbs-Ctrl, hFbs-PDPN-WT and hFbs-PDPN-Del.IC using G-LISA. In contrast to the basal level of active RhoA (RhoA-GTP) in hFbs-Ctrl, the activity of RhoA increased by 2.7-fold in hFbs-PDPN-WT ($P = 0.03$). The limited activation of RhoA was also found in hFbs-PDPN-del.IC (1.9-fold Fig. 3A).

3.4. hFbs expressing constitutively active (G14V) RhoA mutant enhance tumor formation

We hypothesized that the activity of RhoA in fibroblasts affected the tumor formation of A549. To clarify this hypothesis, we generated hFbs expressing a constitutive active form of RhoA (G14VRhoA). The activity of RhoA increased by 4.1-fold in hFbs-G14VRhoA (Fig. 3B). Supplemental Fig. 1 showed the result of Western Blot analysis. In hFbs expressing wild-type RhoA, an enhanced band at around 25 kDa can be detected (positive control). The same enhanced molecular weight protein can be detected in G14VRhoA-transfected hFbs. PDPN-WT transfectants did not exhibit an increase in endogenous RhoA protein. Flow cytometry analyses revealed that hFbs-Ctrl and hFbs-G14VRhoA express similar levels of PDPN at their cell surface (Fig. 3C).

A549 cells were subcutaneously co-injected with either hFbs-Ctrl or hFbs-G14VRhoA into SCID mice. The co-injection of G14V-RhoA-expressing hFbs and A549 cells resulted in a tumor formation rate of 75% at week two ($n = 12$) whereas tumor formation was only observed in 33% ($n = 12$) of the animals co-injected with control hFbs (Fig. 4A). At 3 weeks after the injection, however, the tumor formation rates were almost equal. The growth kinetics of the two groups of tumors were similar once the tumors became detectable (data not shown).

In an *in vitro* colony assay, no differences were observed in the large colony number for A549 cells co-cultured with hFbs-Ctrl or hFbs-G14VRhoA (Fig. 4B).

4. Discussion

In the current study, we first explored how PDPN in stromal fibroblasts is molecularly involved in the enhancement of tumor formation. PDPN expressing stromal fibroblasts exhibit higher levels of RhoA activity, promoting tumor formation in mouse subcutaneous tissue. These results provide insight into the molecular basis for both the clinicopathological importance of PDPN-positive

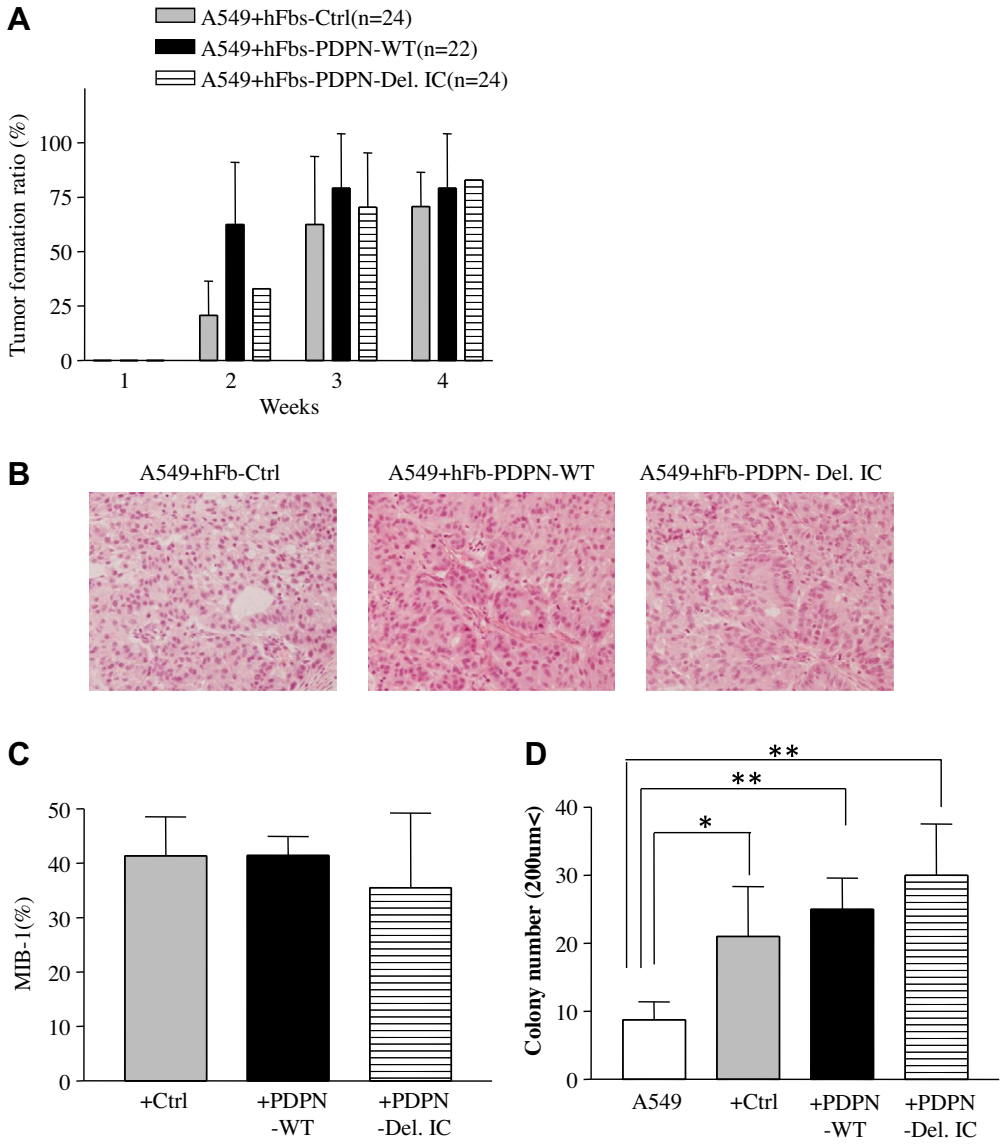

Fig. 2. (A) Tumor formation rates (mean ± SD) after the subcutaneous injection of A549 cells with hFbs into SCID mice. (B) Representative hematoxylin-and-eosin-stained sections of the tumors formed by A549+hFbs-Ctrl, A549+hFbs-PDPN-WT, and A549+hFbs-PDPN-Del.IC. (C) Proliferative activity of engrafted tumor cells confirmed by MIB-1 staining. The error bars show the mean ± SD. (D) Colony formation assay. Colonies with a diameter greater than 200 μm were counted for A549 cells alone or for cells seeded with hFbs. The error bars show the mean ± SD. The data shown are representative of multiple experiments. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ compared with A549 alone.

stromal fibroblasts as a prognostic factor [16,17,21] and our previously reported *in vivo* animal model findings [18].

The elucidation of tumor microenvironment remodeling mechanisms is an important area of cancer research. Several extracellular matrix (ECM) remodeling mechanisms of stromal fibroblasts have been reported [22–24]. RhoA is a master regulator of various cellular processes such as cytokinetics, cytoskeletal regulation, and cell migration [25] and the RhoA activation of stromal fibroblasts can greatly influence ECM remodeling. Rho-mediated matrix alignment reportedly plays a role in an early step in the invasion process [26]. A recent report revealed that the silencing of p190RhoGAP, an endogenous inhibitor of Rho, in mouse embryonic fibroblasts promoted stroma-dependent cancer cell invasion and metastasis using an animal model [27], suggesting that Rho activity in stromal fibroblasts may affect tumor progression. RhoA also regulates gene expression through various transcription factors such as nuclear transcription factor kappa-B (NF-κB), which activate the transcription of inflammatory cytokines [25]. Evidence indicates that the presence of inflammatory cytokines at the tumor site promotes tumor progression inducing cell survival, proliferation, invasion and metastasis [28,29]. In the current study, the enhancement of tumor formation was clearly observed only in our *in vivo* animal model, and PDPN did not function in the anchorage independent growth of cancer cells in an *in vitro* colony assay. This results suggests that the subcutaneous microenvironment, in which the fibroblasts can move and interact with A549, may be important for tumor formation. Biomechanical remodeling of the microenvironment may also be involved in the regulation of tumor engraftment.

Although the transfection of hFbs-PDPN-Del.IC decreased the ratio of tumor engraftment to a level similar to that of hFbs-Ctrl, the possibility that inside-out signals may induce a conformational change in PDPN, resulting in an ineffectual signal transduction via PDPN ligands in tumor cells, cannot be excluded.

We were able to demonstrate that PDPN knock down by ShRNA resulted in a decrease in the *in vitro* colony formation of A549 [18];

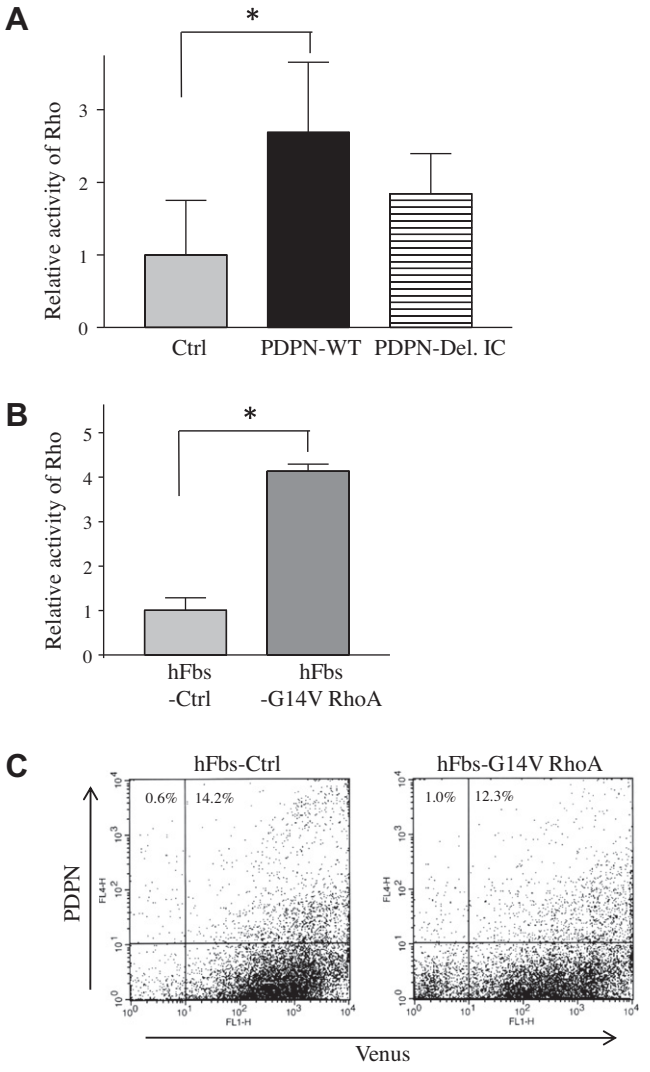

Fig. 3. (A) Levels of RhoA activity in hFbs-Ctrl, hFbs-PDPN-WT, and hFbs-PDPN-Del.IC. The error bars show the mean ± SD ($n = 4$). Asterisk, $P < 0.05$. (B) Levels of RhoA activity in hFbs-Ctrl and hFbs-G14VRhoA. The error bars show the mean ± SD ($n = 3$). Asterisk, $P < 0.01$. (C) Expression of PDPN on the cell surface (hFbs-Ctrl and hFbs-G14VRhoA) as analyzed using flow cytometry.

however, we were unable to obtain corresponding (reverse) results based on overexpression experiments in the current study. In soft agar, the expression of endogenous PDPN in hFbs may be necessary and sufficient to provide an environment conducive to the anchorage independent growth of cancer cell, whereas overexpression may have minimal phenotypic effects despite knockdown lead observable effects.

Not all the primary cultured hFbs exhibited dramatic increase in RhoA activity after the overexpression of PDPN-WT. This variation may be caused by differences in the intrinsic capacity of PDPN to activate RhoA. Alternatively, in cases where signals other than PDPN-mediated signals, such as a CD44 mediated signal, contribute largely to RhoA activation, the effect of exogenously expressed PDPN may be subtle.

In conclusion, we explored how PDPN, a functional protein expressed on fibroblasts, promotes tumor engraftment via increasing RhoA activity. Considering the current results and previous reports indicating that RhoA is over-expressed and/or highly activated in many solid cancer cells [30], the malignant potential of the cancer microenvironment may be associated with the activity of RhoA in both cancer cells and cancer associated fibroblasts. Our results also suggested that the targeting of RhoA in both cancer cells and stromal fibroblasts expressing PDPN may be effective for cancer therapy. Further evaluation of the roles of the PDPN–RhoA axis in stromal fibroblasts may reveal a mechanism explaining how the microenvironment supports tumor progression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.158.

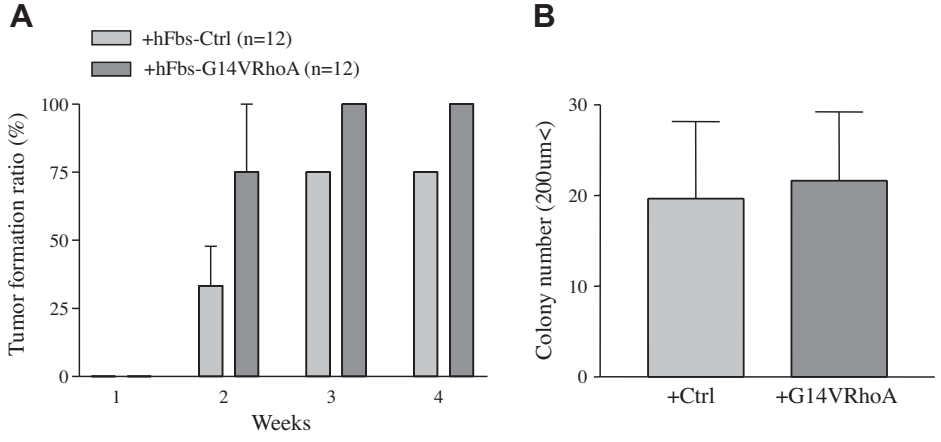

Fig. 4. (A) Tumor formation rates (mean ± SD) after the subcutaneous injection of A549 cells with hFbs-Ctrl or hFbs-G14VRhoA. (B) Colony formation assay. Colonies with a diameter greater than 200 μm were counted for A549 cells alone or for cells seeded with hFbs. The error bars show the mean ± SD.

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