PDZK1-interacting protein 1 (PDZK1IP1) traps Smad4 protein and suppresses transforming growth factor-β (TGF-β) signaling

Received for publication, May 24, 2018, and in revised form, December 27, 2018. Published, Papers in Press, February 4, 2019, DOI 10.1074/jbc.RA118.004153

Souichi Ikeno1, Naoko Nakano3, Keigo Sano3, Takashi Minowa5, Wataru Sato5, Ryosuke Akatsu4, Nobuo Sakata3, Nobutaka Hanagata5, Makiko Fujii6, Fumiko Itoh7, and Susumu Itoh2

From the Laboratory of Biochemistry, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan, Nanotechnology Innovation Station, National Institute of Materials Science, Tsukuba, Ibaraki 305-0047, Japan, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Hiroshima 734-8553, Japan, and Laboratory of Cardiovascular Medicine, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

Edited by Xiao-Fan Wang

Transforming growth factor (TGF)-β signaling in humans is stringently regulated to prevent excessive TGF-β signaling. In tumors, TGF-β signaling can both negatively and positively regulate tumorigenesis dependent on tumor type, but the reason for these opposite effects is unclear. TGF-β signaling is mainly mediated via the Smad-dependent pathway, and herein we found that PDZK1-interacting protein 1 (PDZK1IP1) interacts with Smad4. PDZK1IP1 inhibited both the TGF-β and the bone morphogenetic protein (BMP) pathways without affecting receptor-regulated Smad (R-Smad) phosphorylation. Rather than targeting R-Smad phosphorylation, PDZK1IP1 could interfere with TGF-β- and BMP-induced R-Smad/Smad4 complex formation. Of note, PDZK1IP1 retained Smad4 in the cytoplasm of TGF-β-stimulated cells. To pinpoint PDZK1IP1’s functional domain, we created several PDZK1IP1 variants and found that its middle region, from Phe40 to Ala49, plays a key role in its Smad4-regulating pathway. PDZK1IP1 knockdown enhanced the expression of the TGF-β target genes Smad7 and prostate transmembrane protein androgen-induced (TMEPAI) upon TGF-β stimulation. In contrast, PDZK1IP1 overexpression suppressed TGF-β-induced reporter activities, cell migration, and cell growth inhibition. In a xenograft tumor model in which TGF-β was previously shown to elicit tumor-promoting effects, PDZK1IP1 gain of function decreased tumor size and increased survival rates. Taken together, these findings indicate that PDZK1IP1 interacts with Smad4 and thereby suppresses the TGF-β signaling pathway.

PDZK1-interacting protein 1 (PDZK1IP1),3 alternatively termed MAP17, DD96, and SPAP, was originally identified as an epithelium-specific molecule (1, 2). Furthermore, it was found to be expressed at high levels in various human carcinomas (3, 4). Indeed, overexpression of PDZK1IP1 inhibited tumor necrosis factor-α–induced G1 arrest via impairment of p21 induction (3). PDZK1IP1 also decreases the c-Myc–mediated caspase3-like activity in Rat1 fibroblasts in low serum to retain the phosphatidylinositol 3-kinase/Akt signaling pathway. Consistently, PDZK1IP1-expressing cells proliferate in nude mice (5). Furthermore, an increase in reactive oxygen species has been reported to correlate with tumorigenicity mediated by PDZK1IP1, whereas treatment of PDZK1IP1-expressing cells with antioxidants led to loss of tumorigenicity (6). Recently, PDZK1IP1 was shown to activate Notch signaling to regulate a cancer stem cell pool (7). Furthermore, it was reported that the expression of PDZK1IP1 is correlated with tumorigenicity in lung adenocarcinoma (8). Conversely, overexpression of PDZK1IP1 in a colon carcinoma cell line decreased cell growth in vivo and in vitro (1, 2), and patients with laryngeal carcinoma, in which PDZK1IP1 is highly expressed, had prolonged laryngoesophageal dysfunction–free survival after chemotherapy (9). Thus, PDZK1IP1 might be involved in tumorigenicity either negatively or positively dependent on the tumor type.

The abbreviations used are: PDZK1IP1, PDZK1-interacting protein 1; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; TMEPAI, transmembrane protein androgen-induced; SLC, solute carrier; TβRII, TGF-β type II receptor; TβRI, TGF-β type I receptor; AR-Smad, TGF-β-activin R-Smad-Smad; SBE, Smad-binding element; BRE, BMP-responsive element; ca, constitutively active; PLA, proximity ligation assay; HA, hemagglutinin; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PEI, polyethyleneimine; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; qPCR, quantitative PCR; PFA, paraformaldehyde; DAPI, 4’,6-diamidino-2-phenylindole; co-IP, coimmunoprecipitation.

This work was supported by Grant-in-aid for Young Scientists (B) 15K18866 (to N. N.) and the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) (to S. Itoh) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT); the Takeda Science Foundation (to S. Itoh); the Daiichi-Sankyo Foundation of Life Science (to S. Itoh); the Naito Foundation (to S. Itoh); the Vehicle Racing Commemorative Foundation (to S. Itoh); the Mitsubishi Foundation (to S. Itoh); the Science Research Promotion Fund (S. Itoh); the Research Foundation for Pharmaceutical Sciences (to N. N.); the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (Grant 15S131012) (to N. N. and S. Itoh); and a grant-in-aid for young scientists of Showa Pharmaceutical University (to S. Ikeno and N. N.). This work was also supported by the Joint Usage/Research Program of the Medical Research Institute, Tokyo Medical and Dental University and by the Core-to-Core program “Cooperative International Framework in TGF-β Family Signaling” of the Japan Society for the Promotion of Science. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Fig. S1.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: Laboratory of Biochemistry, Showa Pharmaceutical University, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194-8543, Japan. Tel: 81-41-721-1558; Fax: 81-41-721-1588; E-mail: sitoh@ac.shoyaku.ac.jp.

3 The abbreviations used are: PDZK1IP1, PDZK1-interacting protein 1; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; TMEPAI, transmembrane protein androgen-induced; SLC, solute carrier; TβRII, TGF-β type II receptor; TβRI, TGF-β type I receptor; AR-Smad, TGF-β-activin R-Smad-Smad; SBE, Smad-binding element; BRE, BMP-responsive element; ca, constitutively active; PLA, proximity ligation assay; HA, hemagglutinin; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PEI, polyethyleneimine; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; qPCR, quantitative PCR; PFA, paraformaldehyde; DAPI, 4’,6-diamidino-2-phenylindole; co-IP, coimmunoprecipitation.
Besides its action in tumorigenicity, PDZK1IP1 interacts with several PDZ domain–containing molecules, including NHERFs (sodium-hydrogen antiporter 3 regulator) 1–4 and NaPiIIa and Na+/H+ hydrogen exchanger 3 (NHE3; alternatively termed solute carrier family 9A3 (SLC9A3)) (10). Furthermore, PDZK1IP1 can clearly contribute to the internalization of sodium-dependent phosphate transport protein 2b (NaPiIIa; alternatively termed SLC34A1) in the trans-Golgi network (10). In addition, PDZK1IP1 showed stimulation of Na-dependent transport of mannose and glucose in Xenopus oocytes and mammalian cell lines because PDZK1IP1 acted as a required β-subunit for sodium-dependent glucose cotransporter 2 (SGLT2) (11–13). Therefore, PDZK1IP1 is thought to participate in enhancement of the endogenous uphill transport system in the kidney as well.

Cancer cells are originally generated from a normal cell via several mutation steps in its genome. Recently, Hanahan and Weinberg (14) proposed that the process of tumorigenicity requires 10 kinds of hallmarks. Among these hallmarks, transforming growth factor-β (TGF-β) is known to suppress cell growth through G1 arrest during the cell cycle and/or apoptosis in normal and premalignant cells, whereas this cytokine can promote tumorigenicity in malignant and metastatic cells via TGF-β–mediated immunosuppression, growth factor production, motility, and angiogenesis when cancer cells lose tumor-suppressive responses to TGF-β (15, 16). TGF-β is also involved in embryogenesis and tissue homeostasis (17, 18). Thus, TGF-β is considered to be a multifunctional cytokine.

TGF-β signaling is mainly mediated via the Smad-dependent pathway. This pathway is initiated through ligand binding to TGF-β type II receptor (TβRII). In the Smad-dependent pathway, constitutively active serine/threonine kinase in the cytoplasmic region of TβRII can phosphorylate the glycine/serine-rich domain in the juxtamembrane region of TGF-β type I receptor (TβRI or ALK5) to activate TβRII serine/threonine kinase. Then the active TβRII kinase catalyzes the phosphorylation of TGF-β/activin receptor–regulated-Smads (AR-Smads; i.e., Smad2 and Smad3). The two phosphorylated AR-Smads form a ternary complex with Smad4 to translocate to the nucleus where the complex interacts with myriad transcriptional factors and cofactors to control TGF-β target genes (19–21). To date, it has been reported that dysregulation of TGF-β signaling is implicated in development of various diseases, including cancer, fibrosis, and vascular disorders (22). To avoid excessive TGF-β signaling in cells, therefore, TGF-β signaling is governed by a great number of gatekeepers present from the extracellular microenvironment to the nucleus (23–26). However, how global fine-tuning of TGF-β signaling in cells is controlled by each molecule is not still understood. In the present study, we explored the role of PDZK1IP1 in the TGF-β pathway and found that PDZK1IP1 is a novel interacting partner with Smad4 to perturb TGF-β signaling.

Results

Inhibition of TGF-β signaling by PDZK1IP1

We investigated whether PDZK1IP1 affects the TGF-β signaling using the TGF-β/activin–induced Smad-driven transcriptional (CAGA)12–luc reporter (27). PDZK1IP1 dose-dependently inhibited the activity of the luciferase reporter when cells were stimulated with TGF-β (Fig. 1A). We also observed inhibitory actions of PDZK1IP1 on TGF-β–induced reporter activities using p800-Luc (Fig. 1B) and pGL3ti-850-luc (Fig. 1C), both of which possess the enhancer regions of TGF-β target genes (28, 29). Curiously, we could see the efficiently inhibitory effect of PDZK1IP1 on TGF-β–mediated luciferase activity of both p800-Luc and pGL3ti-850-luc reporters compared with that of the (CAGA)12–luc reporter. Unlike the (CAGA)12–luc reporter that is an artificial promoter including only binding elements for Smad3 and Smad4, both p800-Luc and pGL3ti-850-luc reporters possess natural promoters where other transcription factor(s) except for Smad3 and Smad4 can possibly lie. Thus, it is possible that PDZK1IP1 might also inhibit the function of other transcription factors that can bind to the promoter regions of both p800-Luc and pGL3ti-850-luc reporters. The introduction of Smad4 into MDA-MB468 cells that genetically lack functional Smad4 genes improved TGF-β responsiveness, whereas PDZK1IP1 perturbed TGF-β signaling in MDA-MB468 cells carrying Smad4 (Fig. 1D). Because Smad4 is required for not only the TGF-β/Smad pathway but also the BMP/Smad pathway, the (SBE)4–luc reporter, which is also activated by BMP, was transfected together with PDZK1IP1 in HepG2 cells to investigate whether PDZK1IP1 perturbs the BMP signaling. As seen in Fig. 1E, PDZK1IP1 could counteract BMP signaling as it did TGF-β signaling. Consistently, PDZK1IP1 inhibited the activities of other BMP-induced luciferase reporters, Id-1–luc (Fig. 1F) and (BRE)2–luc (Fig. 1G), upon BMP stimulation. Furthermore, overexpression of PDZK1IP1 (Fig. 1H) revealed marginal inhibition of TGF-β–induced cell migration (Fig. 1I) and significant inhibition of TGF-β–mediated cell growth arrest (Fig. 1J) in A549 cells. These results demonstrated that PDZK1IP1 has the ability to interfere with TGF-β family signaling.

Interaction of PDZK1IP1 with Smads

Because PDZK1IP1 inhibits TGF-β family signaling, we attempted to determine whether PDZK1IP1 influences Smad phosphorylation induced by the TGF-β family. The results showed that PDZK1IP1 affected neither constitutively active ALK5 (ALK5ca)-mediated Smad2 nor constitutively active ALK6 (ALK6ca)-mediated Smad1 phosphorylation (Fig. 2, A and B). However, PDZK1IP1 interacted with Smad2, Smad3, and Smad4 dependent on the ALK5 activation. Particularly, we could observe a strong interaction of PDZK1IP1 with Smad2 and Smad4 (Fig. 2C). Furthermore, its interaction with Smad8 and Smad4 could be detected upon BMP receptor activation (Fig. 2D). Thus, we supposed that PDZK1IP1 mainly interacts with Smad4 to interfere with its downstream signaling. For the above reason, in the following experiments, we took particular note of Smad4 because of its requirement for both the TGF-β and the BMP signaling pathways. Because we were not successful in showing endogenous interaction between PDZK1IP1 and Smad4 using the communoprecipitation assay, we adopted a proximity ligation assay (PLA) to observe their interaction. Without TGF-β stimulation, we could not see any of the red dots that reveal interaction between the two proteins. However,
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treatment of cells with TGF-β exhibited a remarkable number of red spots (Fig. 3A). When PDZK1IP1 was knocked down in PC-3 cells (Fig. 3B), their interaction was lost even though the cells were treated with TGF-β (Fig. 3C). To confirm their interaction further, we transiently introduced PDZK1IP1/HA into A549 cells and then stained the cells with anti-Smad4 (green) and anti-HA (red) antibodies. Their colocalization could be detected in the cytosol with or without TGF-β stimulation.
Although Smad4 is localized in the nucleus upon TGF-β stimulation in the absence of PDZK1IP1, overexpression of PDZK1IP1 retained Smad4 in the cytosol (arrows) (Fig. 3D). These results prompted us to examine whether PDZK1IP1 disturbs the interaction between Smad2 and Smad4 upon TGF-β stimulation. Expectedly, PDZK1IP1 dissociated the ALK5ca-mediated formation of a complex between Smad2 and Smad4 (Fig. 3E). By the same token, ALK6ca-mediated interaction between Smad5 and Smad4 was disrupted by PDZK1IP1 (Fig. 3F).

**Determination of the functional domain in PDZK1IP1**

To evaluate the functional domain(s) in PDZK1IP1 in terms of its inhibitory activity on TGF-β signaling, we made three mutants: PDZK1IP1Δ(I65–M114), PDZK1IP1Δ(V36–M114), and PDZK1IP1Δ(V36–M114, I65–M114).
Inhibition of TGF-β family signaling by PDZK1IP1

(A) TGF-β PLA DAPI Merge

(-)

(+) 10 μm

(B) PC-3 cell

Cont sh-4 sh-5

IB: PDZK1IP1

23 14

IB: β-actin

41 32

(C) TGF-β PLA DAPI Merge

(-)

(+) 10 μm

(D) TGF-β (i) Smad4 (ii) PDZK1IP1 (iii) Merge (iv) DAPI

(-)

(+) 10 μm

(E) 6 x Myc-Smad4

Flag-Smad2 - + + + + +
ALK5ca/HA - - - + + +
PDZK1IP1/V5 - - - - - - (kDa)

IP: Flag
IB: Myc
IB: Myc
IB: Flag
IB: PS2
IB: HA
IB: V5

(F) 6 x Myc-Smad4

Flag-Smad5 - + + + + +
ALK5ca/HA - - - + + +
PDZK1IP1/HA - - + - + -

IP: Flag
IB: Myc
IB: Myc
IB: Flag
IB: PS1
IB: V5
IB: HA

Smad4

Myc

Flag

PS1

V5

PDZK1IP1

Smad4

Myc

Flag

PS1

V5

PDZK1IP1
and PDZK1IP1Δ(M1–A49) (Fig. 4A). Among them, PDZK1IP1Δ(M1–A49) lost the ability to inhibit TGF-β signaling, indicating that the N-terminal region of PDZK1IP1 plays a key role in the inhibitory action of TGF-β signaling (Fig. 4B). To further narrow down its functional domain, we made several N-terminal deletion mutants of PDZK1IP1 (Fig. 4C). PDZK1IP1Δ(M1–W30) could still inhibit TGF-β-mediated luciferase activity, although this inhibitory activity was lower than that of the WT (Fig. 4D). Similarly, BMP signaling was also disturbed by PDZK1IP1Δ(M1–W30) (Fig. 4E). Thus, the region between Met1 and Ala49 in PDZK1IP1 is possibly needed for PDZK1IP1 to inhibit TGF-β family signaling. Indeed, the association between PDZK1IP1 mutants and Smad4 became weaker in proportion to loss of the N-terminal length of PDZK1IP1 (Fig. 4F). PDZK1IP1Δ(M1–W30) can adequately interfere with the reporter activity induced by both TGF-β and BMP despite its marginal association with Smad4. Therefore, it is possible that the weak interaction is enough for PDZK1IP1 to inhibit TGF-β family signaling to some extent. When PDZK1IP1Δ(F40–A49) (Fig. 4G) was introduced into cells to examine whether this mutant possesses inhibitory activity, it did not reveal obvious inhibitory activity for TGF-β signaling (Fig. 4H). To further evaluate whether this 10-amino acid–long region derived from PDZK1IP1 is sufficient to bind to Smad4, we performed a GST pulldown assay using GST-PDZK1IP1(M1–A49) and GST–PDZK1IP1(F40–A49) (Fig. 4I). Smad4 could obviously bind to both GST-PDZK1IP1(M1–A49) and GST-PDZK1IP1(F40–A49), unlike Smad2 and Smad3. These results demonstrated that this 10-amino acid–long region is enough for PDZK1IP1 to interact with Smad4 (Fig. 4J).

Interaction of PDZK1IP1 with Smad4 MH1

To test which domain of Smad4 is able to interact with PDZK1IP1, we used several Smad4 mutants: MH1 alone (Smad4A), MH1 + linker (Smad4B), linker + MH2 (Smad4C), MH2 alone (Smad4D), and linker alone (Smad4L) (Fig. 5A). Among them, Smad4A and Smad4D could show obvious interaction with PDZK1IP1, although the nonspecific band corresponding to PDZK1IP1 could be seen when Smad4B was transfected. In addition, Smad4D revealed interaction with PDZK1IP1. In contrast, the interaction of PDZK1IP1 with Smad4C and Smad4L could not be detected (Fig. 5B). Thus, the MH1 domain of Smad4 seems a main part to interact with PDZK1IP1, although its MH2 domain somewhat possesses the ability to associate with PDZK1IP1. Therefore, there is a possibility that the linker region of Smad4 hinders the interaction between Smad4 MH1 (or MH2) and PDZK1IP1 because of its steric configuration and that the MH2 domain of Smad4 might support Smad4 to associate with PDZK1IP1. Because the peptide from Phe40 to Ala49 of PDZK1IP1 bound to Smad4 (Fig. 4F), we were prompted to examine whether this peptide were able to catch Smad4 MH1. We definitely showed that only MH1 domain of Smad4 interacts with this peptide (Fig. 5C). Thus, it is possible that the full length of PDZK1IP1 associates with Smad4 MH1 to undertake steric hindrance for the interaction between receptor-regulated Smads (R-Smads) and Smad4.

Loss-of-function analysis of PDZK1IP1

Because overexpression of PDZK1IP1 blocked TGF-β signaling, the decreased expression of PDZK1IP1 supposedly enhances it. For that purpose, we established two cell lines (sh-1 and sh-3) in which PDZK1IP1 expression was drastically lowered (Fig. 6A). Indeed, shRNA-mediated depletion of PDZK1IP1 could enhance the mRNA expression of Smad7 and TMEPAI, which are well-known direct target genes for TGF-β signaling (30, 31) (Fig. 6, B and C). We also tested whether loss of function for PDZK1IP1 augmented TGF-β–mediated cell migration (Fig. 6D). One of the PDZK1IP1–knocked down cell line, sh-1, showed an obvious increase of migration activity in both the absence and presence of TGF-β compared with the pLKO.1-transfected control cell line. Conversely, highly basal migration activity in sh-3–carrying cells was seen, although TGF-β could not further enhance migration activity in sh-3–carrying cells. It is possible that the amount of secreted TGF-β from cells might be enough for sh-3–carrying cells to acquire the ability of cell migration. Thus, they might not be able to migrate in response to exogenous TGF-β. Additionally, E-cadherin expression in PC-3 cells carrying shRNAs for PDZK1IP1 was marginally decreased compared with that in mock PC-3 cells when the cells were stimulated with TGF-β (Fig. S1).

Figure 3. Cytosolic retention of Smad4 by PDZK1IP1. A, endogenous interaction between PDZK1IP1 and Smad4 in PC-3 cells. After PC-3 cells were stimulated with 1 ng/ml TGF-β for 2 h, the cells were fixed. Then rabbit anti-PDZK1IP1 and mouse anti-Smad4 antibodies were added for PLA. The upper and lower panels show cells without and with TGF-β stimulation, respectively. Left panels, the red dots indicate colocalization. Middle panels, nuclear staining with DAPI is indicated in blue. Right panels, merged images are shown. B, PC-3 cells infected with lentiviral vectors expressing shPDZK1IP1 (sh-4 and sh-5 cells) were lysed to detect expression of PDZK1IP1 protein by Western blot analysis. Upper, expression of PDZK1IP1; lower, expression of β-actin. C, loss of colocalization between PDZK1IP1 and Smad4 upon TGF-β stimulation in PC-3 cells infected with lentiviral vectors expressing shPDZK1IP1 (sh-4 and sh-5 cells). The experiments were carried out according to A. The upper and lower panels show cells without and with TGF-β stimulation, respectively. Left panels, the red dots indicate colocalization. Middle panels, nuclear staining with DAPI is indicated in blue. Right panels, Merged images are shown. D, cytosolic retention of Smad4 by overexpression of PDZK1IP1 in TGF-β–stimulated A549 cells. Twenty-four hours after transfection, the cells were stimulated with 5 ng/ml TGF-β for 2 h. Subsequently, the cells were fixed and stained with mouse monoclonal anti-Smad4 and rat monoclonal anti-HA3F10 antibodies. Then Alexa Fluor 488–conjugated goat anti-mouse (i) and Alexa Fluor 555–conjugated goat anti-rabbit IgG antibodies (ii) were used for visualization. Colocalization can be seen in the merged panels as the yellow color (iii). Nuclear staining (blue) was carried out using DAPI (iv). The arrows indicate colocalization between Smad4 and PDZK1IP1. E, disruption of the Smad2/Smad4 complex by PDZK1IP1. COS7 cells were transfected with the indicated plasmids and harvested for co-IP experiments according to Fig. 2C. Upper panel, interaction between Smad2 and Smad4 with anti-Flag antibody for immunoprecipitation and anti-Myc9E10 antibody for Western blot analysis; second panel, expression of Smad4 with anti-Myc9E10 antibody; third panel, expression of Smad2 with anti-Flag antibody; fourth panel, expression of phosphorylated Smad2 with PS2; fifth panel, expression of ALK5ca/HA with anti-HA3F10 antibody; bottom panel, expression of PDZK1IP1/V5 with anti-V5 antibody. F, disruption of the Smad4/Smad5 complex by PDZK1IP1. COS7 cells were transfected with the indicated plasmids and harvested for co-IP experiments according to Fig. 2C. Upper panel, interaction between Smad4 and Smad5 with anti-Flag antibody for immunoprecipitation and anti-Myc9E10 antibody for Western blot analysis; second panel, expression of Smad4 with anti-Myc9E10 antibody; third panel, expression of Smad5 with anti-Flag antibody; fourth panel, expression of phosphorylated Smad5 with PS1; fifth panel, expression of ALK5ca/HA with anti-HA3F10 antibody; bottom panel, expression of PDZK1IP1/V5 with anti-V5 antibody. IB, immunoblotting.
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(A) Inhibition of TGF-β family signaling by PDZK1IP1

(B) Luc assay showing inhibition of TGF-β family signaling

(C) Schematic representation of PDZK1IP1

(D) Luminescence assay showing TGF-β family signaling

(F) Schematic representation of 6 X Myc-Smad4

(G) Schematic representation of PDZK1IP1

(H) Luc assay showing inhibition of TGF-β family signaling

(I) Schematic representation of GST-(M^-A^9)

(J) Schematic representation of pDNA3

(K) Schematic representation of Flag-Smads

(2019) J. Biol. Chem.
Suppression of tumor growth by PDZK1IP1 in a xenograft model

The mesothelioma cell line NCI-H290 cannot proliferate in the presence of the TGF-β receptor kinase inhibitor. Therefore, these cells are sensitive to TGF-β signaling to proliferate (32). When we introduced PDZK1IP1 to NCI-H290 cells (Fig. 7A), TGF-β–induced Smad7 expression decreased, whereas phosphorylation of Smad2 upon TGF-β stimulation was not altered as seen in Fig. 2A (Fig. 7, B and C). After NCI-H290 cells carrying PDZK1IP1 were injected into mouse pleura, we measured the tumor volumes with bioluminescence in vivo. One week after implantation of the NCI-H290 cells, the tumor volumes of NCI-H290 cells carrying PDZK1IP1 were dramatically decreased when compared with those of mock NCI-H290 cells (Fig. 7, D and E). Kaplan–Meier analysis revealed that more than 50% of the mice implanted with NCI-H290 cells carrying PDZK1IP1 could survive for longer than 30 days, whereas all of the mice implanted with mock NCI-H290 cells died within 30 days of implantation (Fig. 7F). These results indicated that PDZK1IP1 can inhibit TGF-β signaling to suppress the growth of NCI-H290 mesothelioma cells because NCI-H290 cells might proliferate via endogenous paracrine and/or autocrine secretion of TGF-β.

Discussion

Smad4 plays a crucial role in canonical TGF-β family signaling because all phosphorylated R-Smads must associate with Smad4 to go to the nucleus and govern transcription of TGF-β family target genes (21, 33). Thus, fine control of the expression and function of Smad4 is critical for cells to transduce canonical TGF-β family signaling accurately. Until now, a great number of Smad4 interactors in cells have been found, but most of them contribute to control of Smad4-dependent transcription in the nucleus (34). Also, in the cytosol, several ubiquitin ligases interact with Smad4 to promote its proteasome-dependent degradation (25). However, apart from E3 ubiquitin ligases and other modification enzymes, little is known about Smad4 interactors that regulate TGF-β family signaling in the cytosol.

As seen in Fig. 2, C and D, PDZK1IP1 was associated with Smad4 at a relatively high affinity in addition to Smad2 and Smad8. In contrast, complex formation between PDZK1IP1 and other R-Smads was detected to a lesser extent. Because PDZK1IP1 could inhibit both TGF-β and BMP signaling (Fig. 1) without the influence of R-Smad phosphorylation, we focused on the inhibitory mechanism of Smad4 function by PDZK1IP1.

When we explored the mechanism by which PDZK1IP1 interferes with TGF-β family signaling via its association with Smad4, we found that PDZK1IP1 trapped Smad4 in the cytosol, including the perinuclear region. Indeed, it has been reported that PDZK1IP1 is located in the cytosol, Golgi apparatus, or cell membrane (2, 3, 6, 10, 35). Because the Golgi apparatus is occasionally localized close to the nucleus, our observation that PDZK1IP1 is present in the perinuclear region together with Smad4 might be convincing. Thus, PDZK1IP1 kicks out phosphorylated R-Smads from their complex with Smad4, resulting in the stable interaction of PDZK1IP1 with Smad4 to cease canonical TGF-β family signaling.

PDZK1IP1 possesses a hydrophobic region consisting of 20 amino acids. In particular, a 10-amino acid–long peptide composed of Phe40–Ala49 is enough for Smad4 to interact with PDZK1IP1. Deficiency of this 10-amino acid–long region from PDZK1IP1 led PDZK1IP1 to lose its ability to interact with Smad4, with the result that it could not inhibit TGF-β signaling. PDZK1IP1 was supposed to be integrated into membranes through its two N-terminal hydrophobic regions (2, 36). However, some reports (3, 6, 10, 35) in addition to The Human Protein Atlas (http://www.proteinatlas.org/ENSG00000162366-PDZK1IP1/cell) (46) support our results (Fig. 3, A and D) showing that PDZK1IP1 is in the cytosol in addition to the perinuclear region. Thus, the 10-amino acid–long hydrophobic region in PDZK1IP1 might recruit the MH1 and/or MH2 regions of Smad4 to anchor Smad4 in the cytosol, with the result that canonical TGF-β family signaling cannot be transduced into the nucleus. Conversely, its C-terminal PDZ-binding motif (STPM) regulates the function of NHE3 as a scaffold protein (10). Therefore, PDZK1IP1 might regulate a variety of signal transductions as well as canonical TGF-β family signaling.

Figure 4. Determination of the functional domain in PDZK1IP1. A, illustration of PDZK1IP1 and its mutants. B, requirement of the N-terminal region of PDZK1IP1 for inhibition of TGF-β signaling. PDZK1IP1 or its mutants were transfected together with (CAGA)12-luc and pCH110 in HepG2 cells. The experiments were carried out according to Fig. 1A. All values represent means ± S.D. (n = 3). Significant differences from the control in the presence of TGF-β are indicated with asterisks. C, illustration of the N-terminal deletion mutants for PDZK1IP1. D, role of the region from Met1 to Ala49 in PDZK1IP1 in inhibition of TGF-β signaling. The experiments were carried out according to B except for stimulation of cells with 25 ng/ml BMP-6. All values represent means ± S.D. (n = 3). Significant differences from the control in the presence of BMP-6 are indicated with asterisks. E, interaction of the N-terminal domain of PDZK1IP1 with Smad4. COS7 cells were transfected with the indicated plasmids and harvested for co-IP experiments according to Fig. 2C. Upper panel, interaction between Smad4 and PDZK1IP1 or its mutants with anti-Flag antibody for immunoprecipitation and anti-Myc9E10 antibody for Western blot analysis; second panel, expression of Smad4 with anti-Myc9E10 antibody; third panel, expression of ALKScav/VS with anti-V5 antibody; bottom panel, expression of PDZK1IP1/Flag or its mutants with anti-Flag antibody. G, illustration of the PDZK1IP1 mutant lacking the region from Phe46 to Ala49. H, requirement of the region from Phe46 to Ala49 in PDZK1IP1 to inhibit TGF-β signaling. PDZK1IP1 or PDZK1IP1/ΔPhe46–Ala49 was transfected together with SBE2-luc and pCH110 in HepG2 cells. The experiments were carried out according to Fig. 1A. All values represent means ± S.D. (n = 3). Significant differences from the control in the presence of TGF-β are indicated with asterisks. I, schematic presentation of GST-PDZK1IP1 mutants. J, requirement of a 10-amino acid–long region in PDZK1IP1 to associate with Smad4. A GST pulldown assay was performed using the GST fusion proteins described in Fig. 1A. Smad2 together with ALKSca/HA, Smad3 together with ALKSca/HA, or Smad4 alone ectopically expressed in COS7 cells was mixed with GST fusion proteins. After separating the protein(s) bound to GST-SEPHAROSE 4B by SDS-PAGE, Western blot analysis was performed using anti-Flag antibody (upper panel). The cell lysates from COS7 cells transfected with pcDNA3 were used as the negative control. As the loading controls, GST and GST fusion proteins were visible with Ponceau S staining (lower panel). K, Smad expression used for the GST pulldown assay. Expression of Smads and ALKSca/HA used in J is shown. Upper panel, expression of Smads with anti-Flag antibody; lower panel, expression of ALKSca/HA with anti-ALKS (V-22) antibody. Error bars indicate S.D. **, p < 0.01; *** p < 0.001. JB, immunoblotting.

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a scaffold protein via its interaction with different proteins using its N-terminal and C-terminal domains.

To show the effect of PDZK1IP1 on tumor growth in a xenograft nude mouse model, we adopted NCI-H290 cells, which reveal enhancement of cell proliferation in the presence of TGF-β/H9252 signaling (32). Mice injected with cells carrying PDZK1IP1 exhibited prolonged survival when compared with control mice. Thus, high expression of PDZK1IP1 in NCI-H290 cells, in which TGF-β acts as a tumor-promoting factor, suppresses tumorigenicity. In contrast, the Human Protein Atlas database (http://www.proteinatlas.org/) reveals that high expression of PDZK1IP1 in pancreatic cancer and glioma significantly lowered the probability of survival. This discrepancy might be explained as follows: cancer cells themselves alter the...
expression of PDZK1IP1 dependent on the way in which TGF-β signaling acts on their survival or death in cancer cells.

In conclusion, PDZK1IP1 is a novel antagonist of canonical TGF-β/H9252 family signaling that preferentially disrupts the complex formation between phosphorylated R-Smad and Smad4 upon ligand stimulation to retain Smad4 in the cytosol. Thus, PDZK1IP1 can limit the duration of Smad-dependent transcriptional control of direct target genes for TGF-β family signaling.

Experimental procedures

Expression plasmids

Human PDZK1IP1 and Smad8 cDNAs were cloned by RT-PCR. All the PDZK1IP1 mutants were made using PrimeStar HS DNA polymerase (Takara Bio). All cDNAs were verified by sequencing after PCR amplification. PDZK1IP1 and its mutants were inserted into pcDEF3 (37), pcDNA3.1-V5-His-A (Invitrogen), pcDNA3-HA (38), pcDNA3-Flag (38), or GEX-4T-1 (GE Healthcare). (CAGA)12-luc, (SBE)4-luc, p800-Luc, pGL3ti-850-luc, Id-1-luc, (BRE)2-luc, Flag-Smad1, Flag-Smad2, Flag-Smad3, Flag-Smad4, Flag-Smad5, 6xMyc-Smad4, ALK5ca/V5, ALK5ca/HA, and ALK6ca/V5 have been described previously (27–29, 31, 39, 40). HA-Smad4 mutants were a generous gift from Dr. T. Imamura (Ehime University). Adenoviruses expressing PDZK1IP1 were generated using the pAd/CMV/V5-DEST vector (Thermo Fisher). The resulting plasmids were transfected into 293T cells, and the adenoviruses were amplified. A lentiviral expression vector for PDZK1IP1 was made with the pLV-CMV-IRES-Puro vector (41) and then cotransfected with VSV, GAG, and REV expression vectors in 293T cells to obtain viral particles. The lentiviruses were simultaneously incubated in DMEM containing 8 μg/ml Polybrene (Sigma) for 2 h and then added to the cell culture dishes. Twelve hours after infection, the cells were washed and cultured in medium. The infected NCI-H290 cells, which became puromycin-resistant, were used for the experiments.

Antibodies

Antibodies were obtained from the following sources: mouse monoclonal anti-Flag-M5 antibody (F4042), Sigma; mouse
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monoclonal anti-DYKDDDK tag antibody (catalog number 012-22384) against Flag epitope and mouse monoclonal anti-V5 antibody (catalog number 017-23593), Wako; mouse monoclonal anti-Smad4 (sc-7966), mouse monoclonal anti-Myc9E10 (sc-40), mouse monoclonal anti-β-actin (sc-69879), and rabbit polyclonal anti-ALK5(V-22) (sc-398) antibodies, Santa Cruz Biotechnology; mouse monoclonal anti-HA12CA5 (catalog number 11583816001) and rat monoclonal anti-HA3F10 (catalog number 11867423001) antibodies, Roche Applied Science; sheep anti-mouse IgG (catalog number NA-931-1ML) and donkey anti-rabbit IgG horseradish peroxidase–linked (catalog number NA934-1ML) antibodies,
GE Healthcare. Rabbit polyclonal phosphorylated Smad1 and Smad2 antibodies, termed PS1 and PS2, were homemade (42). To generate a rabbit anti-PDZK1IP1 antibody, the peptide from Asn97 to Met114 in human PDZK1IP1 protein was used to immunize a rabbit (Sigma). The sera were purified using a Protein A IgG Purification kit (Thermo Fisher).

Cell culture

MDA-MB468, PC-3, A549, 293T, and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nacalai Tesque) containing 10% fetal calf serum (FCS; Biological Industries). RPMI 1640 medium (Nacalai Tesque) containing 10% FCS was used for NCI-H290 cells. HepG2 cells were maintained in minimum essential medium (Wako) containing 10% FCS, nonessential amino acids (Nacalai Tesque), and sodium pyruvate.

Transcriptional reporter assays

One day before transfection, HepG2 cells were seeded at 1.2 × 10⁵ cells/well in 12-well plates. The cells were transfected using polyethyleneimine (PEI; Polysciences). Where indicated, 5 ng/ml TGF-β1 (Peprotech) or 25 ng/ml BMP-6 (Peprotech) was added to the wells 24 h after transfection. Subsequently, the cells were cultured in the absence of FCS for 18 h. In all the experiments, β-gal (pCH110; GE Healthcare) activity was measured to normalize for transfection efficiency. Each transfection was carried out in triplicate and repeated at least twice. In the case of MDA-MB468 cells, the experimental procedures were the same except for seeding the cells at 3.0 × 10⁵ cells/well in 6-well plates.

Immunoprecipitation and Western blot analysis

To detect interactions among proteins, plasmids were transfected into COS7 cells (5 × 10⁵ cells/6-cm dish) using PEI. Forty hours after transfection, the cells were lysed in 500 μl of TNE buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 100 units/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β-glycerophosphate). The cell lysates were precleared with protein G–Sepharose beads (GE Healthcare) for 30 min at 4 °C and then incubated with the indicated antibody for 2 h at 4 °C. The protein complexes were immunoprecipitated by incubation with protein G–Sepharose beads for 30 min at 4 °C and then washed three times with TNE buffer. The immunoprecipitated proteins and aliquots of total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to an Ultra Cruz Nitrocellulose Pure Transfer Membrane (Santa Cruz Biotechnology). The membranes were probed with primary antibodies. The primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies and a chemiluminescent substrate (Takara Bio).

RNA preparation and RT-PCR

Total RNA was extracted using a ReliaPrep RNA Cell MiniPrep system (Promega). Reverse transcription was performed with a High-Capacity RNA-to-cDNA kit (Applied Biosystems). PCR was performed using GoTaq (Promega) according to the manufacturer’s instructions. The following primer sets were used to amplify PDZK1IP1, TMEPAI, Smad7, and β-actin cDNAs: 5’-TGAGCCAGGTTGATTTCGAG-3’ and 5’-AGA-GAGGTTATCGGGGT-3’ for mouse PDZK1IP1, 5’-CTGGAGTTCTGGCAATCTG-3’ and 5’-AAGGTCACTGTCCTGAATGG-3’ for mouse TMEPAI, 5’-GAAGTCAGGATTGTTG-3’ and 5’-GGTCTTCTCTCCACATTGCG-3’ for mouse Smad7, and 5’-GCTCATAGCTTCTTCCAGG-3’ and 5’-TGAACCTAAAGGCCACCCGTG-3’ for mouse β-actin.

Quantitative PCR (qPCR)

qPCR was performed using a KAPA SYBR Fast qPCR kit (Kapa Biosystems). All reactions were carried out on a Thermal Cycler Dice (Takara Bio). Each sample was analyzed in triplicate at least twice for each PCR measurement. The melting curves were checked to ensure specificity. Relative quantification of mRNA expression was calculated using the standard curve method with the β-actin level. Before qPCR, the DNA fragment amplified using each primer set was detected to be a single band with the correct size by agarose gel electrophoresis. The following primer sets were used to amplify human PDZK1IP1, Smad7, TMEPAI, and β-actin cDNAs: 5’-TGAGCCAGGTTGATTTCGAG-3’ and 5’-AGA-GAGGTTATCGGGGT-3’ for PDZK1IP1, 5’-CGTGTGCAAAGTGTTCAGG-3’ and 5’-CCAGATAATTCGTTCCCTCCCT-3’ for Smad7, 5’-GATCATCATCATCGTTGG-3’ and 5’-CAC-TGTGACAGATGGTTCTG-3’ for TMEPAI, and 5’-CAAGA-GATGGCCACGGGTCTG-3’ and 5’-TCTCTTGATCTTGGTCCGG-3’ for β-actin. Each experiment was carried out in triplicate and repeated at least twice.

PLA

Cells on cover glasses coated with 0.1% gelatin were cultured with DMEM and 10% FCS. Then the cover glasses were washed with either control or PDZK1IP1-expressing lentiviruses. After puromycin selection, the cells were lysed for Western blot analysis.

Figure 7. Xenograft implantation of mesothelioma cells carrying PDZK1IP1. A, an expression of PDZK1IP1 in NCI-H290 cells. The NCI-H290 cells were infected with either control or PDZK1IP1-expressing lentiviruses. After puromycin selection, the cells were lysed for Western blot analysis. Upper panel, expression of PDZK1IP1; lower panel, expression of β-actin. B, inhibition of Smad7 mRNA expression in NCI-H290 cells expressing PDZK1IP1. The control or PDZK1IP1-expressing NCI-H290 cells were stimulated with 5 ng/ml TGF-β for the indicated times 12 h after the cells had been starved without FCS. The expression of Smad7 mRNA was detected by qPCR. C, phosphorylation of Smad2 by TGF-β in NCI-H290 cells expressing PDZK1IP1. Control or PDZK1IP1-expressing NCI-H290 cells were stimulated with 5 ng/ml TGF-β for the indicated times 12 h after the cells had been starved without FCS. The cell lysates were prepared for Western blot analysis. Upper panel, expression of phosphorylated Smad2 with PS2; middle panel, expression of total Smad2; lower panel, expression of β-actin. D, representative images for bioluminescence in mice implanted with NCI-H290 cells carrying both PDZK1IP1 and luciferase. The tumor volumes were measured using bioluminescence when mice were implanted with NCI-H290 cells carrying both PDZK1IP1 and luciferase were injected with luciferin. Images were taken every week after the cells had been implanted. E, decreased tumor volumes in mice in mice implanted with NCI-H290 cells expressing PDZK1IP1. Experiments were performed as shown in D. These data are derived from images that were taken 1 week after the cells had been implanted because the control mice died gradually 2 weeks after implantation of the NCI-H290 cells. All values represent means ± S.D. (n = 4 or 5). Significant differences from the control are indicated with asterisks. F, Kaplan–Meier graph to compare mice implanted with NCI-H290 cells and those implanted with NCI-H290 cells carrying PDZK1IP1. Error bars indicate S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001. IB, immunoblotting.
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once with phosphate-buffered saline (PBS), fixed for 10 min with 4% paraformaldehyde (PFA; Wako), washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS (43).

The following procedures were performed according to the manufacturer’s instructions (Duolink Bioscience). To visualize the fluorescence, an immunofluorescence microscope (Nikon) was used.

Immunofluorescence staining

Immunofluorescence staining was performed as described previously (44). Briefly, the cells grown on the cover glasses were transfected with PDZK1IP1/HA. If necessary, the cells were stimulated with TGF-β for 2 h. After treatment, the glasses were washed once with PBS, fixed for 10 min with 4% PFA, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS. The cover glasses were blocked with 5% BSA in PBS at 37 °C for 1 h and incubated with 5% BSA (in PBS) containing rat monoclonal anti-HA3F10 and mouse monoclonal Smad4 antibodies at 4 °C overnight. The cover glasses were then washed three times with PBS, incubated with 5% BSA (in PBS) containing both Alexa Fluor 488–conjugated goat anti-mouse IgG antibody (diluted 1:250) (A11001, Invitrogen) and Alexa Fluor 555–conjugated goat anti-rat IgG antibody (diluted 1:250) (A21434, Invitrogen) at room temperature for 1 h, and washed three times with PBS. The nuclei were stained with DAPI. To visualize the fluorescence, an immunofluorescence microscope was used.

Lentiviral shRNAs for PDZK1IP1

The lentiviral vectors for PDZK1IP1 shRNA were constructed using a pLKO.1 vector (45). The sequences for shPDZK1IP1-1, shPDZK1IP1-3, and shPDZK1IP1-5 were 5'-AACATGTGAAAATGCCCCATATctcgagATTAGGGCATT-TCCATAGTT-3', 5'-GAAACAGGAGTAGGTCCtcgaga-gGACTCATCTGCCTTTTTC-3', and 5'-TGAGAATGCTTATGGAATGfctcgagaCTTCTAGCATGGAATGTC-3', respectively. Each lentiviral vector was transfected into 293T cells together with psPAX2 and pMD2.G. After 48 h of transfection, the media were collected as a source of lentiviruses. Each lentivirus was incubated in DMEM containing 8 μg/ml Polybrene for 2 h and then added to the PC-3 cell culture dishes. Twelve hours after infection, the cells were washed and cultured in medium. The infected PC-3 cells, which became puromycin-resistant, were used for the experiments.

GST pulldown assay

PDZK1IP1 mutants were subcloned in pGEX4T-1 (GE Healthcare). GST fusion proteins from Escherichia coli were purified according to the manufacturer’s instructions (GE Healthcare). Cell lysates were prepared from COS7 cells transfected with Smads and/or ALK5ca/HA precleared with GST immobilized to GSH-Sepharose 4B (GE Healthcare) for 30 min at 4 °C. Subsequently, the above cell lysates were incubated with GST-PDZK1IP1 mutants immobilized to GSH-Sepharose 4B for 2 h at 4 °C and washed three times with 50 mM Tris (pH 7.4) containing 100 mM NaCl, 2 mM MgCl2, 10% glycerol, 1% NP-40, 1 mM PMSF, 5 μg/ml leupeptin, 20 units/ml aprotinin, and 5 mM benzamidine. After the samples had been separated by SDS-PAGE, the proteins were blotted on the Ultra Cruz Nitrocellulose Pure Transfer Membrane and detected with an anti-Flag antibody using a chemiluminescent substrate. To show the quantity of GST fusion proteins in each sample, Ponceau S staining was performed after blotting.

Migration assay

Cell migration assays were performed using a Boyden chamber. Costar nucleopore filters (8-μm pore diameter) were coated with 10 μg/ml fibronectin (Sigma) overnight at 4 °C. The chambers were washed three times with PBS. Cells cultured for 12 h without FCS were added to the top of each migration chamber at a density of 1.5 × 10⁵ cells/chamber in 150 μl of DMEM with 0.5% FCS. The cells were allowed to migrate to the underside of the chamber in the presence or absence of 5 ng/ml TGF-β in the lower chamber. After 18 h, the cells were fixed in 4% PFA and stained with hematoxylin (Leica) and eosin (Muto Chemicals). The upper surface was wiped with cotton swabs to remove nonmigrating cells. The cells present on the lower surface were counted. Each experiment was carried out in triplicate and repeated more than twice.

Cell proliferation assay

All animal work was performed with institutional approval by the Animal Care and Use Committee of Showa Pharmaceutical University (approval number P-2018-01-R1 dated April 19, 2018). After the pGL4.50[luc2/CMV/Hygro] plasmid (E131A, Promega) was stably introduced into NCI-H290 cells, the PDZK1IP1-expressing vector was further transfected to luciferase-expressing NCI-H290 cells. Luciferase-expressing NCI-H290 cells (5 × 10⁵ cells) carrying PDZK1IP1 were implanted into the pleural cavity of 7-week-old male BALB/c nu/nu mice purchased from Japan SLC. One week later, the anesthetized mice were intraperitoneally administered with 120 mg/kg d-luciferin potassium salt (Wako). Subsequently, bioluminescence in each mouse was monitored using an Imagine C9100 EM-CCD camera (Hamamatsu Photonics) and AQUA COSMOS imaging software (Hamamatsu Photonics). The mice were housed in the animal facilities of Showa Pharmaceutical University under specific pathogen-free conditions at constant temperature and humidity and fed a standard diet. The study was not blinded. Mice were treated in accordance with the institutional guidelines of the Animal Care and Use Program of Showa Pharmaceutical University.
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