PODXL1 promotes metastasis of the pancreatic ductal adenocarcinoma by activating the C5aR/C5a axis from the tumor microenvironment

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Abstract

Pancreatic invasive ductal adenocarcinoma (PDAC) is a representative intractable malignancy under the current cancer therapies, and is considered a scirrhus carcinoma because it develops dense stroma. Both PODXL1, a member of CD34 family molecules, and C5aR, a critical cell motility inducer, have gained recent attention, as their expression was reported to correlate with poor prognosis for patients with diverse origins including PDAC; however, previous studies reported independently on their respective biological significance. Here we demonstrate that PODXL1 is essential for metastasis of PDAC cells through its specific interaction with C5aR. In vitro assay demonstrated that PODXL1 bound to C5aR, which stabilized C5aR protein and recruited it to cancer cell plasma membranes to receive C5a, an inflammatory chemoattractant factor. PODXL1 knockout in PDAC cells abrogated their metastatic property in vivo, emulating the liver metastatic mouse model treated with antiC5a neutralizing antibody. In molecular studies, PODXL1 triggered EMT on PDAC cells in response to stimulation by C5a, corroborating PODXL1 involvement in PDAC cellular invasive properties via specific interaction with the C5aR/C5a axis. Confirming the molecular assays, histological examination showed coexpression of PODXL1 and C5aR at the invasive front of primary cancer nests as well as in liver metastatic foci of PDAC both in the mouse metastasis model and patient tissues. Hence, the novel direct interaction between PODXL1 and the C5aR/C5a axis may provide a better integrated understanding of PDAC biological characteristics including its tumor microenvironment factors.

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Introduction

Invasive ductal adenocarcinoma of the pancreas (PDAC) is the most common pancreatic malignancy and has been recently highlighted as a serious issue for cancer treatment, given persistent clinical intractability to known therapeutic countermeasures aimed at improving patient prognosis [1,2]. PDAC shows unique histology, where proliferating cancer nests are surrounded by abundant collagenous stroma with cancer-associated fibroblasts (CAFs), inflammatory cells such as macro-
phages and lymphocytes, and scanty tumor blood vessels from neovascularization. These stromal components form a so-called tumor microenvironment essential to cancer progression in vivo. Hence, tumor microenvironments are complex systems of various cellular components, observed to release diverse inflammatory chemokines that interact with cancer cells resulting in tumor growth, invasion and metastasis.

**Podocalyxinlike 1 (PODXL1)**, is an antiadhesive sialomucin, belonging to a member of the CD34 family harboring the extracellular domain and intracellular domain via the transmembrane portion. It is expressed by normal vascular endothelium, renal podocyte, and hematopoietic progenitors [3–5] and has been reported to be a stem cell marker including embryonic stem cells [6,7]. Noticeably, there have been studies demonstrating correlation between PODXL1 expression and poor prognosis of patients with cancer of the diverse origins including colorectal cancer [8], gastric cancer [9], and pancreatic cancer [10]. Functionally, PODXL1 was reported to promote invadopedia formation and metastasis through Rac1/Cdc42/cortactin signaling in breast cancer cells [11], to induce collective migration of MCF7 cells depending on activity of the actin scaffolding cytoplasmic protein ezrin [12]. Recently, it was revealed that PODXL1 was involved in efficient EMT extravasating via the Ezrin signaling on breast cancer and pancreatic cancer cells [13]. Hence, PODXL1 appears critically involved in causing invasion and metastasis of various cancers and is one of the most important regulators of human malignancies; however, the diverse biological roles of PODXL1 still require elucidation for generating future cancer therapeutics. On the other hand, multiple chemokines have been reported as produced in tumor microenvironment in response to inflammatory reaction and tumor immunity against cancer cells. Among them, C5a, a soluble complement which is cleaved from membrane-bound C5 is reported to promote tumor development and progression including cellu-

**Materials and methods**

**Cell culture and antibodies**

Human PDAC lines, BxPC3 (ATCC: CRL1687), AsPC1 (ATCC: CRL1682), MiaPaCa2 (ATCC: CRMCR1420), Panc1 (ATCC: CRL1469), an immortalized nonneoplastic pancreatic ductal cell, HPNE (ATCC: CRL4023), normal human dermal fibroblast, NHDF (ATCC: PCS201010) were grown in RPMI1640 (Wako, Tokyo, Japan) supplemented with 10% of fetal bovine serum (FBS) in a 5% CO2 incubator. Authentication of each cell line was attached. Antibodies employed in this study were as follows. As a primary antibody, antiPODXL1 (SigmaAldrich, Merk, St. Louis, MO), antiIntegrin 1 (Invitrogen, Thermo Fisher Scientific KK, Japan), antiC5aR (CD88) (Hycult Biotech, Wayne, PA), antiC5a (R&D Systems, Mineapolic, MN), antiMyc (SigmaAldrich), antiFLAGtag (Invitrogen, Thermo Fisher Scientific KK), antiTubulin (CST Japan, Tokyo, Japan), antiECadherin (Invitrogen, Thermo Fisher Scientific KK), antiSnail (CST Japan), antiVimentin (CST Japan), antiTRA160 (CST Japan). As a secondary antibody, Alexa488conjugated goat antimouse IgG(H + L), Alexa488goat antirabbit IgG(H + L), Alexa555goat antirabbit IgG(H + L), Alexa555donkey antimouse IgG(H + L), HRP-conjugated goat antinouse IgG(H + L), HRP-conjugated goat antimouse IgG(H + L) for all of these: Invitrogen, Thermo Fisher Scientific KK) were used for the immunohistochemistry, immunofluorescence and immunoblot assays.

**Patients tissues, immunohistochemistry and immunofluorescence**

Paraffin-embedded surgical specimens fixed with 10% buffered formalin and made from the patient tissues of the pancreatic invasive ductal adenocarcinoma were obtained from the department of pathology, Niigata Cancer Center Hospital under approval by the ethics committees of Niigata University and Niigata Cancer Center. Ten cases of primary PDAC tissues (well differentiated; 3 cases, moderately differentiated; 4 case, poorly differentiated; 3 cases) and four cases of liver metastasis of the corresponding PDAC from the same individual among these ten patients were examined for expression for the proteins of the interest by immunohistochemistry and immunofluorescence, using antiPODXL1, antiC5aR, and antiC5a, antiTRA160 antibodies. All specimens were autoclaved prior to incubation with the primary antibody (at 4 ceels for 12hours) for immunohistochemistry or immunofluorescence.

**Plasmid construction for a panel assay of the chemokine receptors**

For the panel screening of chemokine receptors as a binding partner of PODXL1, the plasmids encoding, in total, forty human chemokine receptor were constructed, respectively. These were cotransfected with human PODXL1 and subjected to immunoprecipitation assay. In detail, for the express plasmid construction, cDNAs of interest were subcloned into the pIDTSMART (CTSC) vector to obtain efficient gene expression in a transient manner [18]. The prepared cDNAs covering the entire ORF were as follows: human cDNAs encoding PODXL1 (transcript variant 1; GenBank NM_001018111) and chemokine receptors (C3aR, C5aR, CXCR1, CX3CR1, XCR1, CCR11, BLT1, 2, LTR1, 2, EP14, DP1, 2, FP, IP, TP, and LPAR16). PODXL1 was designed to express as a Hermlinal 3xMyc6xHis-tagged form. Chemokine receptors were all designed to express as Hermlinal 3xFlag6xHis-tagged forms.

**Immunoblot and immunoprecipitation**

All immunoblot analyses were performed with the cell lysates prepared with MPER mammalian protein extraction reagent (Thermo Fisher Scientific) under standard conditions using each of the specific antibodies mentioned above. For the coinmunoprecipitation assay, HEK293T cells were transiently transfected with the PODXL1 expression (PODXL1 with 3xMyc6xHis-tagged at the Cterminal end). Chemokine receptors were all designed to express as Hermlinal 3xFlag6xHis-tagged forms. Combined with a series of chemokine receptorexpressors mentioned above using FuGENEHED (Promega), respectively. Twentyfour hours posttransfection, cell pellets were harvested and lysed in MPER buffer. The prepared lyses were then incubated with agarose beads conjugated with monoclonal antiMyc tag antibody (MBL, Nagoya, Japan). The resultant immunoprecipitates were subjected to immunoblot probed with antiFlag tag antibody. To examine direct binding between PODXL1 and C5aR, PODXL1 was purified from the cell lysate of the PODXL1-HA(x3)His(x6)tagged expressotransfected HEK293T cells using antiHistag antibodyconjugated beads (#D2918, antiHistag mAbagarose beads; MBL Co., Ltd., Japan). C5aR protein was also isolated from the lysate of the C5aRFlag(x3)His(x6)tagged expressotransfected 293 T cells using antiHistag mAb beads. Each protein was mixed together, incubated for 30 min at RT, then the sample was precipitated using beads conju-
gated with antiHA tag antibody (#5618; antiHA tag pAbAgarose beads; MBL Co. Ltd., Japan) for pulldown of PODXL1. The pulldown sample was subjected to SDS-PAGE, blotted onto membrane after fractionation, and immunoblotted with antiHA antibody (#3724; HATag C29F4; Cell Signaling Technology, Danvers, MA) or with antiFlag antibody (#F1804; antiFlag M2 mAb; Sigma Aldrich Japan, Tokyo, Japan) was performed, respectively. As a control, purified PODXL1HAHis alone and PODXL1HAHis incubated with heat-degraded C5aFlagHis protein were employed.

**Generation of PODXL1′ clones by the CRISPR/Cas9 system**

PODXL1 knockout PDAC cell lines (for MiaPaCa2, AsPC1, and Panc1) were generated using CRISPR/Cas9 system. Both plasmids, hCas9 (#41815 Addgene, Watertown, MA), and gRNA (guide RNA) Cloning Vector (#41824), were obtained from Addgene. The gRNA vector including PODXL1 target sequence (GCACAGATGGCGTGCGGCGGcgg) located in part of the Exon 1 was prepared following the manufacturers instruction with tgg sequence as a Protospacer Adjacent Motif (PAM). The hCas9 and PODXL1 gRNA vector were cotransfected into cells using ViaFect Transfection Reagent (#E4981, Promega, Madison, WI). Twentyfour hours posttransfection, the cells were cultured with RPMI medium containing 500 g/ml of Genetin (#1013135, Gibco, Thermo Fisher Scientific, Waltham, MA) for isolating the Genetin-resistant clones. PODXL1 expression deficient clones from each PDAC line were confirmed by lack of PODXL1 protein, using immunoblot analysis with antiHA tag antibody. Genetic mutation of PODXL1 in the knockout clone was also examined by genomic DNA sequencing of PCR amplified product, using the specific primers for PODXL1 target sequence and the adjacent genomic DNA. The primers used for the sequencing were the following: Pod1.Ex1.check.Fw2: 5GCGGTGCGGTCTCCXTTTTCTTCTTTTCTT. 

**Image based protein–protein interaction (PPI) analysis (Fluoppi assay)**

The protein-protein interaction between PODXL1 and candidate proteins (EP4, CXCR2, C5AR, LTßR, CTTN and CXCR1) was detected using the Fluoppi system (MBL, Nagoya, Japan), in which PPIs were visualized as fluorescent foci [19,20]. PODXL1 was subcloned into a pAshMNL ver.2 plasmid to fuse with an Ash (homo oligomerized protein assembly helper) tag (PODXL1Ash). Candidates were subcloned into a pMontiRedMNL plasmid to fuse with a MontiRed (homo tetramer red fluorescent protein from Montipora sp.) tag (CTTNred or C5arRed). These plasmids of PODXL1Ash and candidatesRed were cotransfected into HeLa cells using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers instructions, respectively, and intracellular fluorescent foci with red color were observed by inverted fluorescence microscope (Olympus IX71, Olympus Co. Tokyo, Japan) and detected as an image using cellSens imaging software (Olympus Co.).

**Invasion assay**

Cells were starved by serum free medium for 24 h prior to invasion and migration assays. Cell invasion and migration assays were both evaluated by the Boyden chamber method using the filter inserts (pore size, 8 μm), precoated (for invasion assay) or not precoated (for migration assay) with Matrigel in 24well plates (BD Biosciences, Franklin Lakes, NJ, USA). Each pancreatic cancer cell line (7.5 × 10⁵ cells/insert for MiaPaCa2, 2 × 10⁵ cells/insert for AsPC1, or 4 × 10⁴ cells/insert for Panc1) was seeded on the top chamber. The top chamber was filled with serum-free medium and the bottom chamber was filled with 10% FBS medium. The recombinant chemokines were then set in the bottom chamber at a final concentration of 25 ng/ml for CXCL8 (PeproTech, Rocky Hill, NJ, USA), and 0.5 M for PGE2, 5 ng/ml for CX3CL1 (R&D Systems, Minneapolis, MN, USA) and C5a (ProSpecTany Technology Ltd, Ness Ziona, Israel). After incubation for 8 h in the case of Panc1, or 48 h in the case of Mia PaCa2 and AsPC1 cells, cells that passed through the filter and were attached to the lower surface of the filter were counted by staining with 0.01% crystal violet in 25% methanol. Cells attached to the lower surface of the filter membrane were then quantified by cell counting in five nonoverlapping fields at 100 magnification.

**RNA interference assay using siRNA**

Predesigned siRNAs for human C5aR (ID D00544200005) and control siRNA (ID D0018101005) were purchased from Dharmacon (Lafayette, Colorado, USA). siRNAs (final concentration 50 nM) were transfected using Lipofectamin RNAiMAX reagent (Thermo Fisher Scientific). Fortyeight hours postintroduction of each siRNA, the cells were subjected to the invasion assay described above.

**In vivo mouse liver metastasis model**

1 × 10⁶ cells of MiaPaCa2, AsPC1, or Panc1 were injected into 6 weekold nude mouse spleen exteriorized through a left flank incision, respectively, followed by splenectomy 2 min later. The same number of the PODXL1′ PDAC cells corresponding to each cell line were injected in the same way with the same timing. Approximately 30 days postinjection, the mice grew metastatic liver tumors derived from the injected PDAC cells, and they were then autopsied and compared for tumor formation pairwise with the mice implanted with PODXL1′ cells under the stereo microscope. Total number of mice examined in each group was four (n = 4). Only in the case of Panc1 splenic xenograft, number of mouse examined was two (n = 2). In the C5a blocking assay, each mouse with splenic injection of 1 × 10⁶ of MiaPaCa2 cells was treated with intravenously administrated 50 g/mouse of antiC5a neutralizing antibody on Day 0 (the day of cellimplant), Day 7, Day 14 (in total three times, and once every week), and autopsied and examined on Day 30 pairwise with mice (control group) treated with the same dose of goatIgG. Totally, four mice (n = 4) were examined in each group. Excised tumors in all animal experiments were histologically examined with paraffinembedded specimens by HE staining and immunohistochemistry using antihuman cytokerin, CAM5.2 (BD Biosciences, San Jose, CA) to demonstrate human PDAC cells (MiaPaCa2, AsPC1, and Panc1, respectively) origin.

**Statistical analysis of metastatic tumors in mouse models**

Based on macroscopic finding in combination with microscopic examination of the tissue specimens, number of liver metastasis was counted in each group of mouse splenically injected with MiaPaCa2, AsPC1 in every animal experiment. Average number of metastasis per liver in each case was statistically evaluated by t test (shown as graph with P value).

**Results**

**Characteristic expression of PODXL1 on human PDAC tissues**

PODXL1 expression on PDAC tissues has been reported in previous studies that demonstrated PODXL1 preferentially expressed on the cancer nests in comparison with the nonneoplastic pancreatic acinus and duct, with the expression correlating to the patients poor prognosis [21]. Immunohistochemistry on representative primary PDAC patient tissues using antiPODXL1 antibody revealed that strong membranous PODXL1
expression with or without cytoplasmic expression was observed mainly at the small collective cell forming cancer nests at the invasive front of the PDAC tumor in examined cases (1); well differentiated type, 2; moderately differentiated type, 4; poorly differentiated type, respectively (Figure 1A), but a small number of strong PODXL1-positive cancer cells were observed among the individual tumor glands adjacent to the small invasive nests (Supplementary Figure S1A). PODXL1 expression was not dependent on the differentiation type of PDAC but was detected in all types examined. It has been also reported that the glycosylated form of PODXL1 was recognized as TRA160 antigen [22], an embryonic stem cell and iPSC cell marker. TRA160 expression was detected in similar patterns to that of PODXL1, where TRA160 was strongly positive in small cancer nests at invasive foci in PDAC patient tissues under immunohistochemistry (Supplementary Figure S1B, upper panel) Immunofluorescence using antiPODXL1 and antiTGB1 (Integrin 1, CD29) antibodies highlighted the budding tumor cell from the neoplastic gland acquiring strong expression of PODXL1 as well as ITGB1, indicating PODXL1 might be required for epithelialmesenchymal transition (EMT) of the PDAC cells (Figure 1B and Supplementary Figure S1B, lower panel). Accordingly, the budding single PDAC cell was also detected by immunofluorescence using TRA160 antibody (Supplementary Figure S1B, lower panel). The robust expression of PODXL1 was also observed not only in PDAC but also various cancers, as for example, its expression on invasive nests of colorectal tubular adenocarcinomas (Supplementary Figure S1C).

**PODXL1 is critically involved in metastasis of PDAC cells in vivo**

To elucidate the biological role of PODXL1 in PDAC cells in vivo, generation of PODXL1-targeted PDAC cell clones was achieved by using the CRISPR/Cas9 genome editing system. First, endogenous expression of PODXL1 was examined in four different human PDAC lines established from different patients. As nonneoplastic counterparts, human immortalized pancreatic ductal epithelium (HPNE) and normal kidney epithelial cells were also examined for its expression. Consequently, all four PDAC lines showed strong expression of PODXL1 including the supershifted (hyperglycosylated) form (Figure 2A), and HPNE showed comparably weak PODXL1 expression with a similar pattern, but the glycosylated form was not detected in kidney cells (Figure 2A). These PDAC lines showed membraneous expression of PODXL1 as well as its cytoplasmic expression (Figure 2B, upper panel), representing lamellipodia and plasma membrane localization in AsPC1 and MiaPaCa2 cells (Figure 2B, lower panel). Based on this finding, genome editing to knockout PODXL1, including variant 1 and 2, was performed using the CRISPR/Cas9 system with the designed guideRNA (gRNA) recognizing the part of the exon 1 both on MiaPaCa2 and AsPC1, respectively (Figure 2C, left panel). Four respective PODXL1 (PODXL1) clones were obtained both in MiaPaCa2 (clone #3, 9, 15, 37) and AsPC1 (clone #2, 3, 9, 13), all of which were confirmed as to specific impairment of PODXL1, utilizing immunoblots and genome sequencing (Figure 2C, right panel and Supplementary Figure S2A). Using these knockout (KO) clones, the murine pancreatic tumor model of liver metastases was established by hemispleen injection of the MiaPaCa2 cells lacking PODXL1 expression (KO clone #9) and AsPC1 PODXL1KO cells (KO clone #2), and by using the wild type MiaPaCa2 and AsPC1 cells for comparison, respectively. After 30 days postinjection, the wild type MiaPaCa2 implanted mice developed multiple metastatic foci in the liver, whereas no metastatic foci were observed in the mice implanted with the PODXL1KO #9 clone (Figure 2D, upper panel). Similarly, loss of liver metastasis was observed in mice injected with the AsPC1 PODXL1KO #2 clone in contrast to the cases with the wild type cell injection (Figure 2D, lower panel). Number of the intrahepatic metastases per liver between group of implanted PDAC cells bearing PODXL1 wild type and those of PODXL1 knock (n = 4 in each group) was summarized in graph which showed statistical significance (Figure 2D). Similarly, loss of mesenteric lymph nodal metastasis was also observed in the mice treated with the KO clone of MiaPaCa2 (n = 4 in each group, Supplementary Figure S2B), and the PODXL1KO clone of Panc1 PDAC cell showed the similar phenomenon (Supplementary Figure S2C). Histological examination of the intrahepatic and lymph nodal metastatic tumors demonstrated that metastatic tumors were derived from hemisplenically injected human PDAC cells in the mice treated with tumor cells bearing PODXL1 wild type, and no micrometastatic lesion was observed even microscopically in the liver of the mice injected with tumor cells harboring PODXL1 knockout (Supplementary Figure S3A, 3B, and 3C).

**PODXL1 captures diverse chemokine receptors including C5aR**

The next issue determined was how PODXL1 is critically involved in PDAC invasion/metastasis. Recently, the tumor microenvironment has received much attention because many current studies reveal its significant impact on cancer progression in terms of tumor growth, invasion and metastasis. The tumor microenvironment is a complex system comprised of fibrous stroma, tumor angiogenesis, tumor immunity and inflammatory reaction. Among these, we focused on chemokine receptors which affect the chemotactic cellular motility through interaction with soluble inflammatory chemotactic ligands in the tumor microenvironment. A panel screening for over forty chemokines was performed to find the specific association between PODXL1 and these molecules by employing immunoprecipitation. The results showed that PODXL1 bound multiple chemokine receptors such as C5aR (C5aR1), CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CX3CR1, CCR1, CCR3, CCR4, CCR8, CCR9, BLT1, BLT2, EP2, EP4, DP1, DP2, FP, IP, LPAR1, LPAR2, LPAR4, LPAR5, and LPAR6 (Figure 3A). Among these, we further examined expression of selected chemokine genes semiquantitatively by RTPCR, which revealed enhanced coupling to PODXL1. Essentially all selected chemokines were more or less expressed on PDAC cells and C5aR receptor was the strongest and most consistently expressed on them (Figure 3B). Prominent membranous C5aR expression was detected on all five representative PDAC lines, BxPC3, PK8, Panc1, MiaPaCa2, and AsPC1 (Figure 3C).

**PODXL1 accelerates cellular motility by regulating functional localization and stabilizing C5aR, and triggers EMT via the C5aR/C5a axis on PDAC cells**

Based on these results, the biological role for protein coupling of PODXL1 and C5aR was investigated on PDAC cells bearing the wild type PODXL1, compared with the PODXL1KO cells. Confirmed specific binding of PODXL1 to C5aR was observed only in wild type PODXL1 (PODXL1WT) MiaPaCa2 cells but not in the PODXL1KO cells (Figure 4A); intracellular localization of C5aR was examined with or without PODXL1 as to whether or not this would be affected by the presence of PODXL1. In image based protein–protein interaction (PPI) analysis, the so called Fluoppi system, aggregated fluorescent dots appears only when two proteins of the interest directly binds to each other. Based on the result of Fluoppi analysis using HeLa cells, C5aR was successfully recruited to lamellipodia at the cell membrane on the PODXL1-cotransfected cells which was recognized as aggregated red dots, as in the case of Contactin (CTNN)PODXL1 cotransfected cells (Figure 4B) and Supplementary Figure S4A). On the other hand, it was still retained at the cytoplasm without PODXL1 expression (only with pAsh) (Figure 4B). Other chemokine receptors, such as EP4 and CX3CR1 seen as candidate binding partner to PODXL1, also displayed changes in intracellular localization to their functional site in the presence of PODXL1 (Supplementary Figure S4B). Thus, PODXL1 appeared essential for targeting
C5aR to its functional site by cellular assay. Direct coupling between PODXL1 and C5aR was also demonstrated by pulldown assay using anti-HA conjugated beads to isolate binding partner of 3xHA6xHistagged PODXL1 protein (Supplementary Figure S4C). Based on the immunoprecipitation assay results – establishing the diverse chemokine receptors as binding partner with PODXL1 – CXCL8 (ligand to CXCR2), CX3CL1 (ligand to CX3CR1), C5a (ligand to C5aR), and PEG2 (ligand to EP4), all of which showed augmented association, were incubated with MiaPaCa2 cells and quantified for the increase in cellular motility, using transwell chamber assay (Boyden chamber assay), between the PODXL1-WT and the PODXL1-KO cells. The data indicated that the ligand stimulation by C5a robustly increased PODXL1/WT MiaPaCa2 cellular motility in comparison with that of C5astimulated PODXL1/KO clone (Figure 4C). This phenomenon was specific because the MiaPaCa2 cells treated with C5aR siRNA markedly decreased the cellular motility even when incubating with C5a (Supplementary Figure S4A). The response to C5a seemed to be common to the other PDAC cells such as AsPC1 and Panc1 (Supplementary Figure S4B). We found that another feature of PODXL1/C5aR interaction was the pronounced attenuation of C5aR expression in the PODXL1-KO PDAC cells. In the immunofluorescence results, C5aR expression was much weaker in PODXL1-KO MiaPaCa2 and AsPC1 in contrast to the PODXL1/WT cells, and we made similar observations for PODXL1-deficient Panc1 cells (Figure 4D and Supplementary Figure S6A). Total protein for C5aR was examined by immunoblot using anti-C5aR antibody, and its comparative reduction in the PODXL1-deficient cell was corroborated, using whole cell lysates, between

Figure 1. Expression of PODXL1 on human PDAC tissues from the patients. (A) IHC using anti-PODXL1 Ab on well differentiated type (1), moderately differentiated type (2, 3), and poorly differentiated type PDAC (4). Hatched box indicates the area for hyperview in each case (4, 20, 60). (B) Double IF using anti-PODXL1 Ab (red) and ITGB1 (green) (left panel). Schematic representation of the PODXL1-expressing budding cancer cells from the tumor gland were highlighted.
Figure 2. Loss of liver metastasis in the PODXL1-deficient PDAC cells in vivo. (A) Endogenous PODXL1 expression in six PDAC lines (BxPC3, PK-8, Panc-1, MiaPaCa-2 and PL45), HPNE and kidney epithelial cells as a non-neoplastic by IB using anti-PODXL1 Ab. Note the glycosylated form of PODXL1 showed as supershifted. (B) Expression of PODXL1 detected by IF. Hyperview of AsPC-1 and MiaPaCa-2 showed expression of PODXL1 at the surface membrane as well as at the cytoplasm. (C) Schematic representation of the guide RNA design for the knockout, recognizing a part of the exon 3 (black box) within the PODXL1 genome (left). Confirmation of the KO clones of MiaPaCa-2 (#3, #9, #15, #37) and AsPC-1(#2, #3, #9, #13) by IB using anti-PODXL1 Ab. The 293T lysate transfected with HA-tagged full-length PODXL1 variant 1 was used as a positive control. α-β-tubulin indicated the IB using anti-total tubulin Ab. (D) Development of liver metastasis 30 days after splenic injection of the PODXL1-WT or the KO cells in Balb/c nu/nu mice (n = 4 in each group). Attached Graphs showed average number of liver metastasis/mouse in MiaPaCa-2-injected or AsPC-1-injected mice (n = 4 each WT or KO group, means and s.d. of quadruplicate were shown).
the WT cells and the KO cells, in MiaPaCa2 and AsPC1, respectively (Figure 4E, left panel). Furthermore, immunoblot of the cellular fraction revealed that the majority of C5aR was detected in the membrane fraction of PODXL1WT AsPC1 and MiaPaCa2 cells, respectively, while in the PODXL1KO cells the majority of C5aR was contained in the cytosol fraction (Figure 4E, right panel). These results implied that lack of PODXL1 might impair the protein stability and the functional localization of C5aR in PDAC cells. Given the aggregate findings, we examined whether the PODXL1C5aR interaction was involved in EMT of PDAC cells. Total cell lysates were prepared from each PDAC cell sample with or without C5a stimulation for 12 hours in the PODXL1WT and the KO phenotype of AsPC1 and MiaPaCa2. The sample from the C5atreated PODXL1WT cells showed marked reduction of ECadherin in contrast to an increase in Snail and Vimentin, whereas the KO phenotype displayed no significant quantitative fluctuations of these proteins (Figure 4 F). The PODXL1C5aR/C5a axis was therefore demonstrated to be one of the critical pathways for triggering EMT of the PDAC cells.

**Significance of C5aR/C5a axis for the PDAC metastasis in the presence of PODXL1 in vivo**

With the specific interaction of PODXL1 and C5aR in PDAC cells thus confirmed, the biological impact of C5aR/C5a signaling for promot-
ing PDAC metastasis was examined using the experimental PDAC liver metastasis model in nude mice. Animals injected in the spleen with 1 \(10^6\) of MiaPaCa2 cells were either treated three times, in total, with antiC5a neutralizing antibody (50 g/every 7 days), or with the same dose of goat IgG as a control following the schedule shown in Figure 5A. Thirty days after splenic injection of MiaPaCa2 cells, mice were freshly autopsied and examined for liver metastasis. Significant differences were thereby observed in number of metastatic tumor foci (representative histology for these metastatic tumors was shown in Supplementary Figure S6B) as shown in the stereoscopic image and the summarized graph, where the neutralizing antibody treated mice were poorly showing in metastatic foci (Figure 5A). Thus, the activation of C5aR/C5a signal significantly contributed to liver metastasis formation of PDAC cells \textit{in vivo}. Immunohistochemistry indicated that metastatic cancer cells were positive for C5a, C5aR, and PODXL1 (murine hepatocytes were also positive for C5a and C5aR), and immunofluorescence revealed the coexpression of

**References**

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**Figures**

A. Whole cell extract

B. CTNN-red

C. MiaPaCa-2

D. AsPC-1 and MiaPaCa-2

E. AsPC-1 and MiaPaCa-2

F. AsPC-1 and MiaPaCa-2
PODXL1 and Vimentin as an EMT marker on cancer cells at the metastatic foci (Figure 5B).

Detection of C5aR, C5a and PODXL1 in human PDAC patient primary tissues and liver metastasis

Since the interaction between PODXL1 and C5aR/C5a axis was established in the mouse PDAC xenografted model, we then investigated the endogenous expression of these molecules in human patient tissues. Strong C5aR expression was detected on the invasive nests of primary PDAC tissues as well as PODXL1 expression (Figure 5A), and the merged image of double immunofluorescence detecting PODXL1 and C5aR displayed a few double positive cells inside the invasive cancer nest with membranous colocalization (Figure 5B). In liver metastasis from the same PDAC patient, C5aR and PODXL1 were constantly expressed on the same individual cancer cells as well as in the primary site (Figure 6C), and C5a, as a ligand to C5aR, was strongly positive both on nonneoplastic hepatocytes (Figure 6D, upper panel) and metastatic PDAC cells (Figure 6D, lower panel, hyperview). C5aR expression was also maintained at the lymph nodal metastasis of PDAC (Supplementary Figure S7).

Discussion

In previous studies, expression of PODXL1 had been reported to reflect adverse prognosis for the patient with diverse cancer origins including breast, colorectal, gastric, and pancreatic adenocarcinoma [4,8–10,23]. Similarly, C5aR/C5a expression is known to correlate with clinical prognosis for various lines of cancer [14,16,17]. Moreover, both PODXL1 signaling and C5aR/C5a signaling were proven molecularly to facilitate tumor cell invasion in vitro by activating specific kinases [13,24,25]. For example, PODXL1 triggers invasion by increased matrix metalloproteinases (MMP) 1 and 9 expression and increased activation of MAPK and PI3K activity through ezrin in breast cancer, and by elevating the soluble cat/level/cat signaling through the p38 MAPK/GSK3 pathway in glioblastoma [10,12]. On the other hand, C5aR/C5a signaling was reported to raise the release of MMP from cancer cells – similarly to PODXL1 – to induce PI3K/AKT activation and inhibit p21/pp21 expression, and even to exhibit immunomodulatory effects in facilitating cancer metastasis by suppressing effector CD8(+) and CD4(+) T cell responses [25–27]. Collectively, this information implicates the presently studied molecules as crucially determinant for metastasis, and for resultant poor prognosis, in a wide range of tumors. However, these molecules have also been reported as being independently significant in their biology. In this study, we found the direct molecular connection between PODXL1 and C5aR/C5a axis by demonstrating that PODXL1 is considered to be one of the important binding partner with C5aR, which stabilizes C5aR and recruits it to the functional site (cell membrane) on PDAC cells. Namely, PODXL1 is considered to be essential to activation of C5aR, leading to acquisition of cellular motility imparting invasive and metastatic properties to PDAC cells (Figure 6E). This novel finding may provide a clearcut explanation for the various earlier oncology studies on PODXL1 and C5aR/C5a, although further investigation is necessary for the detailed structure and function of the PODXL1/C5aR complex, and for the mechanism of recruitment of the complex to the plasma membrane. Our experiments also revealed the multifunctional aspects of PODXL1 appearing to allow capture of multiple chemokine/cytokine receptors induced in the tumor microenvironment. We suggest that PODXL1 plays a central role in progression of diverse cancers by activating the soluble factors in response to their unique tumor microenvironment. This notion is supported by earlier reports that the glycosylated form of PODXL1, TRA160 antigen [22,28], was utilized as an important cellular marker for iPSC cells to confirm their full reprogramming status [29], reflecting a wide action permitting multipotent differentiation in response to diverse stimuli.

In a recent trend, multiple oncology studies in tumor medicine have emphasized the importance of the expulsion of cancer stem cells, as they are strongly resistant to known anticancer treatments and hence are sources for recurring malignancy. With that in mind it remains unclear whether PDXL1 expressing tumors contain cancer stemphenotype cells, but the tumors may yet correlate highly with relapse and progression specifically due to the diverse function of PODXL1/TRA160 [30] in the microenvironment, as was demonstrated in this study. Our investigations, importantly, represent a framework for precision therapeutic control of the PDXL1 expressing cancer cell, so as to overcome intractable malignancies such as PDACs.

Conclusions

In this study, we found novel crosstalk between the critical two tumor regulators, PODXL1 and C5aR, in pancreatic cancer (PDAC) which significantly contribute PDAC invasion/metastasis. Through their specific interaction, PDAC acquired accelerated cellular motility and caused aggressive metastasis which was corroborated by in vitro assays of xenografted model using PODXL1 knockout PDAC cell in comparison with the wild type cell. Furthermore, PODXL1 may be multifunctional because it is capable of capturing various chemokine (cytokine) receptors, which suggests diverse role of PODXL1 in the tumor microenvironment.
Figure 5. C5aR/C5a axis is essential to cause liver metastasis of MiaPaCa-2 in vivo. (A) Administration protocol of the C5a-neutralizing Ab (50 g/mouse/time) to the mice splenically injected with MiaPaCa-2 (1 $10^6$ cells). The mice splenically injected with MiaPaCa-2 had in total three intravenous injections of the Ab, once per week, and were autopsied on the Day 30 (upper panel). Development of liver metastasis of implanted MiaPaCa-2 cells with or without C5a Ab-treatment (lower panel). Attached Graphs showed average number of liver metastasis/mouse in the mice treated with control IgG or those treated with anti-C5a Ab ($n = 4$/each group, means and s.d. of quadruplicate were shown). (B) IHC analyses of the metastatic focus of MiaPaCa-2 in the mouse model. Liver isolated from the mice without treatment by anti-C5a Ab shown above was subjected to IHC analysis detecting C5a, C5aR, and PODXL1 expression on metastatic focus. The bottom panel showed coexpression of PODXL1 (red) and Vimentin (green) on an individual cancer cell by double IF.
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Author contributions

E.K. conceived, designed, and supervised the study. K.S., H.I., S.M, W.S. and M.S. performed experiments. E.K. discussed the result with K.S., H.I., M.S. and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.09.003.

Figure 6. Endogenous expression of C5aR, PODXL1 and C5a on the PDAC patient tissues. (A) IHC staining of C5aR on the invasive nests of primary PDAC tissues (two cases of PDAC patients). (B) Double IF showed membranous colocalization of PODXL1 (red) and C5aR (green) on the cancer nest at the invasive front of human PDAC. (C) Coexpression of C5aR and PODXL1 on cancer cells of primary PDAC tissues and the liver metastasis of the same patient. (D) Coexpression of C5aR and C5a at the liver metastatic focus of the PDAC patient. Note that non-neoplastic hepatocytes are also positive for both. (E) Schematic representation of the specific interaction of PODXL1-C5aR/C5a axis in PDAC invasion/metastasis.
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