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Short Communication

Artificial Control of Subtype-Specific Platelet-Derived Growth Factor–Receptor Signaling

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Abstract. Platelet-derived growth factor (PDGF) signaling controls various physiological functions via two receptor subtypes: PDGF receptor (PDGFR) α and PDGFRβ. Nevertheless, our understanding of their roles is limited because of a lack of pharmacological tools to discriminate subtype-specific signaling. We developed a chimeric receptor by combining ligand-binding-domain truncated PDGFRβ with anti-fluorescein single chain antibody, expecting the control of PDGFRβ-specific signaling by oligomerized fluorescein as an artificial agonist. Results show that calcium mobilization, Cdc42 activation, and cell migration were elicited specifically by the artificial ligand in cells expressing the chimeric receptor. Our method is expected to be useful to understand the subtype-specific roles of PDGFRs in various cellular functions.

Keywords: platelet-derived growth factor receptor, chimeric receptor

Platelet-derived growth factor (PDGF) receptor (PDGFR), a receptor tyrosine kinase (RTK) family member, controls various cell functions such as chemotaxis, actin cytoskeleton rearrangement, adhesion, polarity, cell survival, and induction of DNA synthesis (1). Two receptor subtypes –PDGFRα and PDGFRβ– can combinatorially form homodimers and heterodimers of three different kinds. As is the case for most RTK family members, dimerization of two receptor subunits upon binding of their cognate ligands to the extracellular domain of the subunits is a key event that triggers activation of their intrinsic tyrosine kinase activity and reciprocal phosphorylation of tyrosine residues on the intracellular domains (2). The phosphorylated intracellular domains recruit and activate unique sets of downstream signal molecules. Consequently, the subtype-specific variation of cellular signaling, which specifies cell outcomes, is determined on the structural basis of the intracellular domains. Nevertheless, it is often difficult to study the roles of each subtype separately because interactions of ligands and receptors are multiple and complex. Five ligands for PDGFR are known: PDGF-AA, -AB, -BB, -CC, and -DD. Although PDGF-AA can activate a PDGFRα homodimer specifically, all other PDGFs pleiotropically activate more than one type of dimer. It is notable that PDGF-BB activates all three types. In addition, no specific agonists or antagonists exist for discriminating subtypes, precluding conventional pharmacological approaches.

An alternative approach to studying the subtype-specific signaling would be creation of a pair of an artificial ligand and an engineered receptor with an altered specificity because the subtype specificity of signaling is determined only by the intracellular domain, not by other parts of the receptor protein (3). In this study, we developed a chimeric receptor by replacing the extracellular domain of PDGFRβ with a high-affinity anti-fluorescein single-chain antibody (scFv). As an artificial ligand, we prepared an oligomerized fluorescein molecule (OligoFL), expecting that dimerization and activation of the chimeric receptor is elicited by OligoFL. We tested this system in NIH 3T3 cells and found that Ca^{2+} signaling and Cdc42 activation, which are independent signal pathways downstream of PDGFRβ, as well as cell migration, were tightly controlled artificially.

OligoFL was prepared by conjugating fluorescein-NHS (Thermo Scientific, Rockford, IL, USA) with BSA (Sigma Chemical Co., St. Louis, MO, USA), resulting in a valence of eight. A retroviral vector harboring 4M5.3-ΔPRβ/L13 was constructed by inserting cDNAs encoding 4M5.3, the linker sequence (VDEQKLISEEDLN)

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and PDGFRβ without ligand binding domain (ΔPRβ; residue 513 – 1106) into pMX (4), yielding pMX-4M5.3-ΔPRβ/L13. The modification of linker length (L21: DYKDDDDKVDEQKLISEEDLN and L27: GKPIPNPLLGLDSTVDEQKLISEEDLN) was conducted using circular PCR with 4M5.3-ΔPRβ/L13 as a template.

Cells were maintained in DMEM (Nacalai Tesque, Inc., Kyoto) with 10% fetal bovine serum. For viral production, retroviral vector was transfected into GP293 cells. The NIH 3T3 cells were infected with virus particles.

For Ca^{2+} imaging, cells were loaded with fura-2 (Invitrogen Corp., Carlsbad, CA, USA) in medium (5 mM HEPES, 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose, pH 7.4) containing 0.1% BSA. Fluorescence images obtained from 350 and 380 nm excitation were acquired using an imaging system (ARGUS-HiSCA; Hamamatsu Photonics K.K., Hamamatsu) and an objective (×20, NA 0.75; Olympus, Humburg, Germany). For visualization of Cdc42 activity, 4M5.3-ΔPRβ/L21 and FRET-based Cdc42 probe, which is similar to the Raichu probe (5), were coexpressed in NIH 3T3 cells. The FRET image was divided by that of the GFP image to yield a FRET/GFP ratio image for evaluating FRET efficiency. Cell migration assay was conducted using Cell Culture Inserts (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) (6). For counting the migrated cells, fluorescence images of DAPI-stained migrated cells were acquired in a field (2,140 μm × 1,683 μm). Image analyses were conducted using software (ImageJ; NIH, Bethesda, MD, USA).

As portrayed in Fig. 1A, the schematic structure of the chimeric receptor is designated as 4M5.3-ΔPRβ. In 4M5.3-ΔPRβ, HA-tagged anti-fluorescein scFv 4M5.3 (7) was placed at the N-terminal end of extracellular ligand-binding-domain truncated PDGFRβ (ΔPRβ). A short amino acid sequence encoding the myc epitope was inserted between 4M5.3 and ΔPRβ as a spacer for the prototype chimeric receptor (4M5.3-ΔPRβ/L13). We transduced NIH 3T3 cells with retroviral vector harboring a 4M5.3-ΔPRβ/L13 construct and examined its expression using immunostaining with TRITC-labeled anti-HA antibody prior to fixation (Fig. 1B). The chimeric receptor was expressed on the cell surface. To check whether the expressed receptors bind fluorescein, we treated the cells with 10 nM fluorescein-biotin for 10 min; rhodamine-labeled streptavidin was used to detect cell-surface bound fluorescein-biotin. A specific signal was observed in cells expressing the chimeric receptor but not in untransduced cells (Fig. 1C), indicating that scFv retains the ability to bind fluorescein in the context of the chimeric receptor.

Activated PDGFR is known to recruit PLCγ, which produces inositol 1,4,5-trisphosphate, leading to intracellular Ca^{2+} mobilization (8). We measured intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) to test whether the Ca^{2+} signaling pathway is activated via the chimeric receptor. In cells expressing 4M5.3-ΔPRβ/L13, a slight but significant increase in $[Ca^{2+}]_i$ was detected after OligoFL application, although no marked change was observed in untransduced cells (Fig. 1D). When considering that native PDGFRs elicit much larger Ca^{2+} responses (ref. 9 and also see Fig. 1E), the result reflects that the first prototype chimeric receptor 4M5.3-ΔPRβ/L13 is functional but inefficient.

Because the prototype chimeric receptor 4M5.3-ΔPRβ/L13 can bind fluorescein, we reasoned that an impediment to dimerization is likely to cause inefficiency. To improve the dimerization step by adjusting the steric configuration of subunits, we constructed two more chimeric receptors, 4M5.3-ΔPRβ/L21 and 4M5.3-ΔPRβ/L27, which have longer (21 and 27 amino acids in length, respectively) spacers than 4M5.3-ΔPRβ/L13 (13 amino acids in length). All the three constructions were expressed in cells comparably (Fig. 1F). The surface expressions of these chimeric receptors were observed and no significant difference in fluorescence signals was found among the three chimeric receptors (266.9 ± 144.1, 244.0 ± 136.5, and 245.5 ± 143.7 for L13, L21, and L27, respectively; $P>0.9$). In addition, fluorescein-biotin binding ability of 4M5.3-ΔPRβ/L21 and 4M5.3-ΔPRβ/L27 was confirmed. Again, no significant differences were found (371.1 ± 179.9, 382.1 ± 203.1, and 384.9 ± 176.7 for L13, L21, and L27, respectively; $P>0.9$).

We subsequently compared Ca^{2+} responses. The 4M5.3-ΔPRβ/L21-expressed cells showed the best responses upon application of OligoFL (Fig. 1D). Intriguingly, cells expressing 4M5.3-ΔPRβ/L27 possessing the longest spacer showed only marginal responses. These results indicate that proper dimerization and activation requires adequate spacer length and that 4M5.3-ΔPRβ/L21 is the optimal chimeric receptor. We therefore used 4M5.3-ΔPRβ/L21 in subsequent experiments. We examined the dose-dependency of OligoFL-stimulated Ca^{2+} responses. Among 1, 10, and 100 nM OligoFL, 10 nM OligoFL elicited Ca^{2+} responses most efficiently, whereas 100 nM OligoFL provided less effective Ca^{2+} responses (Fig. 1E). This result is consistent with the idea that OligoFL at the higher concentrations tends to bind the chimeric receptor with an equivalent stoichiometry, preventing dimerization. Consistent with the Ca^{2+} responses, the chimeric receptor was found to be tyrosine-phosphorylated upon application of OligoFL (Fig. 1G).

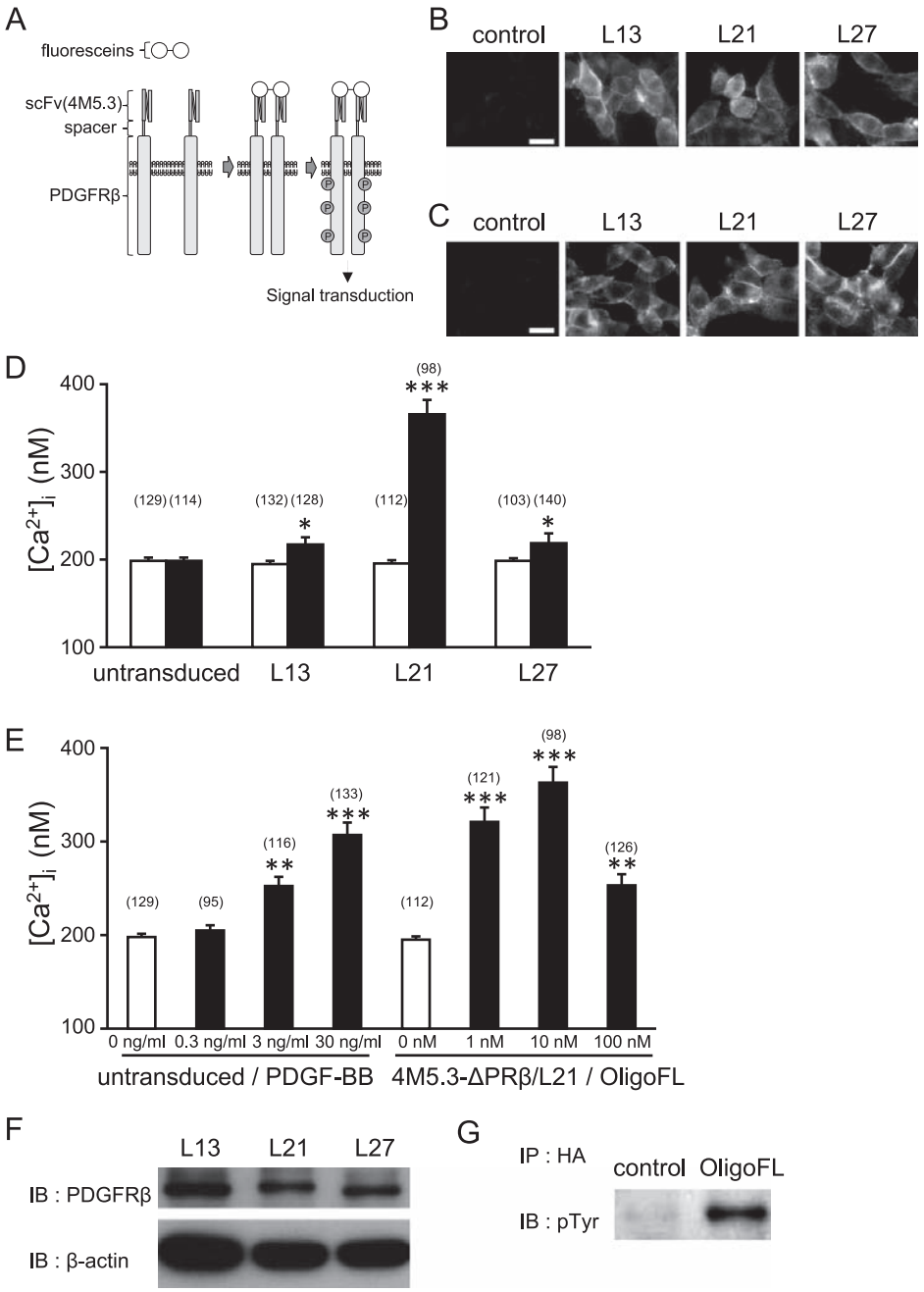


Fig. 1. Function of chimeric receptor triggered by OligoFL. A) Schematic representation of molecular design of chimeric receptor and its mechanism for activation by receptor dimerization. The extracellular ligand binding domain in PDGFRβ is replaced by anti- fluorescein scFv. B) Expression of each chimeric receptor expressed in NIH 3T3 cells detected with anti-HA antibody. L13 (4M5.3-ΔPRβ/L21), L21 (4M5.3-ΔPRβ/L21), and L27 (4M5.3-ΔPRβ/L27) denote the chimeric receptors with a spacer sequence consisting of 13, 21, and 27 amino acids, respectively. The scale bar shows 20 μm. C) Evaluation of fluorescein binding to chimeric receptors. Bound fluorescein-biotin was detected using streptavidin-rhodamine. The scale bar shows 20 μm. D) Evaluation of Ca^{2+} response upon application of 10 nM OligoFL (filled bar) or vehicle alone (open bar). Increase in Ca^{2+} response is evaluated at 6 min after OligoFL application. The number in parenthesis shows the number of cells in each group. Mean ± S.E.M. *P<0.05, ***P<0.001 (Welch's t-test). E) Dose-dependency of PDGF-BB on Ca^{2+} response and OligoFL in NIH 3T3 cells expressing 4M5.3-ΔPRβ/L21 and untransduced cells. The Ca^{2+} response was evaluated at 6 min after application of PDGF-BB or OligoFL (filled bar) or vehicle alone (open bar). The number in parenthesis shows the number of cells in each group. Mean ± S.E.M. **P<0.01, ***P<0.001 (two-tailed multiple t-test with Bonferroni's correction vs. vehicle). F) Expression levels of the chimeric receptors in NIH 3T3 detected with anti-PDGFRβ antibody (Cell Signaling Technology, Beverly, MA, USA). G) Tyrosine phosphorylation level of NIH 3T3 cells expressing 4M5.3-ΔPRβ/L21 after 6 min-stimulation. Cell lysates were immunoprecipitated with the anti-HA-probe antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and subjected to detection with anti-phospho-PDGFRβ Y1021 antibody (Santa Cruz Biotechnology).

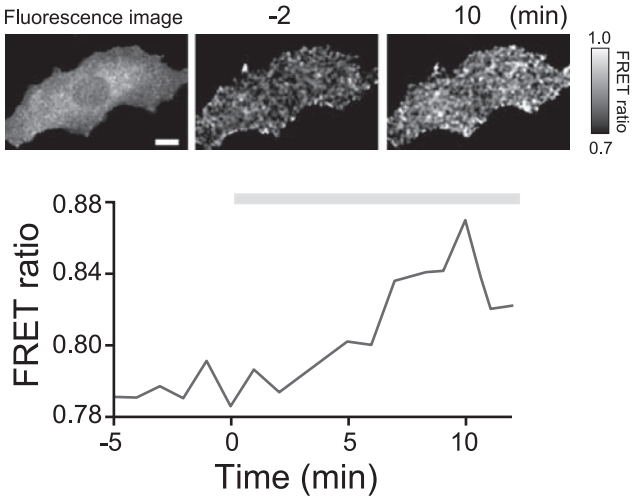


Fig. 2. Cdc42 activation elicited upon OligoFL application. Fluorescent images of a cell coexpressing 4M5.3-*Δ*PR*β*/L21 and FRET-based Cdc42 probe (left). FRET ratio in a cell at 2 min before (center) and 10 min after (right) OligoFL application is shown with the gray scale. The bar shows the period during which 10 nM OligoFL is applied. The scale bar shows 10 μm.

We next evaluated the activity of a Rho-family GTPase, Cdc42, which is known to regulate a number of cell functions, including gene expression, filopodia formation, and cell migration (10). Although detailed mechanisms for Cdc42 activation remain unclear, it has been thought that PDGFR activates Cdc42 via guanine nucleotide exchange factor Vav2 (11). To examine whether Cdc42 is activated upon activation of the chimeric receptor, we monitored activation of Cdc42 using the FRET-based probe in cells expressing 4M5.3-*Δ*PR*β*/L21; activation of Cdc42 was observed 5 min after OligoFL application (Fig. 2). This result, taken together with results of Ca^{2+} signaling, suggests that the chimeric receptor can transmit multiple downstream signaling pathways.

As the final test of functionality of the chimeric receptor, we specifically examined control of cell migration, which is a cellular outcome downstream of PDGFR and which is regulated through integration of multiple signal pathways including Cdc42 and Ca^{2+} signaling pathways (12, 13). The Boyden chamber method was used for cell migration assay (6). Application of 1 – 100 nM OligoFL facilitated migration for cells expressing 4M5.3-*Δ*PR*β*/L21, but not for untransduced cells (Fig. 3). It is notable that the maximum extent of migration by OligoFL was comparable to that by PDGF-BB. These results indicate that OligoFL specifically activates the exogenously introduced chimeric receptor and then properly controls the intrinsic signaling pathways that are necessary for cell migration.

In this study, we developed a chimeric receptor: 4M5.3-*Δ*PR*β*/L21. It can transmit cellular signaling efficiently and tightly. The combination of 4M5.3-*Δ*PR*β*/L21 and its specific agonist, OligoFL, constitutes

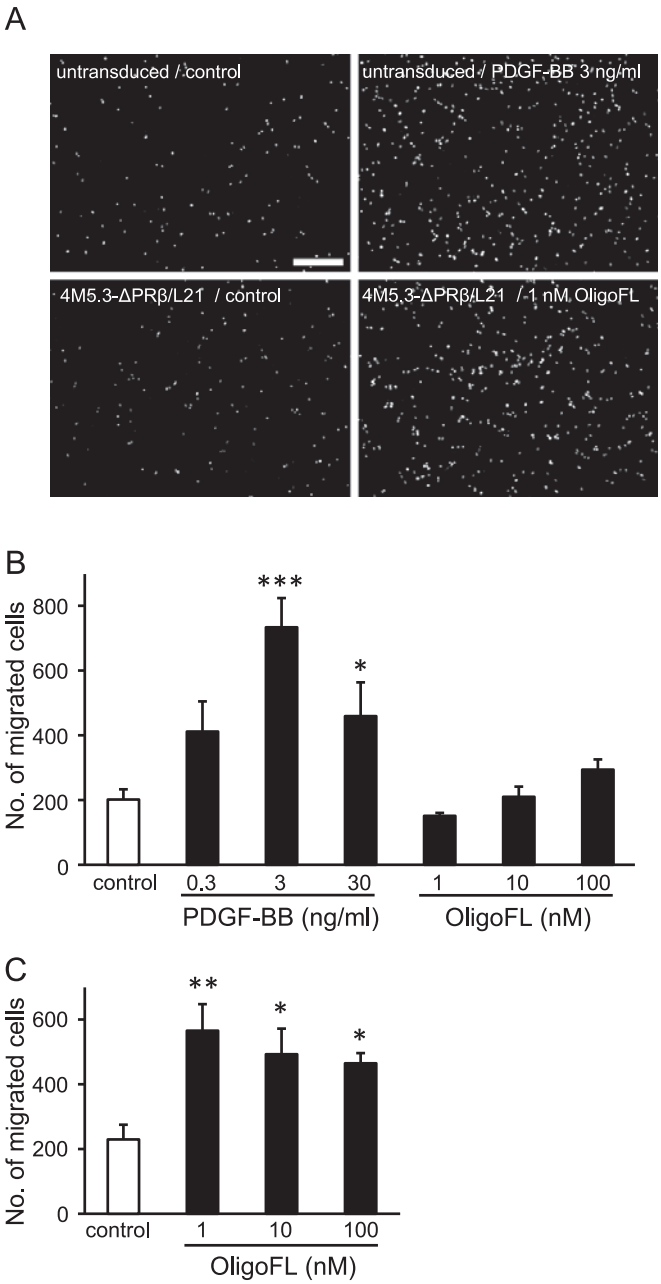


Fig. 3. Cell migration elicited upon OligoFL application to NIH 3T3 cells expressing 4M5.3-*Δ*PR*β*/L21. A) Representative fluorescence images of migrated cells. The scale bar shows 200 μm. B) Dose-dependencies of PDGF-BB and OligoFL on cell migration of untransduced NIH 3T3 cells. Quantities of migrated cells in each field (2,140 μm × 1,683 μm) were evaluated (n = 4). Mean ± S.E.M. **P*<0.05, ****P*<0.001 (two-tailed multiple *t*-test with Bonferroni's correction vs. control). C) Dose-dependency of OligoFL on cell migration of NIH 3T3 cells expressing 4M5.3-*Δ*PR*β*/L21 chimeric receptor. The quantities of migrated cells in each field (2,140 μm × 1,683 μm) were evaluated (n = 4). Mean ± S.E.M. **P*<0.05, ***P*<0.01 (two-tailed multiple *t*-test with Bonferroni's correction vs. control).

a new research platform for studying roles of PDGFR subtypes in various cell functions involving PDGF. A similar strategy can be applied readily to study similar types of tyrosine kinase receptors including vascular endothelial growth factor receptor, epidermal growth factor receptor, and neurotrophin receptors (TrkA, TrkB, and TrkC). Although the ligand in this study, OligoFL, was designed for homo-oligomerization of single receptor subtypes, development of bifunctinal ligands may enable us to examine the role of heterodimers of different receptor subtypes in near future.

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