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Platelet-derived growth factor receptors form complexes with neuropilin-1 during megakaryocytic differentiation of thrombopoietin-dependent UT-7/TPO cells

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ABSTRACT

Neuropilin-1 (NRP-1) is involved in angiogenesis, but the role of NRP-1 in megakaryocytopoiesis is not yet fully understood. In this study, we investigated whether thrombopoietin (TPO) regulates the expression of platelet-derived growth factor (PDGF) and its receptors (PDGFRs) on TPO-dependent UT-7/TPO cells and whether PDGFRs and NRP-1 on UT-7/TPO cells form complexes during megakaryocytic differentiation. When UT-7/TPO cells were starved of TPO for 24 h and then stimulated with 5 ng/ml TPO, the expression of PDGF-B, PDGFRα, and PDGFRβ were significantly up-regulated after the addition of TPO. TPO also induced tyrosine phosphorylation of PDGFRα but not PDGFRβ, and promoted the formation of PDGFRαβ heterodimer complexes. Furthermore, megakaryocytic differentiation of UT-7/TPO cells on treatment with phorbol myristate acetate (PMA) was accompanied by a marked up-regulation of PDGFRβ and NRP-1 protein expression, complex formation between PDGFRs and NRP-1, PDGFRαβ heterodimer complexes, and an increase in PDGF-BB-binding activity. Immunocytochemistry confirmed complex formation between PDGFRs and NRP-1 and PDGFRαβ heterodimer complexes in PMA-differentiated UT-7/TPO cells. Our observations suggest that NRP-1 is involved in megakaryocytopoiesis through complex formation with PDGFRs, and that NRP-1-PDGFR-complexes may contribute to effective cellular functions mediated by TPO and PDGF in megakaryocytic cells.

1. Introduction

Platelet-derived growth factor (PDGF) was first identified as a serum growth factor for fibroblasts, smooth muscle cells, and other cell types [1]. Recent studies have shown that PDGF and its receptors (PDGFRs) play critical roles in mesenchymal cell migration and proliferation, and normal development in embryogenesis [2]. Furthermore, autocrine and paracrine stimulations of PDGF play a role in some human cancers [2,3]. The PDGF family consists of four different polypeptide chains (PDGF-A, -B, -C, and -D) that are linked to form either homo- or heterodimers, of which five have been described: PDGF-AA, -AB, -BB, -CC, and -DD. The PDGF ligands exert their effects on target cells through binding with different specificities to two structurally related protein tyrosine kinase receptors, PDGFRα and PDGFRβ. On ligand binding, PDGFRs homodimerize (PDGFRαα and ββ) or heterodimerize (PDGFRαβ) and trans-phosphorylate each other at specific tyrosine residues, initiating signaling cascades that lead to growth, actin cytoskeleton rearrangements, and chemotaxis [1]. Thus, PDGF-AA induces PDGFRαα homodimers, PDGF-AB induces PDGFRαα homodimers and PDGFRαβ heterodimers, and PDGF-BB can induce all three possible combinations of dimers. The α-granules of platelets are a major storage site for PDGF, and PDGF can be synthesized and secreted by megakaryocytes, leukemia cell lines, and a bone marrow stromal cell line [4–6]. PDGF stimulates megakaryocyte colony formation and cell proliferation of megakaryocytic cells through functional PDGF receptors [7]. These findings suggest that the PDGF/PDGFR system plays a pivotal role in the regulation of megakaryocytopoiesis.

Neuropilin-1 (NRP-1) is a transmembrane glycoprotein that serves as a receptor for two unrelated types of ligand: the class 3 semaphorins in neurons and the vascular endothelial growth factor (VEGF) family in endothelial cells [8,9]. We previously reported that NRP-1 and VEGF receptor 2 (VEGFR-2) form complexes during the

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megakaryocytic differentiation of human thrombopoietin (TPO)-dependent UT-7/TPO cells, resulting in increased VEGF binding activity [10]. However, the participation of NRP-1 in megakaryocytopoiesis is not yet fully understood. In this study, we investigated whether TPO regulates the expression of PDGF and its receptors on TPO-dependent UT-7/TPO cells and whether PDGFRs and NRP-1 on UT-7/TPO cells form complexes during megakaryocytic differentiation. Our observations may help, at least in part, account for the role of NRP-1 in megakaryocytic cells.

2. Materials and methods

2.1. Cell culture and induction of differentiation

UT-7/TPO is a human megakaryocytic cell line completely dependent on TPO for its growth and survival [11]. UT-7/TPO cells were maintained in liquid culture with Iscove's modified Dulbecco's medium (IMDM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; BioWest, Nuaillé, France), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml TPO (a gift from Kirin Pharmaceuticals, Tokyo, Japan). Cells were grown in suspension in a 5% CO_2 humidified atmosphere at 37 °C and passaged every three days. The concentration of viable cells was determined by direct counting with a hemocytometer using the trypan blue exclusion method. UT-7/TPO cells (2.5×10^5 cells/ml) were induced to differentiate into a megakaryocytic lineage by treatment with 10 nM phorbol myristate acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) for 48 h, as described previously [10].

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

UT-7/TPO cells (2.5×10^5 cells/ml) were starved of TPO for 24 h and then stimulated with 5 ng/ml TPO for the indicated time period at 37 °C. Total RNA was extracted from cells and RT-PCR was performed, as described previously [10]. Primer sequences were as follows: PDGF-B forward: 5′-GAT CCG CTC CTT TGA TGA TC-3′, reverse: 5′-GTC TCA CAC TTG CAT GCC AG-3′ (PDGF-B PCR product: 435 base pairs [bp]); PDGFRα forward: 5′-ATC AAT CAG CCC AGA TGG AC-3′, reverse: 5′-TTC ACG GGC AGA AAG GTA CT-3′ (891 bp); PDGFRβ forward: 5′-AAT GTC TCC AGC ACC TTC GT-3′, reverse: 5′-AGC GGA TGT GGA AAG GCA TA-3′ (688 bp); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5′-CGT CTT CAC CAC CAT GGA GA-3′, reverse: 5′-CGG CCA TCA CGC CAC AGT TT-3′ (301 bp).

2.3. Western blot analysis

For the detection of PDGF protein expression and its receptors, UT-7/TPO cells (2.5×10^5 cells/ml) were starved of TPO for 24 h and then stimulated with 5 ng/ml TPO for the indicated time period at 37 °C. Lysates were prepared and Western blot analysis was performed. The amount of protein was determined with a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), and 30 μg was analyzed on the gel. Proteins were electrophoresed by 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P (Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk in 0.1% Tween20-Tris-buffered saline (TBS-T) for 1 h at room temperature and incubated with a 1:250 dilution of rabbit anti-PDGF-A antibody (H-77), anti-PDGF-B antibody (N-30), anti-PDGFRα antibody (951), or anti-PDGFRβ antibody (P-20, each Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at room temperature. After washes with TBS-T, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH, USA) at a dilution of 1:9000 for 2 h at room temperature. In some experiments, the following primary antibodies were used at a dilution of 1:1000: rabbit anti-PDGFRα antibody (D1E1E), anti-PDGFRβ antibody (28E1), anti-NRP-1 antibody (D62C6), and anti-β-Actin antibody (13E5, each Cell Signaling Technology, Danvers, MA, USA). After washes with TBS-T, chemiluminescence detection was performed with SuperSignal West femto Chemiluminescent Substrate (Pierce Biotechnology). For the detection of the mTOR (mammalian target of rapamycin) signaling pathway, UT-7/TPO cells (2.5×10^5 cells/ml) were starved of TPO for 24 h, cultured for an additional 2 h at 37 °C in FBS-free IMDM, and then stimulated with recombinant human PDGF-AA, -AB, or -BB (150 ng/ml, R&D Systems, Minneapolis, MN, USA) for 10 min at 37 °C in the presence or absence of 5 ng/ml TPO. Cell lysates were prepared and Western blot analysis was performed using the mTOR Substrates Antibody Sampler Kit (Cell Signaling Technology) according to the manufacturer's instructions.

2.4. Immunoprecipitation and Western blot analysis

For the detection of receptor complex formation, undifferentiated or PMA-differentiated UT-7/TPO cells (2.5×10^5 cells/ml) were incubated in FBS-free IMDM containing 5 ng/ml TPO for 2 h at 37 °C and then stimulated with recombinant PDGF-AA, -AB, or -BB (150 ng/ml, R&D Systems) for 10 min at 37 °C. After washing the cells with PBS, lysates were prepared, incubated with Protein-G Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences AB, Bjorkgatan, Sweden) for 1 h at 4 °C, and immunoprecipitated with a goat anti-PDGFRα or anti-PDGFRβ antibody (R&D Systems), anti-NRP-1 antibody (C-19, Santa Cruz Biotechnology), or a rabbit anti-VEGFR-2 antibody (55B11, Cell Signaling Technology) overnight at 4 °C. Western blot analysis was performed for the detection of PDGFRα, PDGFRβ, NRP-1, or VEGFR-2 using a 1:1000 dilution of rabbit anti-PDGFRα antibody (D1E1E or D13C6), anti-PDGFRβ antibody (28E1), anti-NRP-1 antibody (D62C6), or anti-VEGFR-2 antibody (D5B1, each Cell Signaling Technology), respectively.

2.5. Immunocytochemistry

Undifferentiated UT-7/TPO cells were cytocentrifuged onto a MAS-coated 2-well slide glass (Matsunami Glass Industries, Osaka, Japan) at a density of 2.5×10^4 cells/well. The PMA-differentiated UT-7/TPO cells were plated on a Glass Base Dish (Asahi Glass, Tokyo, Japan) at a density of 2×10^5 cells/ml. The attached cells were rinsed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min on ice, and then permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 5 min on ice three times. The cells were then blocked with PBS containing 0.3% Triton X-100, 5% horse serum, and 0.1% NaN_3 (PBS-TSN) for 1 h on ice, and they were incubated overnight at 4 °C with primary antibodies diluted in PBS-TSN. Goat anti-PDGFRα antibody (1:10, R&D Systems), rabbit anti-PDGFRα antibody (1:10, D13C6, Cell Signaling Technology), goat anti-PDGFRβ antibody (1:50, R&D Systems), and rabbit anti-NRP-1 (1:200, Abcam, Cambridge, MA, USA) were used as primary antibodies. After washing with PBS-T, the cells were incubated for 3 h on ice with secondary antibodies diluted in PBS-TSN. Alexa Fluor®488-conjugated donkey anti-rabbit IgG and Alexa Fluor®647-conjugated donkey anti-goat IgG (each 1:400, Life Technologies) were used as secondary antibodies. Following several washes in PBS-T, the cells were mounted using Prolong Gold antifade reagent with DAPI (Life Technologies) and examined using a Leica TCS-SP5/TIRF confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.6. PDGF-BB-binding assay

The PDGF-BB-binding activity of UT-7/TPO cells was determined by flow cytometric analysis using Fluorokine® biotinylated human PDGF-BB (R&D Systems) according to the manufacturer's instructions. Undifferentiated or PMA-differentiated UT-7/TPO cells (4×10^6 cells/ml) were incubated with 10 μl of biotinylated PDGF-BB reagent or negative control reagent for 1 h at 4 °C. Then, 10 μl of avidin-FITC reagent was added to each sample, and the reaction mixture was incubated for an additional 30 min at 4 °C in the dark. After incubation, the cells were washed twice to remove unreacted avidin-FITC and analyzed by flow cytometry. In the blocking assay, 100 ng/ml of neutralizing anti-PDGF antibody, anti-PDGFRα antibody, or anti-PDGFRβ antibody (each R&D Systems) was first mixed with 10 μl of biotinylated PDGF-BB and incubated for 15 min at room temperature. The mixture was then transferred to a tube containing UT-7/TPO cells, which were preincubated with 1 ng/ml of mouse IgG (R&D Systems) for 15 min at room temperature, and incubated for an additional 1 h at room temperature. In the case of semaphorin (Sema) inhibition, UT-7/TPO cells were incubated with 1 μg/ml of recombinant human Sema3A/Fc chimera (Genzyme-Techne, Minneapolis, MN, USA) for 1 h at 4 °C. After washing, the cells were incubated with 10 μl of biotinylated PDGF-BB reagent for 1 h at 4 °C. The following reaction was the same as described above.

2.7. Statistical analysis

Data are expressed as the mean ± SD. Significance was assessed by Student's *t*-test using the StatView software (Abacus Concepts, Berkeley, CA, USA). A p-value of <0.05 was considered significant.

3. Results

3.1. TPO regulates the expression of PDGF and its receptors and promotes the formation of PDGFRαβ heterodimer complexes in UT-7/TPO cells

When UT-7/TPO cells were starved of TPO for 24 h and then stimulated with 5 ng/ml TPO, both mRNA and protein expression of PDGF-B, PDGFRα, and PDGFRβ was significantly up-regulated after the addition of TPO (Supplementary Fig. 1). To investigate whether PDGFRs on UT-7/TPO cells are functional, we determined PDGFR tyrosine phosphorylation in UT-7/TPO cells. TPO induced the tyrosine phosphorylation of PDGFRα but not PDGFRβ within 1 h after the addition of TPO in UT-7/TPO cells (Fig. 1A). We also investigated whether TPO could promote the formation of PDGFRαβ heterodimer complexes in UT-7/TPO cells. As shown in Fig. 1B, an anti-PDGFRα or anti-PDGFRβ antibody immunoprecipitated PDGFRβ or PDGFRα, respectively, from lysates of UT-7/TPO cells 24 h after the addition of TPO. When anti-PDGFRα (D1E1E) and anti-PDGFRβ (28E1) antibodies were used, we detected two bands at about 190 kd, of which the upper band corresponds to the PDGFR glycosylated. Next, we investigated agonist-induced activation of the mTOR signaling pathway in UT-7/TPO cells. After FBS starvation for 2 h, UT-7/TPO cells were stimulated with recombinant PDGF-AA, -AB, or -BB in the presence or absence of 5 ng/ml TPO, and lysates were subjected to Western blot analysis for the detection of the mTOR signaling pathway. As shown in Fig. 1C, mTOR itself was constitutively activated in UT-7/TPO cells irrespective of stimulation with PDGF isoforms. In the presence of TPO, recombinant PDGF-AA induced the phosphorylation of p-4E-BPI (Thr37/46), whereas PDGF-AB and -BB induced the phosphorylation of p-70S6K (Thr389) in UT-7/TPO cells. These findings suggest that PDGFRs on UT-7/TPO cells are functional and may be involved in the cellular responses through the activation of the mTOR signaling pathway.

3.2. Megakaryocytic differentiation promotes the formation of complexes between PDGFRs and NRP-1 and of PDGFRαβ heterodimer complexes in UT-7/TPO cells

The PMA treatment of UT-7/TPO cells induced them to differentiate into a megakaryocytic lineage with an increased number of multilobulated giant cells and CD61- and CD41-positive cells [10]. The megakaryocytic differentiation of UT-7/TPO cells was accompanied by a marked up-regulation of both PDGFRβ and NRP-1 protein expression (Fig. 2A). Next, we investigated whether megakaryocytic differentiation could promote receptor complex formation between NRP-1 and PDGFRs in UT-7/TPO cells. Undifferentiated or PMA-differentiated UT-7/TPO cells were starved of

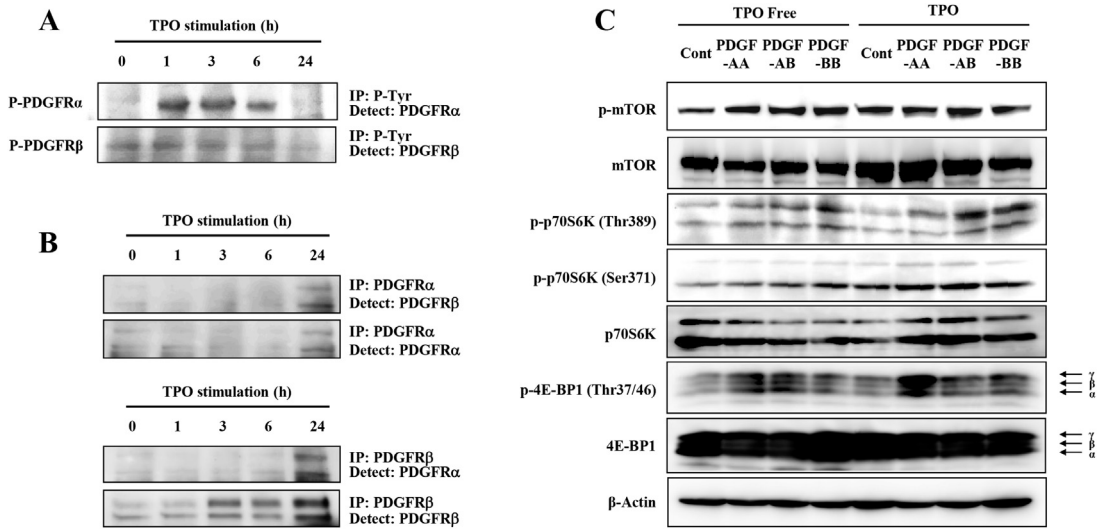

Fig. 1. PDGFRs on UT-7/TPO cells are functional. Lysates were prepared, immunoprecipitated with an anti-phosphotyrosine antibody **(A)** or an anti-PDGFRα antibody or anti-PDGFRβ antibody **(B)**, and subjected to Western blot analysis for the detection of PDGFRα and PDGFRβ. A representative of three independent experiments is shown. **C**: UT-7/TPO cells were starved of TPO for 24 h and then stimulated with recombinant PDGF-AA, -AB, or -BB (150 ng/ml) in the presence or absence of 5 ng/ml TPO for 10 min at 37 °C. Cell lysates were prepared and subjected to Western blot analysis for the detection of the mTOR signaling pathway. A representative of five independent experiments is shown.

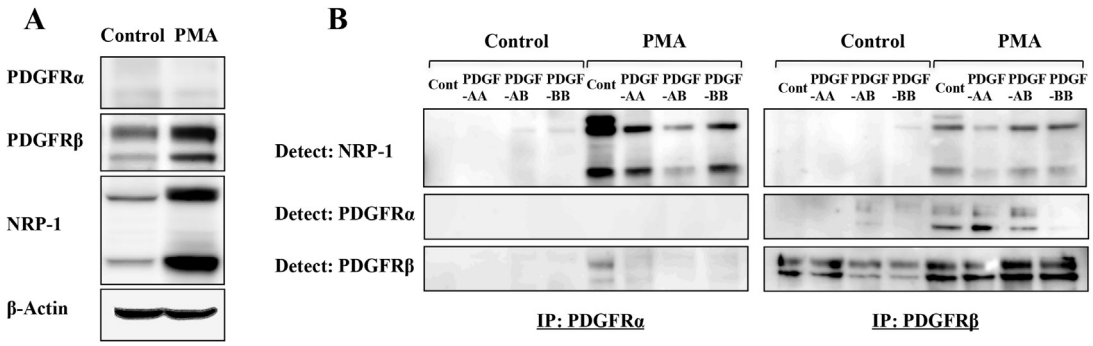

Fig. 2. PDGFRs form complexes with NRP-1 during megakaryocytic differentiation of UT-7/TPO cells. **A**: Cell lysates from undifferentiated (Control) or PMA-differentiated (PMA) UT-7/TPO cells were prepared and Western blot analysis was performed for the detection of PDGFRs and NRP-1 protein expression. **B**: Undifferentiated (Control) or PMA-differentiated (PMA) UT-7/TPO cells were stimulated with recombinant PDGF-AA, -AB, or -BB (150 ng/ml) for 10 min at 37 °C. Cell lysates were prepared, immunoprecipitated with an anti-PDGFRα or anti-PDGFRβ antibody, and subjected to Western blot analysis for the detection of NRP-1, PDGFRα, and PDGFRβ. A representative of three independent experiments is shown.

TPO, stimulated with recombinant PDGF-AA, -AB, or -BB, immunoprecipitated with an anti-PDGFRα or anti-PDGFRβ antibody, and subjected to Western blot analysis for the detection of NRP-1, PDGFRα, and PDGFRβ. As shown in Fig. 2B (left panel), anti-PDGFRα antibody clearly immunoprecipitated NRP-1 from lysates of PMA-differentiated UT-7/TPO cells. Anti-PDGFRβ antibody also immunoprecipitated NRP-1 from lysates of PMA-differentiated UT-7/TPO cells (Fig. 2B, right panel). In addition, anti-PDGFRβ antibody immunoprecipitated PDGFRα from lysates of PMA-differentiated UT-7/TPO cells. It is noteworthy that both anti-PDGFRα and anti-PDGFRβ antibodies co-immunoprecipitated with NRP-1 even in the absence of agonists, indicating that the megakaryocytic differentiation itself promoted complex formation. To investigate whether a concomitant expression of PDGFRs and NRP-1 parallels to that of VEGFR-2 and NRP-1 [10], we performed immunoprecipitation analysis for the detection of PDGFRα, PDGFRβ, NRP-1, and VEGFR-2 simultaneously in UT-7/TPO cells (Supplementary Fig. 2). As might have been expected, anti-NRP-1 antibody immunoprecipitated PDGFRα, PDGFRβ, or VEGFR-2 from lysates of PMA-differentiated UT-7/TPO cells. However, anti-PDGFRα antibody or anti-PDGFRβ antibody did not immunoprecipitate with VEGFR-2 in both undifferentiated and PMA-differentiated UT-7/TPO cells, suggesting that a super complex receptor unit composed of these receptor kinases cannot be formed during megakaryocytic differentiation of UT-7/TPO cells.

To simultaneously visualize receptor complexes between PDGFRs and NRP-1, we performed immunocytochemistry during megakaryocytic differentiation of UT-7/TPO cells. Both PDGFRα and PDGFRβ co-localized with NRP-1 in PMA-differentiated UT-7/TPO cells (Fig. 3A and B). Furthermore, PDGFRα co-localized with PDGFRβ in PMA-differentiated UT-7/TPO cells (Fig. 3C), although the staining patterns were quite different from those of the PDGFRs-NRP-1 complexes. These findings suggest that PDGFRs on UT-7/TPO cells form complexes with NRP-1 and also PDGFRαβ heterodimer complexes during megakaryocytic differentiation.

3.3. Megakaryocytic differentiation increases PDGF-BB-binding activity of UT-7/TPO cells

To investigate whether the formation of receptor complexes could affect PDGF-BB-binding activity in UT-7/TPO cells, we performed a PDGF-BB-binding assay using flow cytometry and biotinylated PDGF-BB. Most undifferentiated UT-7/TPO cells were bound to PDGF-BB (Fig. 4A-a, upper panel), and the PDGF-BB-binding activity was further increased in PMA-differentiated UT-7/TPO cells (Fig. 4B). The PDGF-BB-binding activity of both undifferentiated and PMA-differentiated UT-7/TPO cells was inhibited almost completely by anti-PDGF, anti-PDGFRα, or anti-PDGFRβ antibody (Fig. 4A-b, -c, and -d, upper and lower panels, respectively). Sema3A, which is a competitive inhibitor of NRP-1, partially inhibited the PDGF-BB-binding activity of undifferentiated cells, whereas it failed to inhibit the activity of PMA-differentiated cells (Fig. 4A-e, upper and lower panels, respectively), presumably due to complex formation between NRP-1 and PDGFRs in PMA-differentiated UT-7/TPO cells. These findings suggest that megakaryocytic differentiation increased PDGF-BB-binding activity on UT-7/TPO cells, resulting from the formation of complexes between PDGFRs and NRP-1 and of PDGFRαβ heterodimer complexes.

4. Discussion

The heterodimeric PDGFRαβ complex, which can be preferentially induced by ligand binding with PDGF-AB, has different properties to the corresponding homodimeric PDGFRαα and PDGFRββ complexes. The more efficient Ras activation and mitogenic stimulation are induced by PDGF-AB rather than by PDGF-BB [12], suggesting that the PDGFRαβ heterodimer induces more potent mitogenic effects than the homodimeric complexes. One mechanism for such a difference is that the autophosphorylation of tyrosine residues occurs at different sites in the PDGFRαβ heterodimer from those in the homodimeric complexes [1,13]. In this study, we showed that the PDGFRαβ heterodimer complex was formed in undifferentiated UT-7/TPO cells after stimulation with TPO, as well as during the megakaryocytic differentiation of UT-7/TPO cells. Although TPO up-regulated both PDGFRα and PDGFRβ protein expression in UT-7/TPO cells (Supplementary Fig. 1), TPO induced tyrosine phosphorylation of PDGFRα but not PDGFRβ. It has been reported that the kinase-inactive receptor was phosphorylated by the kinase-active receptor after the formation of PDGFRαβ heterodimer complexes, resulting in the phosphorylation within the receptor dimer occurring in trans between the components, and the fact that the heterodimeric receptor complex mediates a stronger mitogenic response than either of the homodimeric complexes [13]. Our observations suggest that the PDGFRαβ heterodimer complex may function to transduce the signals of TPO more efficiently in megakaryocytic cells, and that TPO regulates the function of megakaryocytic cells through PDGFRs, as well as Mpl receptor.

Neuropilins are involved in neuronal axon guidance in embryonic development and play pivotal roles in angiogenesis [8,9]. Recent studies have revealed a much broader spectrum in signaling

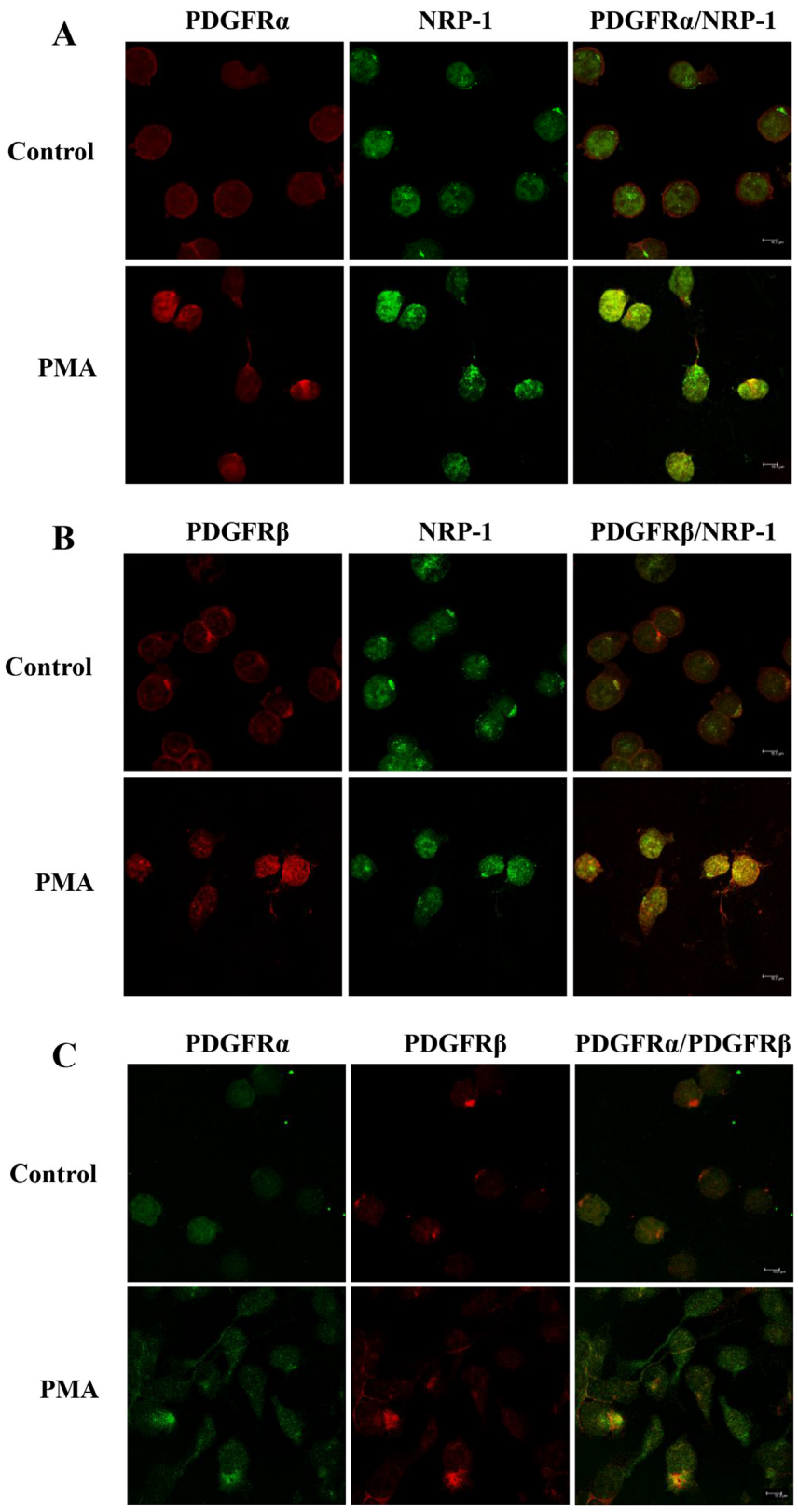

Fig. 3. PDGFRs form complexes with NRP-1 and PDGFRαβ heterodimer complexes during megakaryocytic differentiation of UT-7/TPO cells. **A, B**: Undifferentiated (Control) or PMA-differentiated (PMA) UT-7/TPO cells were double stained for PDGFRα or PDGFRβ (red) and for NRP-1 (green), and then viewed and photographed using a confocal microscope. **C**: Undifferentiated (Control) or PMA-differentiated (PMA) UT-7/TPO cells were double stained for PDGFRα (green) and PDGFRβ (red), and photographed. A representative of three independent experiments is shown.

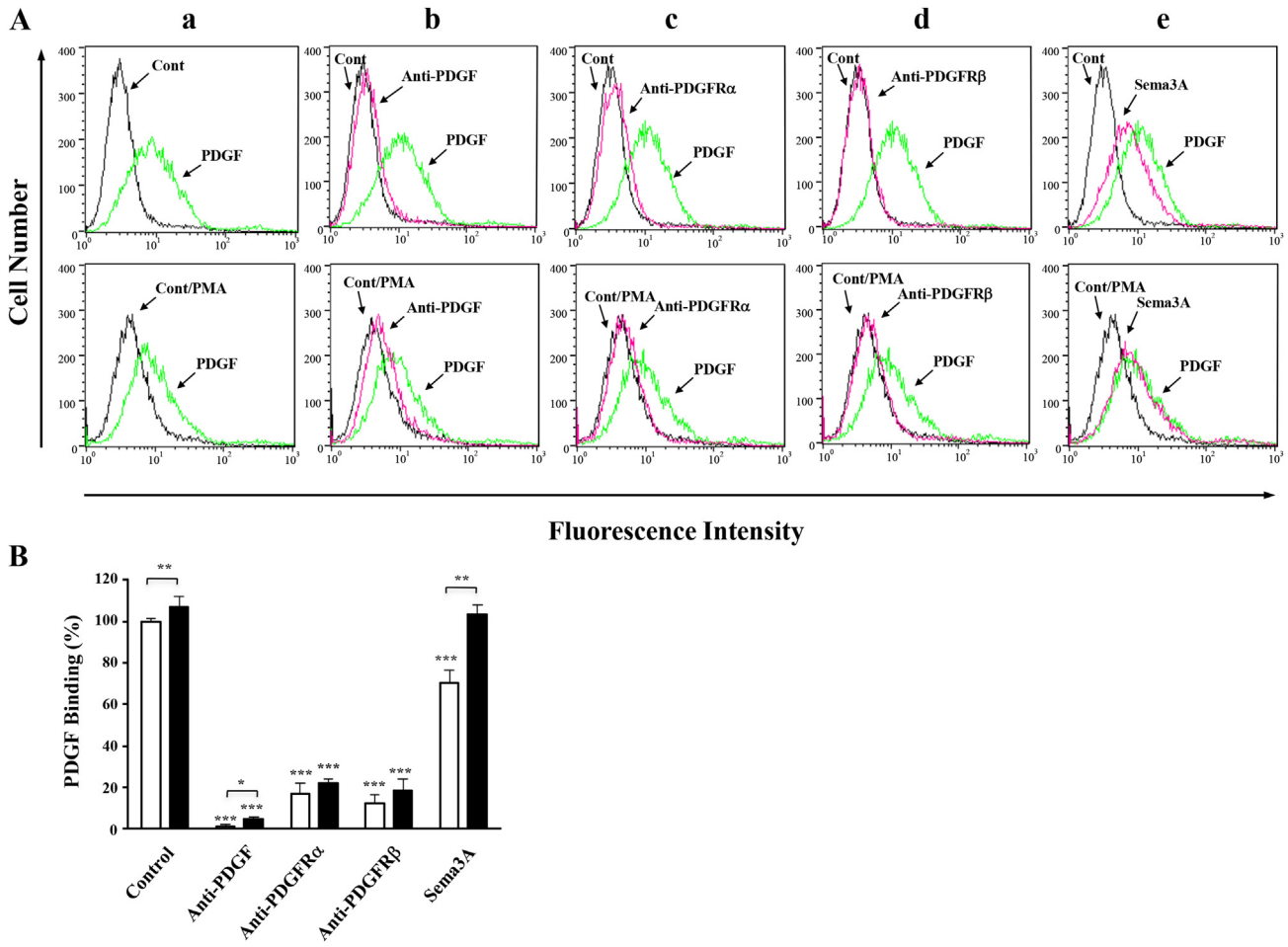

Fig. 4. Megakaryocytic differentiation promotes an increase in PDGF-BB-binding activity of UT-7/TPO cells. **A**: Undifferentiated (upper panel) or PMA-differentiated (lower panel) UT-7/TPO cells were stained with PDGF-biotin and avidin-FITC, and analyzed by flow cytometry. Green lines depict PDGF–BB binding of UT-7/TPO cells. **b, c, d, e**: Red lines depict PDGF–BB binding of UT-7/TPO cells that were treated with an anti-PDGF (**b**), anti-PDGFRα (**c**), or anti-PDGFRβ (**d**) antibody for 15 min at room temperature, or with Sema3A (**e**) for 1 h at 4 °C. A representative of three independent experiments is shown. **B**: Undifferentiated (open column) or PMA-differentiated (closed column) UT-7/TPO cells were treated with medium alone (Control), an anti-PDGF, anti-PDGFRα, or anti-PDGFRβ antibody for 15 min at room temperature, or with Sema3A for 1 h at 4 °C, and then PDGF-BB-binding activity was assessed by flow cytometry. The data represent the mean ± SD of three independent experiments (**$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induced by other growth factors, including transforming growth factor β1 and its receptors, hepatocyte growth factor and its receptors, PDGF and PDGFRs, fibroblast growth factors, and integrins [14,15]. NRP-1 knockdown significantly reduced PDGFR activation in mesenchymal stem cells and vascular smooth muscle cells [16,17]. Furthermore, NRP-1 co-localized with PDGFRβ in hepatic stellate cells, NRP-1 knockdown attenuated PDGF-induced chemotaxis, while NRP-1 overexpression increased cell motility [18]. However, the role of NRP-1 in megakaryocytic cells is not yet fully understood. In this study, we showed that the increased expression of both NRP-1 and PDGFRβ was accompanied by an increase in the PDGF-BB-binding activity in PMA-differentiated UT-7/TPO cells, where the complexes between NRP-1 and PDGFRβ were formed. The PDGF-BB-binding activity of UT-7/TPO cells was abrogated almost completely by anti-PDGF, anti-PDGFRα, and anti-PDGFRβ neutralizing antibody in both undifferentiated and PMA-differentiated cells, suggesting that PDGF–BB binding in UT-7/TPO cells was mainly mediated by PDGFRs. However, it was partially abrogated by Sema3A in undifferentiated UT-7/TPO cells, whereas Sema3A failed to inhibit PDGF–BB binding in PMA-differentiated cells. The formation of complexes between NRP-1 and PDGFRβ may promote the more rigid binding of NRP-1 on megakaryocytic cells. These findings suggest that the formation of complexes between NRP-1 and PDGFRs plays a role in regulating PDGF signaling in megakaryocytic cells.

The binding of TPO to the Mpl receptor activates various types of intracellular signaling pathway that play important roles in the regulation of megakaryocytopoiesis, such as phosphatidylinositol 3-kinase (PI-3K). The TPO-induced PI-3K activity is essential for optimal cell survival and cell cycling of megakaryocyte progenitors [19]. One downstream target of PI-3K is mTOR, which is a Ser/Thr kinase that regulates cell growth and cell-cycle progression [20,21]. The mTOR signaling pathway is involved in the regulation of megakaryocytopoiesis and regulates cell growth through two effector proteins critical for ribosomal biogenesis and translational initiation, p70S6K1 and 4E-BP1 [22,23]. Activated p70S6 kinase initiates the translation of a class of mRNAs containing a tract of polypyrimidine in their 5′ untranslated region. Upon phosphorylation, 4E-PB1 is inactivated and releases eIF4E, a protein that will be recruited to the translation initiation complex regulating cap-dependent translation [24]. In this study, we showed that PDGF-AA induced the phosphorylation of p-4E-BPI (Thr37/46) in the

presence of TPO, whereas PDGF-AB and -BB induced the phosphorylation of p-70S6K (Thr389) in UT-7/TPO cells. These findings suggest that the agonist-induced activation of PDGFRs on UT-7/TPO cells may be involved in the mTOR signaling pathway and regulated by the different downstream signaling proteins of mTOR.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.124.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.124.

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