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Integral roles of a guanine nucleotide exchange factor, FARP2, in osteoclast podosome rearrangements

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ABSTRACT **Podosomes are recently rediscovered highly dynamic actin-rich structural and functional modules that form close contact with the surrounding substrate. They play a role in the control of migration, tissue invasion, and matrix remodeling of highly motile cells, including lymphocytes, macrophages, dendritic cells, and osteoclasts. In osteoclasts, the compaction of podosomes induces the formation of a tight adhesive contact, the sealing zone, which defines a subosteoclastic environment specialized for bone resorption. Integrins and the Rho family small GTPases are key regulators of podosome rearrangements. However, it remains to be determined how the activation of integrins and Rho family GTPases is regulated during osteoclast podosome rearrangements. Here, we demonstrate a crucial role for the FERM domain-containing guanine nucleotide exchange factor (GEF), FARP2, in osteoclast podosome rearrangements and resorbing activity. We determine by live cell imaging and biochemical assays that FARP2 is required for localized activation of GTP-bound Rac1 into podosome-ring like structures. In addition, FARP2 is relevant to integrin β3 activity during osteoclastogenesis. Furthermore, FARP2 deficiency results in reduced formation of multinucleated osteoclasts and resorption pits compared to wild-type osteoclasts (controls). Collectively, our findings reveal an integral role of FARP2 for regulation of Rac1 and integrin β3 throughout podosome rearrangement in osteoclastogenesis.—Takegahara, N., Kang, S., Nojima, S., Takamatsu, H., Okuno, T., Kikutani, H., Toyofuku, T., Kumanogoh, A. Integral roles of a guanine nucleotide exchange factor, FARP2, in osteoclast podosome rearrangements. *FASEB J.* 24, 4782–4792 (2010). www.fasebj.org**

Key Words: small GTPases · integrin · bone resorption

PODOSOMES ARE PROMINENT actin-based cellular structures that serve as unique structural and functional modules to mediate cellular adhesion, trafficking, and matrix remodeling (1, 2). Podosomes are observed not only in highly motile cells, including lymphocytes (3), macrophages (4, 5), dendritic cells (6, 7), and osteoclasts (8), but also in endothelial cells and smooth muscle cells (9, 10). Podosome turnover is regulated by the dynamic organization of the actin cytoskeleton. Localized and finely tuned activities of Rho family of small GTPases are crucial for regulation of actin rearrangements and maintenance of podosome structures (11–14). In addition, integrin engagement with the extracellular matrix (ECM) is required for podosome formation through regulation of actin dynamics (15, 16). Although dynamic remodeling of the actin cytoskeleton through Rho family GTPases and integrins is crucial for spatiotemporal regulation of podosomes, the regulatory mechanisms for integrins and Rho family small GTPases during podosome rearrangements still remain unclear.

FARP2 is a Dbl family guanine nucleotide exchange factor (GEF) specific for Rac1 (17). GEFs from the Dbl family integrate extracellular signaling with appropriate activation of Rho GTPases in specific subcellular regions (18). FAPR2 contains a FERM domain, characteristic of the plasma membrane proteins linker, and associates with the cytoplasmic regions of plexin-A family members through its FERM domain (19). Previous studies suggest a regulatory role of FARP2 in transduction of semaphorin-induced repulsive cues in axons, *via* regulation of Rac1 activation and inhibition of integrins by suppressing PIPKIγ661, a phosphatidylinositol kinase that has been implicated in promoting the assembly of integrin-containing focal adhesions (17, 19). Of note, the expression of FARP2 has been reported to be induced by stimulation of receptor

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activator of NF-κB ligand (RANKL), a key cytokine for development of osteoclasts (20). Osteoclasts are highly motile, bone-resorbing cells; their activity largely depends on their adhesion to the bone surface by podosomes (21). Although these findings suggest a regulatory role for FARP2 in cytoskeletal reorganization through regulating Rac1 and integrins during osteoclastogenesis, the functional significance of FARP2 in osteoclasts still remains to be determined.

In this work, we explored the mechanisms for the podosome dynamics regulated by FARP2 during osteoclastogenesis. Using live cell imaging and biochemical assays, we provide evidence that FARP2 is required for localized activation of GTP-bound Rac1 into podosome-ring structures. In addition, FARP2 is relevant to integrin β3 activity during osteoclastogenesis. Furthermore, we here generate FARP2-deficient mice, thereby present that FARP2 deficiency results in reduced formation of osteoclasts and resorption pits. Collectively, our findings reveal an integral role of FARP2 for regulation of Rac1 and integrin β3 throughout podosome rearrangement in osteoclastogenesis.

MATERIALS AND METHODS

Mice

To construct the FARP2 targeting vector, a 5.2-kb fragment containing the ninth and tenth exon of the FERM domain was replaced with a neo resistance cassette, and the herpes simplex virus thymidine kinase (HSV-*tk*) gene was inserted for selection against random integration. The linearized targeting plasmid DNA was transfected into embryonic stem (ES) cells by electroporation. After double selection with G418 and gancyclovir, 96 resistant clones were screened for homologous recombination of the *FARP2* targeted allele by PCR and Southern blot analysis. Two clones with homologous recombination were identified and isolated. ES cells from the two independent *FARP2* mutant clones were injected separately into blastocysts from C57BL/6 mice. The blastocysts were transferred to pseudopregnant ICR foster mothers, and chimeric males were then backcrossed to C57BL/6 females for 5 generations. Germline transmission and the genotype of *FARP2*-targeted allele were assayed further by genomic PCR analysis, RT-PCR analysis, and Western blot analysis. For genomic PCR analysis, genomic DNA was isolated from tails of mice. PCR was performed with 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The following oligonucleotide primers were used to identify the rearranged *FARP2* locus: primer 1 (5′-ATCAAACTCCACCCTGAGGTCCATG-3′), primer 2 (5′-TTTGTAAACTGCAGCGTTTCTCTTC-3′), and primer 3 (5′-CTTCTGAGGGGATCGGCAAT-3′). For RT-PCR analysis, RNA was isolated from bone marrow-derived dendritic cells using RNeasy kits (Qiagen, Venlo, The Netherlands) and treated with DNase I (Invitrogen, Eugene, OR, USA) to eliminate genomic DNA. cDNA was synthesized using a SuperScript II cDNA synthesis kit (Invitrogen), and RT-PCR was performed with 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s using the follwing primers: primer a, 5′-CCTTCTGCAGTCGGAAATCG-3′; and primer b, 5′-TTGGTGGTACCCTGGAACAC-3′. For Western blot analysis, we obtained anti-FARP2 polyclonal antibody by immunizing rabbits with mouse FARP2 peptides coded by exon 12. Brain lysates of wild-type (+/+) or FARP2$^{-/-}$ (−/−) mice were prepared and immunoprecipitated with protein A-sepharose plus rabbit anti-FARP2 polyclonal antibody. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with rabbit anti-FARP2 polyclonal antibody. Mice were maintained in a specific pathogen-free environment. All experimental procedures were consistent with our institutional guidelines.

Establishment of stable transfectants

Stable RAW264.7 transfectants, which express either the intact form of FARP2 (FL-FARP2) or a RacGEF domain-deleted form of FARP2 (ΔGEF-FARP2), were established by introducing V5-tagged FL-FARP2 or V5-tagged ΔGEF-FARP2 expression constructs with a pMCIneo vector using Nucleofector (Amaxa, Koeln, Germany) according to the manufacturer's protocol. Transfectants expressing V5-tagged FL-FARP2 or V5-tagged ΔGEF-FARP2 were selected in the presence of G418, screened using a monoclonal anti-V5 antibody (Invitrogen) and cloned. These cells were stimulated in the presence or absence of LPS, and the production of TNF-α in the culture supernatant was measured by ELISA.

In vitro osteoclast differentiation

RAW264.7 transfectants were cultured in α-MEM containing 10% FCS for 72 h in the presence of soluble mouse RANKL (20 ng/ml) (Peprotech, Rocky Hill, NJ, USA). Mouse bone marrow-derived osteoclasts were induced as described previously (22). In brief, bone marrow progenitor cells were cultured with mouse M-CSF (20 ng/ml) (Peprotech) in α-MEM containing 10% FCS. After 48 h, culture medium was replaced with M-CSF plus RANKL, and cells were cultured for an additional 96–120 h. The resulting cells were fixed and stained with tartrate-resistant acid phosphatase using a TRAP-staining kit (TaKaRa, Shiga, Japan). TRAP solution assay was performed as described previously (23). In brief, cells were solubilized by lysis buffer (0.2% Nonidet-P40) and stained with TRAP solution. The TRAP activity was measured at 405 nm of absorbance using a microplate reader (Arvo MX; PerkinElmer, Waltham, MA, USA).

Confocal microscopy

For actin staining, OCLs were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were permeabilized in 0.1% Triton X-100 in PBS for 5 min, washed with PBS, and then incubated in PBS containing Alexa-Fluor546-phalloidin (Invitrogen) and SYTO11 (Invitrogen) for 30 min. For immunostaining, OCLs were fixed in 4% paraformaldehyde in PBS for 30 min, blocked in 30% normal goat serum for 1 h, and then incubated overnight in Can Get Signal solution A (Toyobo, Osaka, Japan) containing rabbit anti-FARP2 antibody. After washing with PBS, cells were incubated in 2% BSA-PBS containing AlexaFluor647-F(ab′)$_2$ fragment of goat anti-rabbit IgG (Invitrogen) and Alexa-Fluor546-phallodinin for 1 h. After washing with PBS, cells were visualized using an inverted confocal microscope (LSM5 exciter with HAL 100; Zeiss, Heidelberg, Germany).

FRET analysis

To monitor the activation of small GTPase, Rac1, RAW264.7 transfectants were transiently transfected with a FRET probe, Raichu-Rac1 (24), a gift from Michiyuki. Matsuda (Kyoto University, Kyoto, Japan), using Nucleofector (Amaxa). After 24 h incubation, CFP-positive cell population was sorted and cultured in α-MEM containing 10% FCS in the presence of

RANKL. FRET imaging and phase contrast time-lapse microscopic imaging were performed using a Nikon Biostation IM imaging system and NIS-Element Advanced Research software (Nikon Instech, Tokyo, Japan). For imaging, the cells were imaged every 5 min for ≥1 h.

Cell adhesion assay

RAW264.7 transfectants were cultured either on plastic plates or on calcium phosphate-coated osteologic discs (BD Biosciences, Franklin Lakes, NJ, USA) in the presence of RANKL (20 ng/ml). After 120 h, plates or discs were washed carefully, and the remaining adherent cells were solubilized in buffer containing CyQuant GR Dye (Cell Biolabs, Denver, CO, USA). Fluorescence [relative fluorescence units (RFU)] was measured with a fluorescence plate reader at 480/520 nm (Arvo MX; PerkinElmer).

RT-PCR analysis

Total RNA was extracted from cultured RAW264.7 cells or bone marrow-derived osteoclasts using an RNeasy mini kit (QIAGEN) and treated with DNase I (Invitrogen) to eliminate genomic DNA. cDNA was synthesized using a SuperScript II cDNA synthesis kit (Invitrogen). RT-PCR was performed by using gene-specific primers.

Western blot analysis

RAW264.7 transfectants were solubilized in buffer containing 1% Nonidet-P40, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM Na_3VO_4, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and protease inhibitor cocktail (Roche, Basel, Switzerland). After adjusting the amount of proteins among cell lysates (BCA protein assay kit; Pierce, Rockford, IL, USA), the lysates were separated by SDS-PAGE and immunoblotted with antiphospho integrin β3 (phospho tyrosine 773, reacts with mouse and human phospho integrin β3; Abcam, Cambridge, UK), anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA), or anti-integrin β3 (Cell Signaling Technology, Danvers, MA, USA).

Analysis of bone phenotype

Lumber spines from male 32-wk-old wild-type and FARP2$^{-/-}$ mice were fixed in 70% ethanol, embedded in glycolmethacrylate, and sectioned in 3-μm slices. The specimens were subjected to histomorphometic analyses using a HistomeryRT Camera (System-supply, Nagano, Japan). Examinations were performed using methods described previously (22).

Retroviral gene introduction into bone marrow osteoclasts

FARP2 was cloned into the vector pMX-IRES-GFP. pMX-IRES-GFP or pMX-FARP2-IRES-GFP was cotransfected with pCG-VSV-G into Plat-E cells using Lipofectamine 2000 (Invitrogen). After 48 h, culture supernatants containing retrovirus were collected and filtered through a 0.45-μm polysulfonic filter. Bone marrow cells were cultured with M-CSF for 48 h and infected with virus solution. After 24 h, culture medium was replaced with RANKL-containing medium.

Statistical analysis

We analyzed statistical significance using an unpaired, 2-tailed Student's t test. Values of $P < 0.05$ were considered significant.

RESULTS

FARP2 is involved in multinucelation of osteoclasts

To determine the role of FARP2 in osteoclastogenesis, we first examined the expression of FARP2 in RAW264.7, a murine macrophage-like cell line, which can differentiate into osteoclast-like cells (OCLs) in response to stimulation with RANKL. Before stimulation with RANKL, the expression of FARP2 was hardly detectable (Supplemental Fig. 1*A*). After stimulation with RANKL for 72 h, RAW264.7 cells differentiated into multinucleated OCLs, and the expression of FARP2 increased (Supplemental Fig. 1*A*). To examine the functional importance of FARP2 during osteoclastogenesis, we established RAW264.7 cell transfectants that expressed an intact form of FARP2 (FL-FARP2) or a RacGEF domain-deleted dominant-negative form of FARP2 (ΔGEF-FARP2) (19) (Supplemental Fig. 1*B*). The transfectants proliferated at the same rate as parental RAW264.7 cells and produced comparable amounts of TNF-α in response to LPS (Supplemental Fig. 1*C, D*). In addition, no differences were found among transfectants and controls with respect to the expression levels of cell surface adhesion molecules, including integrins and CD44 (Supplemental Fig. 1*E*). When these cells differentiated into OCLs in the presence of RANKL, tartrate-resistant acid phosphatase (TRAP)-positive large multinuclear OCLs were generated both from parental RAW264.7 cells and FL-FARP2-expressing cells. In contrast, TRAP$^+$ large multinuclear OCLs were hardly detectable in ΔGEF-FARP2-expressing cells (**Fig. 1*A, B***). It is noteworthy that mononuclear or smaller multinucleated TRAP$^+$ cells could be observed in ΔGEF-FARP2-expressing cells (Fig. 1*A, B*), suggesting that FARP2 is required for efficient multinucleation during osteoclastogenesis. Consistent with this, total TRAP activity was comparable among parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells (Fig. 1*C*). In addition, the expression of NFATc1, a master regulator of osteoclast development (25), and osteoclast marker genes (including TRAP) were induced in ΔGEF-FARP2-expressing cells after stimulation with RANKL (Fig. 1*D*). Although the expression levels of FARP2 in FL-FARP2-expressing cells seemed to be slightly higher than those in parental cells, no apparent differences were found in RANKL-induced OCL differentiation between parental and FL-FARP2-expressing cells. Collectively, these results indicated that FARP2 is required for late-stage osteoclastogenesis, in which mononuclear osteoclasts differentiate into large multinucleated osteoclasts but is dispensable for mononuclear osteoclast development.

FARP2 is required for podosome formation and rearrangements

We next examined the actin cytoskeleton in RAW264.7 cells, since arrangement of actin-based cytoskeletal structures are crucial for osteoclast development (26). After stimulation with RANKL for 72 h, OCLs from FL-FARP2-expressing cells displayed podosome-ring

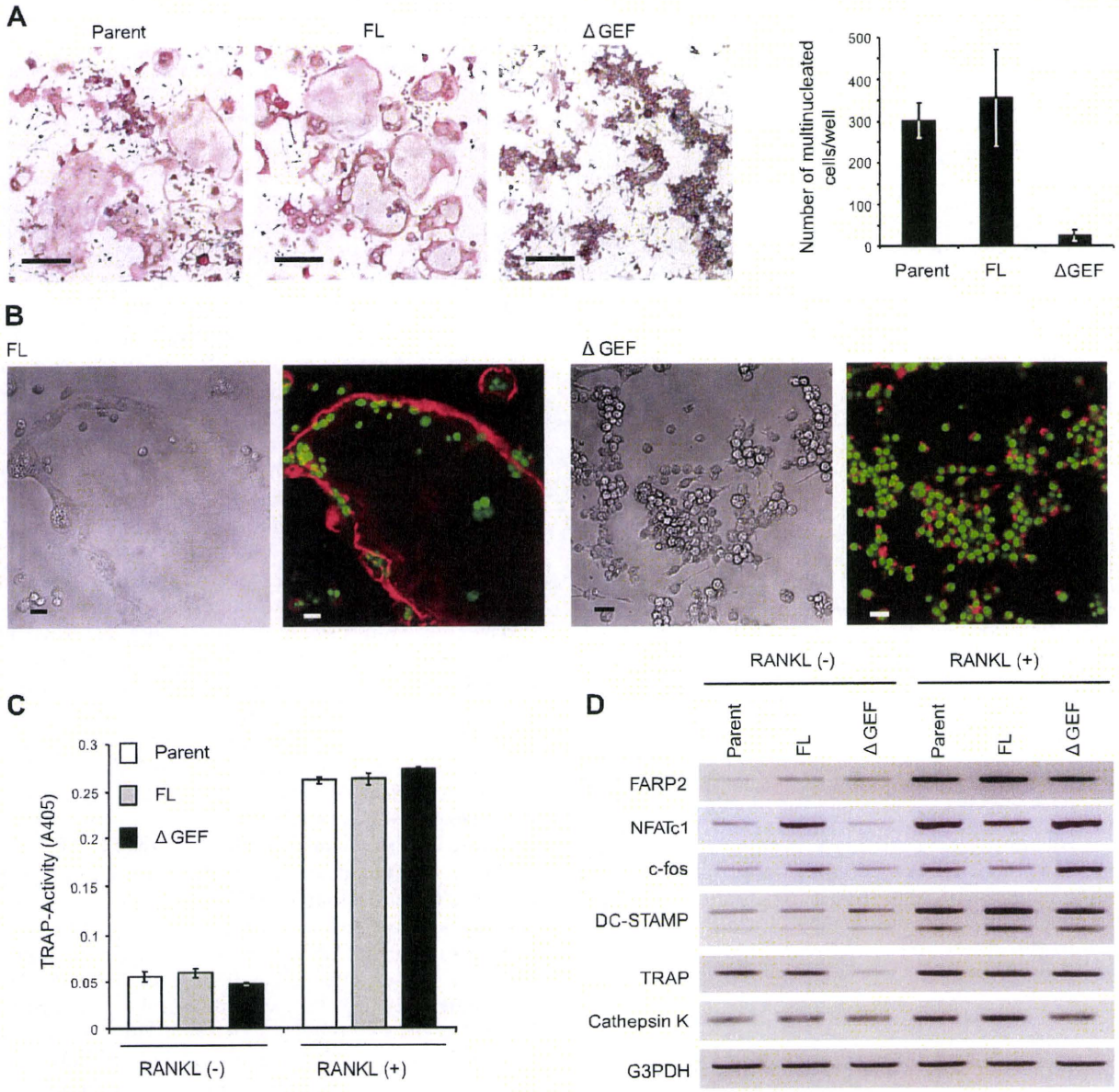

Figure 1. FARP2 is involved in RANKL-induced osteoclastogenesis. *A*) *In vitro* osteoclast differentiation of RAW264.7 cells. Parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells were cultured in the presence of RANKL (20 ng/ml). TRAP staining and number of multinucleated TRAP-positive OCLs are shown. Values are mean ± SE values for each group. Results are representative of 5 independent experiments. *B*) Confocal micrographs showing the F-actin structures and nuclei in OCLs. FL-FARP2-expressing cells or ΔGEF-FARP2-expressing cells cultured in the presence of RANKL were stained with AlexaFluor546-phalloidin for F-actin staining and SYTO11 for nuclear staining. *C*) TRAP solution assay of parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells before and after differentiation into OCLs. Parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells were cultured in the presence or absence of RANKL for 96 h. TRAP activity of these cells was measured. Values are means ± SD. Results are representative of 3 independent experiments. *D*) Expression profiles of FARP2 or osteoclast marker genes in OCLs. Parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells were cultured in the presence or absence of RANKL for 96 h. Expression of the indicated genes was determined by RT-PCR. Scale bars = 200 μm (*A*); 20 μm (*B*).

structures with typical actin cores. However, ΔGEF-FARP2-expressing cells displayed disturbed actin organization and impaired podosome-ring structures. ΔGEF-FARP2-expressing cells exhibited significantly reduced percentages of podosome positive cells (FL-FARP2-expressing cells, 49.2±14.1%; ΔGEF-FARP2 expressing cells; 4.3±3.4%; P=0.00012; **Fig. 2*A***). In addition, FL-FARP2-expressing cells formed resorption pits on calcium phosphate-coated discs, whereas no resorption pits were observed when ΔGEF-FARP2-expressing cells were cultured on the discs (Fig. 2*B*). No apparent differences were found in the actin structures and resorbing activity between parental cells and FL-FARP2-expressing cells (data not

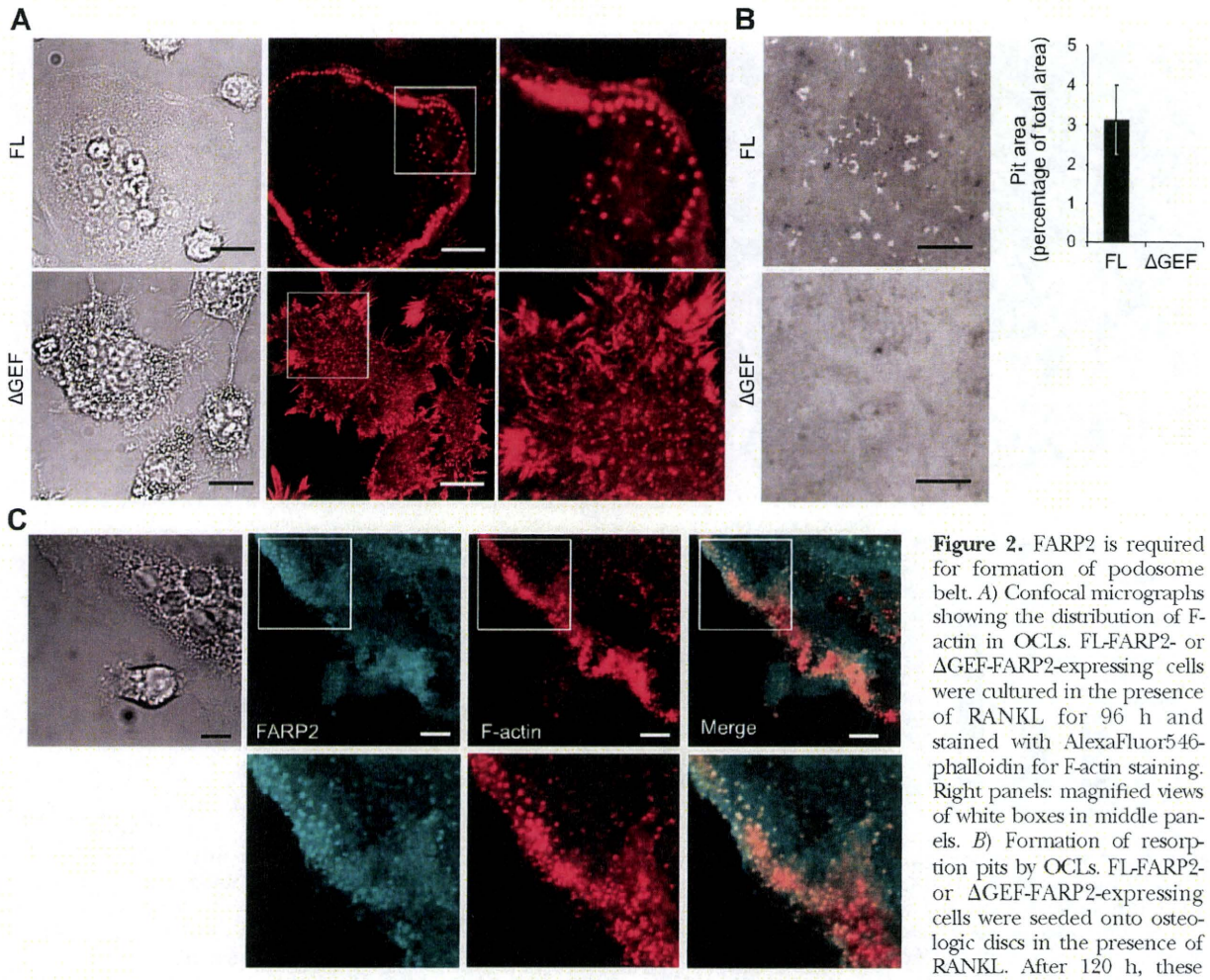

Figure 2. FARP2 is required for formation of podosome belt. *A*) Confocal micrographs showing the distribution of F-actin in OCLs. FL-FARP2- or ΔGEF-FARP2-expressing cells were cultured in the presence of RANKL for 96 h and stained with AlexaFluor546-phalloidin for F-actin staining. Right panels: magnified views of white boxes in middle panels. *B*) Formation of resorption pits by OCLs. FL-FARP2- or ΔGEF-FARP2-expressing cells were seeded onto osteologic discs in the presence of RANKL. After 120 h, these discs were washed to remove cells, and the area of resorption pits was measured. Values are means ± SE for each group. Results are representative of 3 independent experiments. *C*) Confocal micrographs showing the distribution of FARP2 in OCLs. Parental cells were cultured in the presence of RANKL for 96 h, and stained with anti-FARP2 antibody and AlexaFluor546-phalloidin for F-actin staining. Bottom panels: magnified views of white boxes in top panels. Scale bars = 20 μm (*A*); 500 μm (*B*); 10 μm (*C*).

shown). Of note, FARP2 colocalized with dense actin cores (Fig. 2*C*). These results strongly suggested that FARP2 is required for formation of podosome-ring structures.

FARP2 regulates localized-activation of Rac1 during osteoclastsogenesis

FARP2 has a RacGEF domain in its carboxy-terminal region (Supplemental Fig. 1*B*), and the Rho family of small GTPases, including Rac1, has been proposed to be essential for formation and regulation of posodome-ring structures in osteoclasts (14, 27). We therefore examined the RacGEF activity of FARP2 during osteoclastogenesis. We performed a pulldown assay using a fusion protein containing the Cdc42/Rac interactive binding (CRIB) domain of p21-activated kinase (PAK), which selectively binds to Rac-GTP. However, a pulldown assay using whole cell lysates did not show any differences in Rac1 activity among parental, FL-FARP-expressing, and ΔGEF-FARP2-expressing cells (data not shown). We next determined the spatiotemporal changes in Rac1 activity in living cells using a fluorescence resonance energy transfer (FRET) system. We used the FRET monitor probe, Raichu-Rac1, which consisted of Rac1, CRIB of PAK, and the pair of YFP and CFP. When Rac1 is bound to GTP, intramolecular binding to CRIB brings YFP into proximity with CFP, which increases FRET from CFP to YFP (24). Raichu-Rac1 was transiently transfected into parental, FL-FARP2-expressing, or ΔGEF-FARP2-expressing cells, which were then stimulated with RANKL, and changes in FRET signals were monitored by an incubator-integrated time-lapse fluorescence microscope. In parental cells, FRET signals from Raichu-Rac1 were observed in ring shaped-structures, and morphologies of these structures changed throughout the whole imaging processes (**Fig. 3*A***), which could be observed in FL-FARP2-expressing cells as well (data not shown). Podosomes are dynamic and short-lived structures with an apparent half-life of 2–12 min (15, 28). Although we did not show simultaneous dynamics of actin filaments, the distribution of FRET signals changed during each 5 min interval,

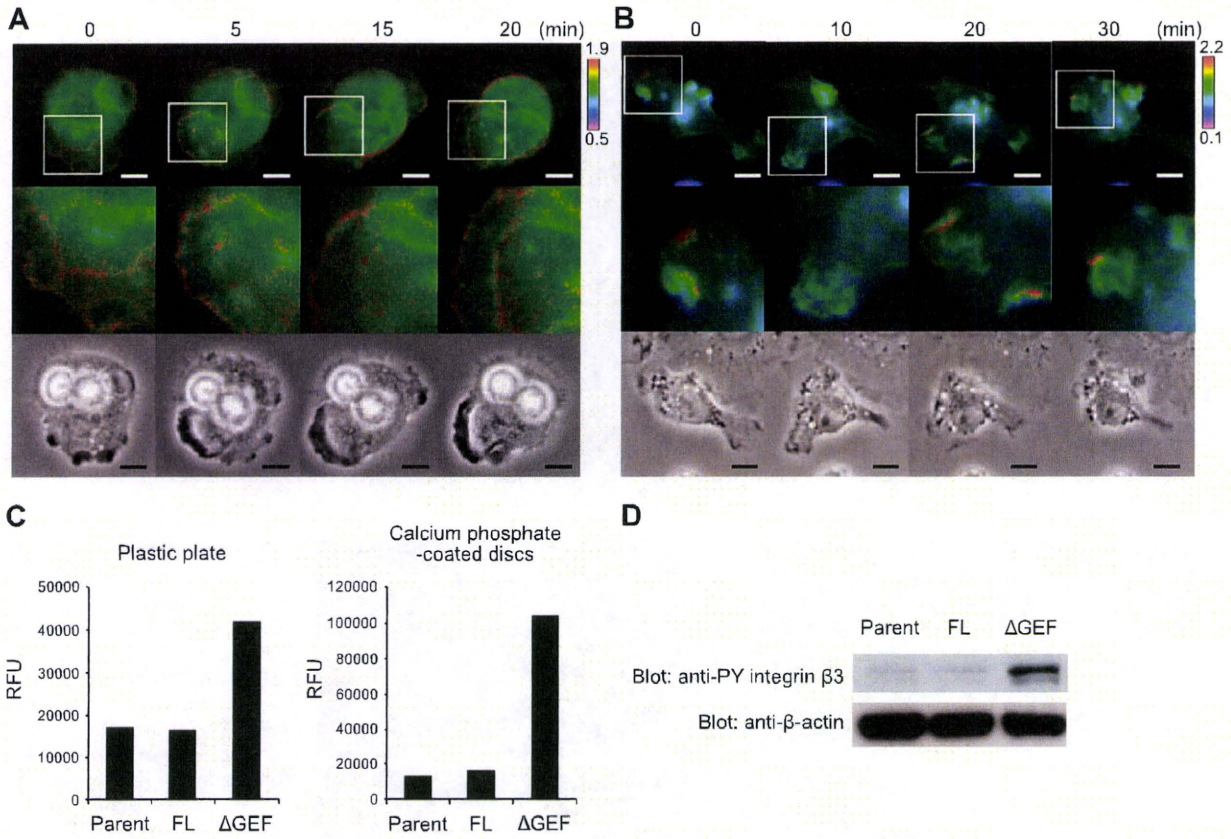

Figure 3. FARP2 is required for regulation of Rac1 and integrin β3. *A, B*) Imaging of Rac1 activities during osteoclast differentiation. Parental (*A*) or ΔGEF-FARP2-expressing cells (*B*) transiently transfected with Raichu-Rac1 expression vector were cultured with RANKL for >65 h. CFP fluorescence, YFP fluorescence and phase-contrast images were taken every 5 min. YFP/CFP ratio images were created to measure FRET efficiency. Experiments were done ≥5 times; representative results are shown. *C*) Adhesion activities of OCLs. Parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells were seeded onto culture plates or osteologic discs and cultured with RANKL. After 120 h, plate or discs were washed, and adherent cells were measured. Results are representative of 3 independent experiments. RFU, relative fluorescence unit. *D*) Phosphorylation of integrin β3 in parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing cells. Lysates from parental, FL-FARP2-expressing, and ΔGEF-FARP2-expressing were immunoblotted with the indicated antibodies. Phosphorylation of tyrosine 773 on integrin β3 activates the receptor and promotes cell adhesion (43–45).

implying the idea that the FRET signal is identical to podosome-rings. In contrast, ring-shaped FRET signals could not be detected in the ΔGEF-FARP2-expressing cells, although polarized FRET signals from Raichu-Rac1 were observed in the extending lamellipodia in the cell periphery (Fig. 3*B*). These results suggested that Rac1 activation is concentrated in podosome-ring like structures and that FARP2-mediated localized Rac1 activation is relevant to the podosome-ring structures.

FARP2 regulates integrin activation and cellular adhesion

In addition to the impaired regulation of GTP-Rac1 during RANKL-induced osteoclastogenesis, we found that ΔGEF-FARP2-expressing cells exhibited abnormally enhanced adhesion activities relative to control cells (Fig. 3*C*). Integrin-mediated mechanical contact with membrane-matrix proteins is important for podosome formation and organization. Osteoclasts express several integrins as membrane matrix receptors: αvβ1, α2β1, and αvβ3 (29, 30). In particular, integrin αvβ3 is shown to be important for osteoclast biology (16, 31). FARP2 regulates integrin activities *via* its amino-terminal FERM domain (19). We therefore examined whether the expression of ΔGEF-FARP2 affects the activation status of integrin β3. A Western blot assay using whole-cell lysates revealed that ΔGEF-FARP2-expressing cells exhibited high levels of phosphorylation of integrin β3 relative to parental and FL-FARP2-expressing cells (Fig. 3*D*). Of note, ΔGEF-FARP2-expressing cells exhibited enhanced adhesion activities against the ECM even in the absence of RANKL (Supplemental Fig. 2), suggesting that FARP2 regulates integrin activation in a RANKL-independent manner. Collectively, these results suggested that FARP2-mediated regulation of integrins is important for cellular adhesion to the ECM.

Cell spreading and resorptive activities are diminished in FARP2$^{-/-}$ osteoclasts

To confirm the function of FARP2 in osteoclasts further, we generated mice with a null mutation in

the FARP2 locus (Supplemental Fig. 3*A*). The percentage of osteoclast precursor cells, relative to the total number of bone marrow cells, was comparable between FARP2$^{+/+}$ and FARP2$^{-/-}$ mice (Supplemental Fig. 3*B*). In addition, FARP2$^{-/-}$ and FARP2$^{+/+}$ bone marrow-derived monocytes expressed similar levels of cell surface adhesion molecules (Supplemental Fig. 3*C*). However, when bone marrow-derived mononuclear cells were cultured in the presence of macrophage-colony stimulating factor (M-CSF) and RANKL, FARP2 deficiency resulted in a decrease in the surface area covered by osteoclasts and their resorptive activity. (**Fig. 4**). The number of FARP2$^{-/-}$ multinucleated osteoclasts (>3 nuclei/cell) and the area covered by multinucleated osteoclasts were smaller than those of FARP2$^{+/+}$ osteoclasts (Fig. 4*A*, *B*), and the defect was restored by retrovirally introducing FARP2 (Supplemental Fig. 4). Although the number of FARP2$^{-/-}$ multinucleated osteoclasts increased with increasing concentrations of RANKL (Fig. 4*C*), the FARP2$^{-/-}$ multinucleated osteoclasts covered a smaller area than FARP2$^{+/+}$ osteoclasts (Fig. 4*C*, *D*). In accordance with this observation, FARP2$^{-/-}$ cells exhibited reduced resorption pits on calcium phosphate-coated discs, relative to FARP2$^{+/+}$ cells (Fig. 4*E*), although the expression of osteoclast markers including NFATc1 was observed in FARP2-deficient cells after stimulation with RANKL (Fig. 4*F*). On the other hand, in high concentrations of RANKL, the surface area covered by FARP2$^{-/-}$ multinucleated osteoclasts was comparable to that of FARP2$^{+/+}$ multinucleated osteoclasts (Supplemental Fig. 5). It is possible that FARP1 (also known as chondrocyte-derived ezrin-kile domain containing protein; CDEP), another FERM domain-containing GEF protein whose structure is closely related to that of FARP2 (32), compensates for FARP2 deficiency. However, the expression of FARP1 was hardly detected in osteoclasts (Supplemental Fig. 6), suggesting that molecules other than FARP1 compensate for the FARP2 deficiency in osteoclasts at high concentrations of RANKL. Taken together, these findings supported

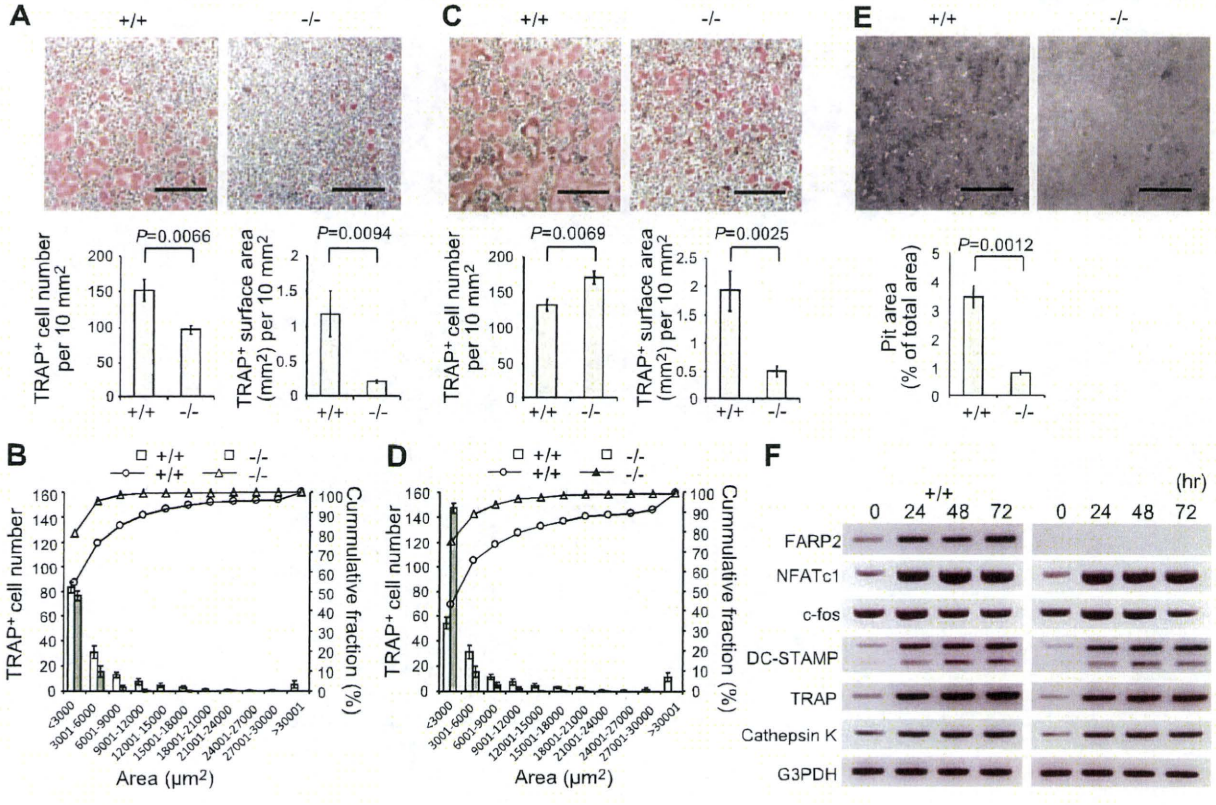

Figure 4. FARP2 deficiency results in impairment of osteoclastogenesis. *A, C) In vitro* osteoclastogenesis in the absence of FARP2. Bone marrow-derived monocytes from wild-type (+/+) or FARP2$^{-/-}$ (−/−) mice were cultured with M-CSF plus RANKL (*A*, 5 ng/ml; *C*, 20 ng/ml) for 96 h. Top panels: TRAP staining. Bottom panels: number of TRAP$^+$ cells and total surface area covered by TRAP$^+$ cells. Results are representative of 3 independent experiments. Scale bars = 500 μm. *B*) Distribution of number and area of TRAP$^+$ cells in panel *A*. *D*) Distribution of number and area of TRAP$^+$ cells in panel *C*. *E*) Formation of resorption pits by osteoclasts. Bone marrow-derived monocytes from wild-type or FARP2$^{-/-}$ mice were cultured onto osteologic discs in the presence of M-CSF (20 ng/ml) plus RANKL (20 ng/ml) for 120 h. Area of resorption pits on discs was measured. Values are means ± SE for each group. Results are representative of 3 independent experiments. *F*) Expression of FARP2 or osteoclast markers. Bone marrow-derived-monocytes from wild-type or FARP2$^{-/-}$ mice were cultured in the presence of M-CSF (20 ng/ml) and RANKL (20 ng/ml) for the indicated times. Expression of the indicated gene transcripts was determined by RT-PCR.

that FARP2 is required for the efficient formation of large multinucleated osteoclasts.

FARP2 is required for the effects of semaphorins on osteoclastogeneis

We have previously shown that FARP2 associates with the intracellular region of plexin-A proteins (19). Class III and class VI semaphorins utilize plexin-A proteins as receptor components (33–35). In RAW264.7 cells, we observed the expression of Sema6B and Sema6D, but not the class III semaphorin, Sema3A, before and after differentiation into OCLs (**Fig. 5A**). Consistent with this finding, incubation of RAW264.7 cells with either recombinant soluble Sema6B or Sema6D promoted osteoclast differentiation, whereas stimulation with the recombinant proteins did not enhance osteoclast differentiation in ΔGEF-FARP2-expressing cells (Fig. 5*B–D*). Furthermore, in the case of primary bone marrow-derived precursor cells in which the expression of Sema6B and Sema6D could be observed (data not shown), incubation of FARP2$^{+/+}$ precursor cells with either recombinant soluble Sema6B or Sema6D promoted osteoclastogenesis (Fig. 5*E–G*); these effects were completely abolished on FARP2$^{-/-}$ cells (Fig. 5*E–G*). Of note, recombinant Sema6D induced dephosphorylation of β3 integrin in

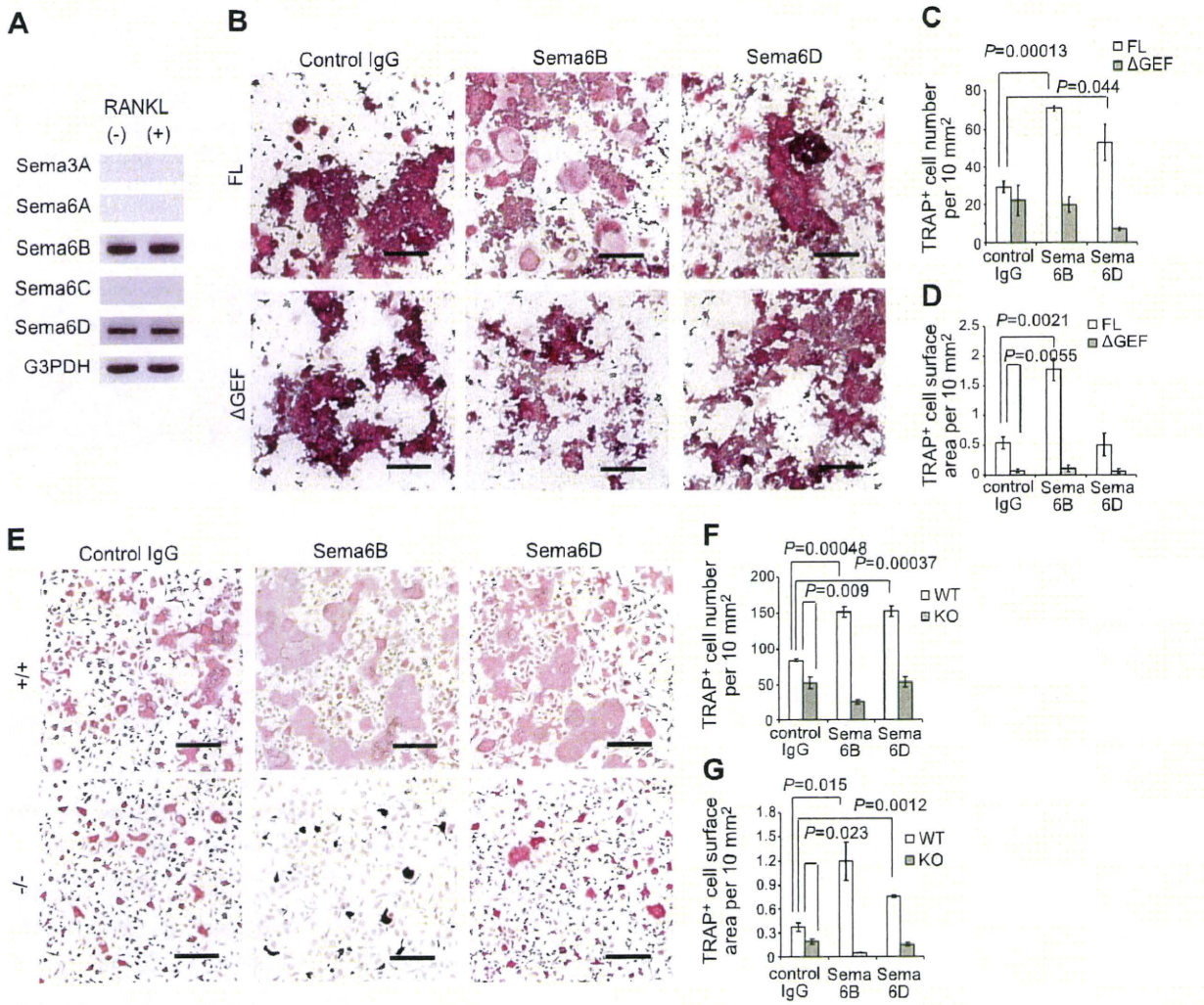

Figure 5. Class VI semaphorins are involved in FARP2-mediated osteoclastogenesis. *A*) Expression profiles of semaphorins in OCLs. RAW264.7 cells were cultured in the presence or absence of RANKL for 96 h. Expression of transcripts of Sema3A, Sema6A, Sema6B, Sema6C, Sema6D, and G3PDH was determined by RT-PCR. *B–D*) Recombinant class VI semaphorins, Sema6B or Sema6D, promote osteoclast differentiation from RAW264.7 cells. FL-FARP2-expressing and ΔGEF-FARP2-expressing cells were seeded onto culture plates coated with the indicated recombinant proteins, and cultured with RANKL (5 ng/ml) for 72 to 96 h. *B*) TRAP staining. *C*) Number of TRAP$^+$ cells. *D*) Total surface area covered by TRAP$^+$ cells. *E–G*) Recombinant class VI semaphorins, Sema6B or Sema6D, promote osteoclast differentiation from bone marrow-derived monocytes. Bone marrow cells from wild-type (+/+) or FARP2$^{-/-}$ (−/−) mice were cultured with M-CSF (20 ng/ml) for 48 h. Cells were harvested and seeded onto culture plates coated with the indicated soluble recombinant semaphroins, and cultured with M-CSF (20 ng/ml) and RANKL (5 ng/ml) for 96 h. *E*) TRAP staining. *F*) Number of TRAP$^+$ cells. *G*) Total surface area covered by TRAP$^+$ cells. Results are representative of 3 independent experiments. Scale bars = 200 μm.

parental and FL-FARP2-expressing cells, whereas Sema6D-stimulation did not induce dephosphorylation of β3 integrin in ΔGEF-FARP2-expressing cells (Supplemental Fig. 7), supporting the involvement of FARP2 in semaphorin-signaling in osteoclastogenesis. Taken together, these results indicated that FARP2 is involved in class VI semaphorin-induced enhancement of osteoclast cell spreading and development.

DISCUSSION

Podosomes are highly dynamic, actin-rich structures that mediate cell attachment, migration, and matrix degradation of highly motile cells. In particular, osteoclasts require podosome structures to exert bone resorbing activity. Here, we highlighted not only a crucial role of FARP2 in podosome rearrangements in osteoclasts but also its importance in osteoclast development and functions. Indeed, defects in FARP2 in osteoclast precursors resulted in impairment of podosome-ring structures during osteoclastogenesis, leading to diminished formation of multinucleated osteoclasts and resorptive activity. In contrast, the expression of osteoclast differentiation markers was not affected by the defects of FARP2. These findings strongly suggested that FARP2 is dispensable for initiation of osteoclastogenesis but is required for osteoclast maturation. It is also plausible that podosome organization is related to efficient multinucleation during osteoclast differentiation. The suppressed resorptive activity in FARP2-deficient osteoclasts is reminiscent of the phenotypes observed in mutant cells that lack Vav3, a Rho family GEF from Dbl family (36), further supporting the importance of GEF-mediated Rac1 regulation in osteoclast functions.

During osteoclastogenesis, integrin engagement with the ECM is required for formation of podosomes, with the signal mediated through induction of activation of tyrosine kinases, such as Src (28). Among several integrins expressed on osteoclasts, integrin αvβ3 is important for osteoclast biology, because integrin β3-deficient osteoclasts display defective resorptive activities due to abnormal cytoskeleton and impaired formation of actin rings (31). On the other hand, finely tuned activities of Rho family of small GTPases are crucial for the maintenance of podosome structures. Moreover, activities of Rho family small GTPases counterbalanced osteoclast spreading (13). Both integrins and Rho family small GTPases are critical for podosome turnover. Here, we showed integral roles for FARP2 for Rac1 and integrin β3 during dynamic arrangements of podosome structures in osteoclasts: FARP2 is required not only for localized activation of GTP-bound Rac1 in podosome-ring like structures but also for integrin regulation in osteoclasts. Our time-lapse imaging data showed that GTP-bound Rac1 is concentrated in the podosome-ring like structures, and localization of GTP-bound Rac1 in podosome-ring like structures requires FARP2. Furthermore, FARP2 may participate in regulating integrin β3 activity in osteoclasts. Although the molecular mechanism underlying the role for FARP2 in integrin regulation in osteoclast podosome rearrangements still remains to be determined, it is possible that down-regulation of integrin activities by FARP2 is crucial for rapid turnover of podosome structures in osteoclasts. Indeed, we have previously reported that, in neurons, FARP2 regulates Rac1 activation and suppresses integrin activities and focal adhesion by inhibiting an isoform of type-I phosphatidylinositol phosphate kinase, PIPKIγ661 (19). We also demonstrated that FARP2 is required for class VI semaphorin-mediated enhancement of osteoclastogenesis. Semaphorin signaling has been reported to be involved in axonal repulsion through regulation of small G proteins and myosin heavy chain in neurons (37–39). These findings support the idea that semaphorin signaling is required for cytoskeletal rearrangement in a broad range of cells including osteoclasts.

Phenotypes observed in ΔGEF-FARP2-expressing or FARP2-deficient cells were similar in the context of defective osteoclast differentiation and impaired resorptive activity. This finding suggests that ΔGEF-FARP2 plays a dominant negative role in these cells. It seems that the expression levels of FL-FARP2 were relatively low compared to those of ΔGEF-FARP2 in RAW264.7 cell transfectants. However, in accordance with the observation on ΔGEF-FARP2, FARP2-deficinet mice exhibited impairments in the bone (Supplemental Fig. 8). Of note, it seems that ΔGEF-FARP2-expressing cells exhibited more severe phenotypes than FARP2-deficient cells. Differences between a dominant negative mode of action *vs.* straight elimination of a protein might account in part for the differences between ΔGEF-FARP2-expressing cells and FARP2-deficient cells. It is also worthy of note that the expression of FARP2 is detectable in carvarial cell-derived osteoblasts (Supplemental Fig. 6), suggesting the involvement of FARP2 in osteoblasts. Further detailed analysis of osteoclasts and osteoblasts in FARP2-deficient mice will be required to address our understanding of the function of FARP2 in both osteoclasts and osteoblasts.

The membrane-proximal cytoplasmic regions of plexin-A proteins contain amino acid sequences to which the FERM domain binds, through which FARP2 can associate with plexin-A proteins (19); thus, FARP2 links semaphorin-plexin signals to cytoskeletal reorganization during osteoclast development. Indeed, the phenotypes observed in plexin-A1$^{-/-}$ osteoclasts were similar to that of FARP2$^{-/-}$ osteoclasts (22). In addition, either Sema6B or Sema6D could promote osteoclastogenesis, and these effects were completely abolished on FARP2$^{-/-}$ cells. We previously demonstrated that plexin-A1 forms a receptor complex with TREM-2 and DAP12 (22), which is involved in activation of calcium signals that lead to induction of NFATc1 during osteoclastogenesis (40). However, we found comparable levels of calcium oscillation among parental RAW264.7 cells and ΔGEF-FARP2-expressing cells (unpublished observation), indicat-

ing that FARP2 and TREM-2/DAP12 have distinct roles in plexin-A-mediated osteoclastogenesis. TREM-2/DAP12-mediated signals are required for the early phase of osteoclastogenesis, during which NFATc1 is induced. However, a FARP2-mediated signal is required for the late phase of osteoclastogenesis, during which podosomes are dynamically rearranged. Of note, CD44, one of components of podosome structures (41), contains a region to which the FERM domain of ezrin binds (42). Thus, it is possible that FARP2 is also involved in other receptor systems which are required for osteoclastogenesis.

In summary, we have revealed an important role for FARP2 as a regulator of Rac1 and integrin β3 during osteoclast development. The present findings contribute to our understanding of the basic mechanisms of podosome formation and osteoclast development. Podosomes are actin-rich structures present not only in osteoclasts but also in monocytes, dendritic cells, endothelial cells, and smooth muscle cells. Disruption of podosome formation has been implicated in the pathogenesis of Wiskott-Aldrich syndrome (5, 8) or chronic myeloid leukemia (15). Thus, our findings not only clarify a novel mechanism for podosome formation but also provide a clue to understand the cellular dynamics in both physiological and pathological tissue environments. FJ

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Review

Semaphorins and their receptors: Novel features of neural guidance molecules

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Abstract: Semaphorins were originally identified as axon guidance cues involved in the development of the nervous system. In recent years, it is emerging that they also participate in various biological systems, including physiological and pathological processes. In this review, we primarily focus on our cumulative findings for the role of semaphorins and their receptors in the regulation of the immune system, while also summarizing recent progress in the context of cardiovascular system.

Keywords: Semaphorin, immune cell interactions, cardiac morphogenesis, plexins

1. Introduction

Cumulative findings indicate that the nervous and immune systems have considerable overlap and links.[1] For example, some axon guidance molecules, such as slits and ephrins, have been shown to regulate immune cell migration.[2),3] In addition, T-cell-antigen-presenting cell contact sites, the so-called 'immunological synapse', are structurally similar to the 'neurological synapse' that connects pairs of neurons. These shared molecules and interactions play critical roles in inducing proper immune responses.

Semaphorins were named for properties that are analogous to the system of flags and lights that is used in rail and maritime communication. They were initially identified as repulsive axon guidance factors that direct neuronal axons to their appropriate targets.[4] To date, more than 20 types of semaphorins have been identified, and they have diverse functions in many physiological processes, including cardiogenesis,[5] angiogenesis,[6] vasculogenesis,[7] tumor metastasis,[8] osteoclastogenesis,[9] and immune regulation.[10] We isolated the cDNA of Sema4D through the search for CD40 (an immune co-stimulatory molecule)-inducible genes by subtractive PCR-cloning. In addition, we isolated the cDNA of Sema6D from the cDNA library of the heart by PCR-cloning using degenerative primers. The identification of Sema4D and Sema6D has clarified the important roles of the semaphorin family in the immune and cardiac systems, respectively. Here, we review the pleiotropic functions of semaphorins, primarily focusing on their roles in immune responses and cardiac morphogenesis.

2. Semaphorins and their receptors

Semaphorins are secreted and membrane-associated proteins that are characterized by a conserved extracellular amino-terminal 'Sema' domain. Based on their C-terminal structures, this diverse group of proteins has been further divided into eight subclasses.[11] Semaphorins in classes I (invertebrate) and IV–VII are membrane-associated, whereas those in classes II (invertebrate), III, and VIII (virally encoded) are secreted. Two groups of proteins,

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Abbreviations: NPs: neuropilins; TIM-2: T-cell immunoglobulin and mucin domain containing protein 2; OTK: tyrosine kinase receptors off-track; VEGFR2: vascular endothelial growth factor receptor 2; TREM-2: triggering receptor expressed on myeloid cell; DAP12: DNAX-activating protein 12; ITIM: immunoreceptor tyrosine-based inhibitory motifs (ITIM); Th1: T helper type 1; Th2: T helper type 1; EAE: experimental autoimmune encephalomyelitis; Treg: regulatory T cells; Foxp3: forkhead box P3; CTLA-4: cytotoxic T-lymphocyte antigen-4 (CTLA-4); Ena: Enabled; GEF: guanine nucleotide exchange factor.

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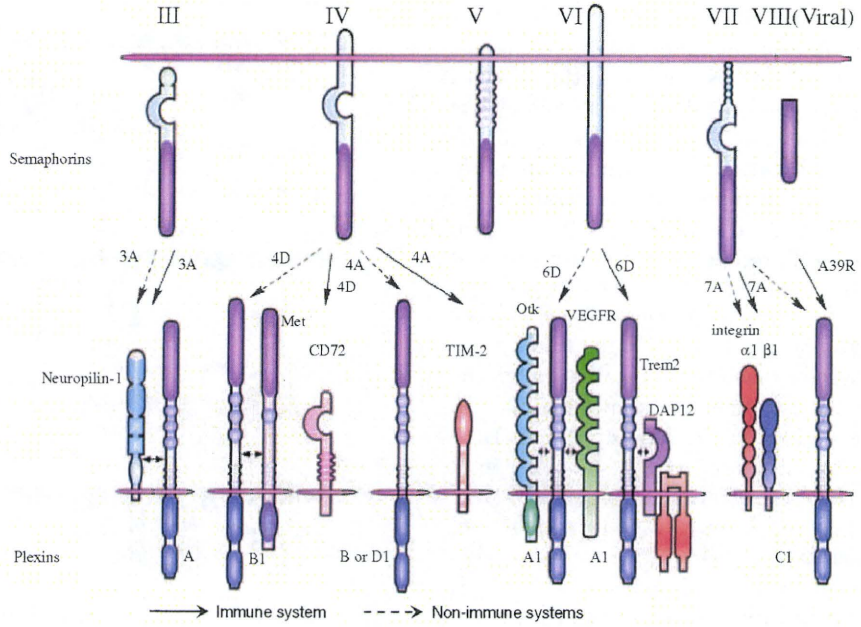

Fig. 1. Representative semaphorins and their receptors. Among the eight subclasses of semaphorins, class I and II semaphorins are found in invertebrates (not shown in figure) and class III–VII are vertebrate semaphorins. Classes II and III and viral semaphorins are secreted, whereas class IV–VI are transmembrane. Class VII represents GPI-anchored proteins. Sema3A directly binds NP-1 and associates with plexin-A1, resulting in inducing plexin-A-mediated signals. Although Sema4D binds to plexin-B1 in neurons, plexin-B1 couples with Met in epithelial cells and induces Sema4D-mediated cell outgrowth. In the immune system, Sema4D binds to CD72, which promotes B cell and DC activation. Sema4A recognizes plexin-B and D1 in the non-immune systems but uses TIM-2 as a receptor for T cell activation in the immune system. During cardiogenesis, plexin-A1 associates with OTK or VEGFR2 at distinct sites. However, plexin-A1 forms a receptor complex with TREM-2-DAP12, which is critical for DC activation and osteoclastogenesis. Sema7A has two types of receptors: $\alpha 1\beta 1$ integrin for macrophage activation and plexin-C1 for inhibition of cell adhesion. Viral semaphorin A39R also recognizes plexin-C1 and modulates dendritic cell function.

plexins and neuropilins (NPs), have been identified as the primary semaphorin receptors.[12) 14)] Most membrane-bound semaphorins directly bind plexins, whereas class III semaphorins require NPs as obligate coreceptors. However, increasing evidence has shown that semaphorin receptor usage is more complex than previously thought.[15)] For example, Sema3E signals independently of NPs through plexin-D1,[7)] while Sema7A uses integrins to exert its functions in both the nervous and immune systems.[16),17)] In addition, two molecules unrelated to plexins and NPs, CD72[18)] and T-cell immunoglobulin and mucin domain containing protein 2 (TIM-2),[19)] functionally interact with Sema4D and Sema4A, respectively, in the immune system (Fig. 1).

Plexins are canonical semaphorin receptors that have large cytoplasmic domains. In the nervous system, plexin-mediated signals have been shown to exert diverse neural functions by regulating GTPase activities and cytoplasmic/receptor-type protein kinases.[20)] These signals are also involved in integrin-mediated attachment.[21) 23)] Of note, plexins can associate with different co-receptors in distinct tissues to allow semaphorins to exert pleiotropic functions. For instance, plexin-A1 is associated with the tyrosine kinase receptors off-track (OTK) and vascular endothelial growth factor receptor 2 (VEGFR2) in heart morphogenesis.[24)] In another context, plexin-A1 forms a receptor complex with triggering receptor expressed on myeloid cell (TREM)-2/DNAX-activating protein 12 (DAP12) during osteoclastogenesis.[9)] Furthermore, plexin-B1 has been shown to associate with the receptor tyrosine kinases Met and ErbB2, inducing invasive growth of epithelial cells.[25),26)] These observations provide insight into the diversity of semaphorin functions (Fig. 1).

3. Immune semaphorins

(1) Sema4D: a semaphorin involved in B cell/dendritic cell (DC) activation (Fig. 2a). Sema4D, also known as CD100, is the first sema-

phorin protein for which immunoregulatory functions were identified. In the immune system, abundant expression of Sema4D is observed in resting T cells. The basal expression of Sema4D in B cells and DCs is very low, but protein levels are considerably higher following cellular activation.[18),27)] Sema4D promotes B cell activation in the context of proliferation and antibody production. Plexin-B1 and CD72 have been identified as Sema4D receptors in the nervous and immune systems, respectively.[14),18),22)] CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic domain,[28)] and is known to function as a negative regulator of B cells through the recruitment of the tyrosine phosphatase SHP-1 to its phosphorylated ITIM.[18)] Ligation of Sema4D to CD72 induces dissociation of SHP-1, resulting in B cell activation (Fig. 2a). Consistently, Sema4D-deficient mice display impaired antibody production, implicating Sema4D in B cell activation. In addition to its involvement in B-cell responses, Sema4D is involved in T cell activation by promoting the activation and maturation of DCs. In fact, Sema4D-deficient mice display impaired generation of antigen-specific T cells.[18),27)] Although Sema4D is a transmembrane protein, its extracellular region is proteolytically cleaved from the surface of activated lymphocytes in a metalloprotease-dependent process.[29)] Sema4D is also cleaved from the surface of platelets by the metalloprotease ADAM17.[30)] Elevated levels of soluble Sema4D protein are detectable in the culture supernatants of activated lymphocytes and in the sera of immunized and autoimmune mice.[29)]

(2) Sema4A: a semaphorin involved in both T cell activation and differentiation. Sema4A is another class IV semaphorin. In the immune system, Sema4A is constitutively expressed on DCs.[19)] The expression of Sema4A is also detectable in activated T cells and T helper type 1 (Th1)-polarized cells. DC-derived Sema4A and T cell-derived Sema4A play different roles during the course of T cell-mediated immunity; in particular, DC-derived Sema4A is crucial for antigen-specific T cell priming, whereas T cell-derived Sema4A is involved in helper T cell differentiation[31)] (Fig. 2b). Indeed, the critical involvement of Sema4A in the differentiation of helper T cells has been demonstrated by the phenotypes of Sema4A-deficient mice. Sema4A deficient mice show impaired responses to heat-killed *Propionibacterium acnes*, a Th1-inducing agent. In contrast, these mice show enhanced T helper type 2 (Th2) responses against infection of *Nippostrongylus brasiliensis*, a

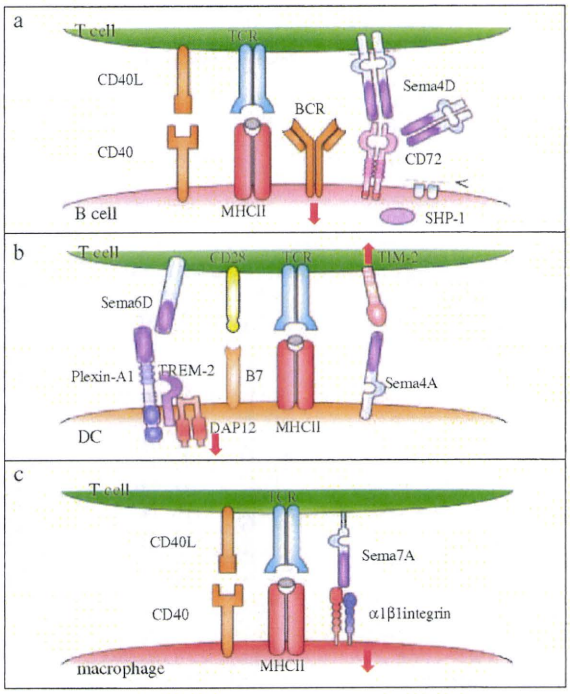

Fig. 2. Semaphorins in immune cell interactions. Semaphorins are involved in various phases of immune cell responses. (a) During T-cell-mediated B-cell activation, engagement of CD72 by Sema4D induces dephosphorylation of CD72 and dissociation from SHP-1, which results in enhancement of BCR signals. Sema4D can also be cleaved proteolytically and function as a soluble form in an autocrine/paracrine manner. (b) Sema6D on T cells can activate DCs through plexin-A1. Sema4A on DCs binds to TIM-2 and activates T cells. (c) T-cell-mediated inflammatory responses require antigen–MHC class II–TCR interaction and CD40L–CD40 signals. However, the interaction between Sema7A and $\alpha 1\beta 1$ integrin is also critical for activation of inflammatory cells such as macrophages.

Th2-inducing intestinal nematode.[31)] Furthermore, Sema4A-deficient mice on a Th2-prone BALB/c background spontaneously develop atopic dermatitis (T.M. unpublished data), indicating that Sema4A is involved in the regulation of Th1/Th2 development. Several lines of evidence support a role of TIM-2, which is induced on activated T cells, as a functional receptor for Sema4A.[19)] The expression of TIM-2 is preferentially upregulated on T cells during Th2 differentiation. Administration of recombinant TIM-2 protein suppresses the development of experimental autoimmune encephalomyelitis (EAE) is suitable mice immunized with proteolipid protein-derived peptide by inhibiting the generation of Th1 cells.[32)] Furthermore, TIM-2-deficient mice show exacerbated lung inflammation accompanied by dysregulated Th2

responses.[33] It thus appears that Sema4A-TIM-2 interactions negatively regulate Th2 responses. However, there are some phenotypic differences between Sema4A-deficient and TIM-2-deficient mice. For example, T cells from TIM-2-deficient mice but not from Sema4A-deficient mice show enhanced basal proliferation. This observation raises the possibility that Sema4A and/or TIM-2 have other binding partners. Indeed, T cells express members of the plexin-B family and plexin-D1, both of which have Sema4A-binding activities.[34]

(3) Sema6D and plexin-A1: an interaction involved in the T cell-dendritic cell interface and osteoclastogenesis. Plexins are the canonical/primary semaphorin receptors during the development of the nervous and cardiovascular systems. Class III semaphorins bind a receptor complex formed by plexin-A1 and NP-1. Additionally, plexin-A1 serves as a direct binding receptor for the class VI semaphorins, Sema6C and Sema6D.[24),35] In the immune system, plexin-A1 is specifically expressed by DCs.[36] The function of plexin-A1 in DCs has been shown using RNA interference and by analysis of plexin-A1-deficient mice.[9),36] 'Knockdown' of plexin-A1 in DCs by short hairpin RNA impairs their ability to activate T cells *in vitro* and *in vivo*. In addition, plexin-A1-deficient DCs are poor at stimulating antigen-specific T cells.[36] *In vivo*, plexin-A1-deficient mice show impaired T cell-priming.[9] These observations indicate that plexin-A1 expression in DCs is required for the initial activation and efficient generation of antigen-specific T cells. It is worthy of note that plexin-A1-deficient mice develop osteopetrosis as a result of decreased bone resorption due to defective osteoclastogenesis.[9] Sema6D was suggested to be a ligand for plexin-A1. Indeed, recombinant Sema6D protein binds to and activates DCs, and this activity is profoundly attenuated in plexin-A1-deficient DCs. These observations suggest that Sema6D-expression on T cells is involved in DC-activation. The expression of Sema6D is also observed in osteoclasts. Recombinant Sema6D protein enhances *in vitro* osteoclastogenesis, suggesting that Sema6D and plexin-A1 might function in osteoclastogenesis in an osteoclast-autonomous manner. Plexin-A1 forms a receptor complex with TREM-2 and the adaptor molecule DAP12 in DCs and osteoclasts.[9] Interestingly, DAP12-deficient mice show impaired T cell responses and develop osteopetrosis,[37] and genetic mutations in human DAP12 or TREM-2 result in a bone fracture syndrome called Nasu-Hakola disease,[38] supporting the idea that plexin-A1 physiologically associates with the TREM-2/DAP12 complex.

(4) Sema7A: a semaphorin involved in inflammatory responses. Sema7A, also known as CD108, is a membrane-associated GPI-anchored protein.[39] In the nervous system, Sema7A has been shown to promote olfactory bulb axon outgrowth and is required for the proper formation of the lateral olfactory tract during embryonic development.[16] Plexin-C1 was initially identified as a receptor for Sema7A.[14] However, in its Sema domain, Sema7A contains an arginine–glycine–aspartate sequence that is a well conserved integrin-binding motif, and it exerts its function through β1 integrin, not through plexin-C1.[16] In the immune system, the expression of Sema7A is induced on activated T cells, and it is involved in T cell-mediated inflammatory immune responses.[17] Recombinant Sema7A protein stimulates monocytes/macrophages through $\alpha 1\beta 1$ integrin, also known as very late antigen-1, inducing the production of proinflammatory cytokines[17] (Fig. 2c). Consistently, Sema7A-deficient mice are resistant to the development of inflammation, including hapten-induced contact hypersensitivity and experimental autoimmune EAE.[17] These observations indicate that interactions between Sema7A and $\alpha 1\beta 1$ integrin are crucial for T cell-mediated macrophage activation at sites of inflammation. Plexin-C1 is also expressed in macrophages; however, recombinant Sema7A protein induces normal production of proinflammatory cytokines in plexin-C1-deficient macrophages (unpublished data). Therefore, at least for T cell–macrophage interactions, $\alpha 1\beta 1$ integrin but not plexin-C1 seems to be the predominant receptor for Sema7A, implying that integrin-mediated signaling is a common mechanism for Sema7A functions in both the nervous and immune systems.

(5) Sema3A and plexin-A4: a semaphorin and semaphorin receptor required for negative regulation of T cell responses. Sema3A was the first semaphorin identified in vertebrates. Sema3A directly binds to NP-1, inducing the activation of plexin-A proteins and the transduction of axon repulsive signals. Several lines of evidence suggest that Sema3A also functions in the immune system (Fig. 1, Table 1). The expression of Sema3A is detected in activated DCs, T cells, and some tumor cells. Sema3A inhibits spontaneous monocyte migration *in vitro*. In addition, Sema3A suppresses T cell proliferation by inhibiting actin cytoskeletal reorganization and downregulating MAPK signaling.[40] Furthermore, Sema3A-deficient T cells exhibit

Table 1. Immune semaphorins, their receptors and diseases

Semaphorins/ receptors	Expression in the immune system	Binding partner	Immunological activities	Related-neurological diseases
Sema3A	N.D.	Plexin A proteins	Inhibition of monocyte migration	Alzheimer's disease
		Neuropilin-1	Inhibition of T-cell activation	Atopic dermatitis
Sema4A	Dendritic cells	Plexin B proteins	T-cell activation	EAE
	Acticated-T cells	Plexin-D1	Promotion of Th1-differentiation	Atopic dermatitis
	Th1 cells	TIM-2		
Sema4D	T cells	Plexin-B1	B-cell activation	EAE
	Activated-B cells	CD72	DC-activation	HAM
	Dendritic cells			
Sema5A	N.D.	N.D.	N.D.	Parkinson's disease
	(Oligodendrocytes)			
Sema6D	T cells	Plexin-A1	DC-activation	
	B cells			
	NK cells			
Sema7A	Acticated-T cells	Plexin-C1	Monocyte/macrophage-activation	Contact hypersensitivity
		Integrin $\alpha 1\beta 1$		EAE
Neuropilin-1	T cells	Class III semaphorins		Alzheimer's disease
	Treg cells			
Plexin-A1	Dendritic cells	Class VI semaphorins	DC-activation	EAE
	(Osteoclasts)			
Plexin-A4	T cells	Class VI semaphorins	Inhibition of T-cell activation	EAE
	Dendritic cells			
	Macrophages			
Plexin-B1		Class IV semaphorins		
TIM-2	Acticated-T cells	Sema4A	T-activation	EAE
	Th2 cells			Airway atopy
CD72	B cells	Sema4D	B-cell activation	
	(Dendritic cells)		DC-activation	
Integrin $\alpha 1\beta 1$	Monocytes	Sema7A	Monocyte/macrophage-activation	EAE
	Macrophages			

enhanced *in vitro* proliferative responses to anti-CD3 antibodies.[41] These observations suggest that Sema3A serves as a negative regulator of T cells. Similar to other plexin-A proteins, plexin-A4 forms a receptor complex with NP-1 to transduce class III semaphorin-mediated signaling or directly binds to Sema6A.[42] Plexin-A4-deficient T cells exhibit hyperproliferation and enhanced TCR signals upon anti-CD3 stimulation. Furthermore, plexin-A4-deficient mice show enhanced T cell priming and exacerbated T cell-mediated immune responses such as EAE.[41] Therefore, plexin-A4 might interact with Sema3A in the immune system and this interaction might negatively regulate T cell responses. However, it remains unclear how plexin-A4 negatively regulates T cells and whether other semaphorins are relevant to plexin-A4-mediated immune responses.

(6) NP-1: a marker for regulatory T cells. As described above, NP-1 was originally identified as a cell surface glycoprotein that acts as a class III semaphorin receptor. NP-1 is also known as human DC-specific antigen (blood DC antigen)-4, a specific plasmacytoid DC marker in humans, and was assigned CD304. In the immune system, the expression of NP-1 was observed in DCs and T cells.[43] NP-1 is thought to be involved in the initiation of primary immune responses through a homophilic interaction at contact sites between T cells and DCs. In addition, NP-1 has been identified as a specific marker for $CD4^+CD25^+$ regulatory T (Treg) cells.[44] Indeed, NP-1 is a member of the group of forkhead box P3 (Foxp3)-inducible genes, which also includes CD25, glucocorticoid-induced tumor necrosis factor receptor-related protein, and cytotoxic T-lymphocyte

antigen-4 (CTLA-4).[44] More recently, it has been suggested that NP-1 in Treg cells contributes to the long contact between Treg cells and DCs compared with the shorter contact between naive T cells and DCs. Treg cells made stable contacts with DCs that preceded the contact of naive T cells with DCs; this might lead to the inhibition of T-cell activation at steady state.[45] The finding that Treg cells are endowed with the ability to have long interactions with DCs mediated by NP-1 supports the idea that NP-1 might contribute to physical interactions between T cells and DCs.

4. Semaphorins in cardiac morphogenesis

Cumulative findings also indicate the significance of semaphorins in the development of other organ systems, such as the cardiovascular system, in which some semaphorins control the migration of endothelial cells, cardiac myocytes, or their precursors.[24),34),46),47)]

(1) Sema6D-Plexin-A1 in cardiac morphogenesis. One of the best characterized roles of a semaphorin in cardiac development is that of Sema6D, which belongs to the class VI transmembrane-type semaphorin subfamily. In mice, the expression of Sema6D is first detected in the cardiac crescent and neural fold of E9 embryos.[24)] The roles of Sema6D in the developing heart have been revealed by a series of studies using the chick embryo system. Inoculation of transfected cells that release a large amount of soluble Sema6D into cultured chick embryos at Hamberger and Hamilton (HH) stage 9 results in enhanced looping of the cardiac tube and enlargement of the ventricular region. In ovo inoculation of Sema6D producing cells or recombinant soluble Sema6D into HH stage 29 embryos results in an expanded ventricular cavity with a thin myocardial layer and an enlarged endocardial cushion. In contrast, RNAi-mediated knockdown of Sema6D inhibits looping of the cardiac tube.[24)] In this context, Sema6D signals are largely mediated through Plexin-A1, which is also expressed in the embryonic heart. Indeed, RNAi-mediated knockdown of Plexin-A1 or expression of truncated Plexin-A1 resulted in shrunken ventricles.[24)] Therefore, the Sema6D-Plexin-A1 axis is critically involved in the dynamic remodeling of the cardiac tube and formation of the ventricle and endocardial cushion.

(2) Sema6D exerts distinct biological activities in endothelial cells in different regions of the cardiac tube. Sema6D inhibits the migration of outgrowing cells from the ventricle segment. In contrast, Sema6D promotes the migration of outgrowing cells from the conotruncal and atrioventricular valve segments, which later fuse to form the endocardial cushion. These biological activities of Sema6D appear to be mediated through Plexin-A1, as they are abrogated by RNAi-mediated knockdown of Plexin-A1 or expression of truncated Plexin-A1 in endothelial cells from the ventricle or conotruncal segments.[24)] Interestingly, we have demonstrated that Plexin-A1 forms a complex with OTK in the endothelial cells of the ventricular region of the cardiac tube, whereas it forms a complex with VEGFR2 in endothelial cells of the conotruncal segments. Further, the Sema6D-induced migration of endothelial cells is suppressed both by RNAi against Plexin-A1 and by RNAi against VEGFR2.[24)] This differential association of Plexin-A1 with additional receptor components allows Sema6D to exert distinct biological activities in adjacent regions, which is critical for complex cardiac morphogenesis.

(3) Reverse signaling of Sema6D in the cardiac ventricle. Among the semaphorin family members, class VI semaphorins are unique because of their relatively long cytoplasmic regions, which may imply the existence of reverse signaling. Indeed, it has been reported that the Sema6A cytoplasmic region can associate with two molecules,[48)] Abl kinase and Enabled (Ena), a member of the Ena/VASP family. Abl kinase and Ena are known to play opposing roles downstream of drosophila Robo, an axon guidance receptor for Slit.[49)] Ena has also been implicated in reverse signaling of drosophila Sema1a.[50)] Upon binding to the ectodomain of Plexin-A1, Abl kinase is recruited to the cytoplasmic tail of Sema6D and activated, resulting in phosphorylation of Ena and dissociation from Sema6D.[46)] In the case of fate-mapping studies using myocardial cells, cells carrying defects in Sema6D reverse signaling arrest in the compact layer, while expression of constitutively active Abl kinase enhances the migration of cells from the compact layer to the trabeculae.[46)] Thus, class VI semaphorins transduce reverse signals through their cytoplasmic domains to regulate biological output.

5. Semaphorin-mediated signals in endothelial cell migration

As described above, the class III semaphorins exert pleiotropic functions through NP-1/Plexin-A receptor complexes, which regulate small GTPases

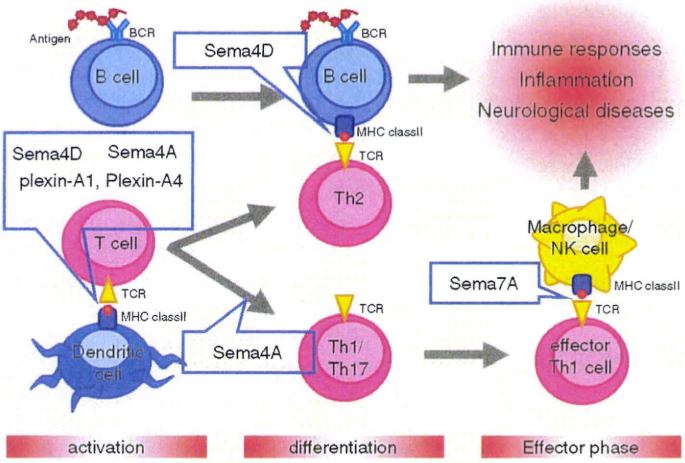

Fig. 3. Involvement of immune semaphorins in various phases of immune responses. In the initial phase of T cell immune responses, Sema4D and Sema6D expressed by T cells activate DCs through their receptors, CD72 and plexin-A1, respectively. Plexin-A1 is also involved as a negative regulator. Sema4A expressed on DCs is also involved in T cell priming through its binding partner, TIM-2, of which expression is induced on activated T cells. Sema4A is required for regulating the differentiation of Th cells. In contrast, Sema4D is upregulated on activated T cells and promotes humoral immune responses through the activation of B cells. Differentiated effector T cells express Sema7A on their cell surface, and interactions between T cell expressing Sema7A and macrophage expressing $\alpha 1\beta 1$ integrin induce macrophage activation, resulting in the promotion of inflammatory responses. BCR, B cell receptor; MHC, major histocompatibility complex; TCR, T cell receptor.

and integrins.[21),51),52)] However, the molecular mechanisms underlying this regulation/integration have remained unclear.

We have determined that the FERM domain-containing guanine nucleotide exchange factor (GEF) FARP2 functions as an immediate downstream signal transducer of the Plexin-A1-NP-1 receptor complex in Sema3A-mediated repulsion of neuronal axons.[23)] Sema3A-induced dissociation of FARP2 from Plexin-A1 and activation of its Rac-GEF activity triggers a series of biochemical events, including Rac activation and the binding of Rnd1, a member of the Rho GTPase family, to Plexin-A1. This binding stimulates the GAP activity of Plexin-A1 for R-Ras, a member of the Ras GTPase family. The down-regulation of R-Ras leads to the cytoskeletal disassembly that is critical for Plexin-A1-mediated growth cone collapse.[22)] In parallel with this event, dissolved FARP2 competes with an isoform of type-1 phosphatidylinositol phosphate kinase, PIPKIγ661, for the FERM domain of talin. PIP_2 generated by PIPKIγ661 in association with talin is important for the stability of integrin-mediated focal adhesions,[53),54)] and the inhibition of PIPKIγ661 kinase activity by FARP2 binding down-regulates integrin function. Thus, semaphorin-Plexin signaling is involved in cardiac morphogenesis by regulating cytoskeletal dynamics and cell-matrix interactions.

6. Perspectives

Accumulating evidence reveals that several semaphorins and their receptors have multiple biological activities. These semaphorins form a family of immunoregulatory molecules, called 'immune semaphorins' (Fig. 3, Table 1). Indeed, the lack of semaphorin family proteins results in several immune disorders, including autoimmune diseases, allergy, and congenital bone disease. Lack of these proteins also causes unresponsiveness to physiological immune responses (Table 1). Therefore, semaphorin family proteins are at least involved in immunological homeostasis, based on sophisticated immune cell communication systems. Furthermore, the patterning and morphogenesis of the heart, as regulated by semaphorins and their receptors are discussed in this review. However, the molecular information available in this context remains fragmented. To understand heart development further, we should complete the definition of the molecular systems required for its formation, and then seek the points of integration between these systems.

Several important issues remain to be resolved. Although semaphorins function to regulate cell motility and morphology by activating plexins, most of the immunological studies of semaphorins have only focused on their costimulatory effects. However, it is plausible that semaphorins exert their functions by affecting the cytoskeleton. Further studies are required to clarify the role of semaphorins in cell-cell interactions, cell morphology, and dynamics, both in the immune system and cardiac morphogenesis. Finally, understanding of semaphorins and their receptors should allow pharmacological modulation of their functions, and may lead to the identification of potential therapeutic targets for several human diseases.

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