The receptor tyrosine kinase Ror2 has recently been shown to act as an alternative receptor or coreceptor for Wnt5a and to mediate Wnt5a-induced migration of cultured cells. However, little is known about the molecular mechanism underlying this migratory process. Here we show by wound-healing assays that Ror2 plays critical roles in Wnt5a-induced cell migration by regulating formation of lamellipodia and reorientation of microtubule-organizing center (MTOC). Wnt5a stimulation induces activation of the c-Jun N-terminal kinase JNK at the wound edge in a Ror2-dependent manner, and inhibiting JNK activity abrogates Wnt5a-induced lamellipodia formation and MTOC reorientation. Additionally, the association of Ror2 with the actin-binding protein filamin A is required for Wnt5a-induced JNK activation and polarized cell migration. We further show that Wnt5a-induced JNK activation and MTOC reorientation can be suppressed by inhibiting PKCζ. Taken together, our findings indicate that Wnt5a/Ror2 activates JNK, through a process involving filamin A and PKCζ, to regulate polarized cell migration.

Ror2 belongs to the Ror family of evolutionally conserved receptor tyrosine kinases (1) and acts as an alternative or coreceptor for Wnt5a, a representative noncanonical Wnt protein (2–4). During mouse development, Ror2 plays essential roles in developmental morphogenesis (5, 6) and is expressed in various cell types that display extensive migratory activities, including neural crest-derived cells and mesenchymal cells (7). Loss- or gain-of-function analyses in mice, Xenopus laevis and Caenorhabditis elegans reveal that, like Wnt5a, Ror2 and/or Ror2 orthologs are required for convergent extension movements during gastrulation, and/or inhibiting the cysteine-rich angiogenic inducer ICAM-1.

It has been proposed that Ror2 mediates Wnt5a signaling by activating the Wnt-c-Jun N-terminal kinase (JNK) pathway, which regulates convergent extension movements in Xenopus gastrulation, and/or inhibiting the β-catenin-TCF pathway (2, 4, 10). Wnt5a stimulation is known to promote cell migration (11–13), which Ror2 seems to mediate through its association with filamin A (FLNa) (3). However, it remains largely unknown how Ror2 and FLNa function in Wnt5a-induced cell migration and whether JNK is involved in this process.

Polarized cell migration is essential for development and wound-healing and requires rearrangements of microtubule (MT) and actin cytoskeletons (14, 15). In directionally migrating cells in wounded monolayers of cultured cells (e.g. fibroblasts) following external stimuli (e.g. epidermal growth factor and lysophosphatidic acid), MT arrays and actin filaments become polarized facing to the wound edge. In such cells, selectively stabilized MTs (post-translationally detyrosinated tubulins or Glu tubulins) orient toward the leading edge and MT organizing center (MTOC) is reoriented to lie between the nucleus and the leading edge (16). At the leading edge, actin cytoskeletons are also reorganized to form lamellipodia, generating driving force for polarized migration (17). On the other hand, it has been established that JNK is involved in wound-healing in Drosophila (18, 19). Erk and p38 as well as JNK have also been implicated in cell migration during wound closure of fibroblastic and/or epithelial cells (20–24). Although JNK is activated following Wnt5a stimulation of cultured cells (4, 25), its role in polarized cell migration remains elusive. Furthermore, it has recently been reported that Wnt5a-induced signaling pathway seems to cooperate with Par/pPKC pathway to mediate polarized reorganization of the microtubule cytoskeleton during a wound response (26), but it is unclear how JNK can interact functionally with components of the Par/pPKC pathway.

We show by using in vitro wound-healing assays that Ror2 mediates polarized cell migration during Wnt5a-induced
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

Wound closure by regulating lamellipodia formation and MTOC reorientation in migrating cells at the wound edge. In fact, these cellular events can be impaired by suppressed expression of Ror2. Furthermore, JNK is activated at the wound edge, in a Ror2-dependent manner, following Wnt5a stimulation, and inhibiting JNK activity abrogates Wnt5a-induced lamellipodia formation and MTOC reorientation. We also show that FLNa and its interaction with Ror2 are required for Wnt5a-induced JNK activation and polarized cell migration. Moreover, we show that Wnt5a-induced JNK activation and MTOC reorientation can be suppressed by inhibiting PKCζ, suggesting a possible functional link between Wnt5a/Ror2/JNK and Par/pPKC pathways. Collectively, these results provide new insights into the mechanism by which Wnt5a/Ror2 regulates JNK activity, through a process involving FLNa and PKCζ, for polarized cell migration.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—The cDNAs for Myc-FLNa and its Δ20 mutant, isolated by restriction enzyme digestion from pMYC-C1 (3), were cloned into the retroviral vector pMXs-puro (a gift from T. Kitamura). An anti-Ror2 antibody was prepared as described (27). Antibodies against phospho-JNK (Thr-183/Tyr-185, Cell Signaling), JNK (FL, Santa Cruz Biotechnology), phospho-ERK (Thr-202/Tyr-204, Cell Signaling), ERK (K-23, Santa Cruz Biotechnology), FLNa (FLMN01, AbCam), PKCζ (C-20, Santa Cruz Biotechnology), bromodeoxyuridine (BrdU) (3D4, BD Biosciences), GFP (IL-8, Clontech), and Myc (A-14, Santa Cruz Biotechnology) were purchased commercially. SP600125, JNK inhibitor I (cell-permeable peptide consisting of HIV-TAT 48-57 and 20-amino acid JNK binding domain of JPl with two proline residues as spacer), and control peptide for JNK inhibitor I (cell-permeable peptide consisting of HIV-TAT 48-57 and two proline residues) were purchased from Calbiochem. Anisomycin, actinomycin D, and BrdU were obtained from Sigma. Mouse-purified Wnt5a was obtained from R&D Systems. For immunostaining, cells were fixed at 4 °C according to the manufacturer's instruction. For phalloidin staining, cells were fixed with 3.7% paraformaldehyde in 0.1 M phosphate-buffered saline, and stained with rhodamine-phallacidin. For immunoblot analysis, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Whole cell lysates were subjected to immunoblot analysis as described (27). For BrdU labeling, cells were incubated with 1 μM BrdU during the course of the wound-healing assay and stained with anti-BrdU antibody according to the manufacturer's instruction. For phaloidin staining, cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline, treated with 0.2% Triton X-100 in phosphate-buffered saline, and stained with rhodamine-phalloidin (Invitrogen) or Alexa Fluor 488-phalloidin (Cambrex Bio Science). For immunostaining, cells were fixed at −20 °C in methanol. Fixed cells were stained with the respective antibodies as described (3). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained using a laser scanning confocal imaging system (LSM510, Carl Zeiss MicroImaging, Inc.) and processed using Photoshop CS (Adobe).

RESULTS AND DISCUSSION

Wnt5a Promotes Polarized Cell Migration in a Ror2-dependent Manner—When exposed to external stimuli that induce cell migration, cells become polarized by reorganizing microtubule and actin cytokinetic results, resulting in reorientation of MTOC and formation of an F-actin-rich membrane protrusion toward the direction of cell movement. Because Wnt5a is one of the stimuli that induces cell migration in various cell types, including fibroblasts (3, 11–13), we examined whether or not Wnt5a could induce microtubule and actin rearrangements to regulate cell polarity by performing in vitro wound-healing assays. To this end, confluent monolayers of NIH3T3 cells, which express Ror2 endogenously, were treated with either control (neo) conditioned medium (CM) or Wnt5a CM. Because serum lipid,
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

A, confluent monolayers of NIH3T3 cells were wounded with a pipette tip and treated with either control (neo) conditioned medium (CM), or Wnt5a CM for 16 h. Cells were fixed and stained with rhodamine-phalloidin. Photographs show representative results (left). The data are expressed as mean ± S.D. of three independent experiments, with the migrating distance of cells without CM treatment set as 1.0 (right). Bar, 500 μm. B, confluent monolayers of NIH3T3 cells were wounded as in A and then treated with DMEM containing 1% FBS in the presence (+) or absence (−) of purified Wnt5a (300 ng/ml) for 16 h. The relative migrating distance of the wound edge was shown as mean ± S.D. of three independent experiments, with the migrating distance of cells without purified Wnt5a treatment set as 1.0. C, wounded monolayers of cells were immediately fixed (0 h, left) or treated with either neo CM (middle) or Wnt5a CM (right) for 0.5 h and then fixed. Cells were stained with rhodamine-phalloidin to visualize F-actin. Arrowheads indicate lamellipodial protrusions of cells at the wound edge. Bar, 50 μm. D, wounded monolayers were treated with neo CM or Wnt5a CM for the indicated times. Cells were costained with anti-γ-tubulin antibody for MTOC (green) and DAPI for nuclei (blue). Representative images at 4 h were shown (top). White lines indicate the 90° sector facing to the wound edge. The percentage of wound-edge cells in which MTOC was within the 90° sector was measured (bottom). The data are expressed as mean ± S.D. of three independent experiments (n > 100 for each time point in each experiment). Bar, 50 μm. E, wounded monolayers were treated with neo CM or Wnt5a CM for 4 h and costained with antibodies against α-tubulin (green) and detyrosinated tubulin (Glu MTs, red) (right) merged images. The percentage of wound-edge cells positive for Glu MTs was measured (bottom). The data are expressed as mean ± S.D. of three independent experiments (n > 100 in each experiment). Bar, 50 μm.

FIGURE 1. Wnt5a stimulates polarized cell migration. A, confluent monolayers of NIH3T3 cells were wounded with a pipette tip and treated with either control (neo) conditioned medium (CM), or Wnt5a CM for 16 h. Cells were fixed and stained with rhodamine-phalloidin. Photographs show representative results (left). The relative migrating distance of the wound edge was shown as mean ± S.D. of three independent experiments, with the migrating distance of neo CM-treated cells set as 1.0 (right). Bar, 500 μm. B, confluent monolayers of NIH3T3 cells were wounded as in A and then treated with DMEM containing 1% FBS in the presence (+) or absence (−) of purified Wnt5a (300 ng/ml) for 16 h. The relative migrating distance of the wound edge was shown as mean ± S.D. of three independent experiments, with the migrating distance of cells without purified Wnt5a treatment set as 1.0. C, wounded monolayers of cells were immediately fixed (0 h, left) or treated with either neo CM (middle) or Wnt5a CM (right) for 0.5 h and then fixed. Cells were stained with rhodamine-phalloidin to visualize F-actin. Arrowheads indicate lamellipodial protrusions of cells at the wound edge. Bar, 50 μm. D, wounded monolayers were treated with neo CM or Wnt5a CM for the indicated times. Cells were costained with anti-γ-tubulin antibody for MTOC (green) and DAPI for nuclei (blue). Representative images at 4 h were shown (top). White lines indicate the 90° sector facing to the wound edge. The percentage of wound-edge cells in which MTOC was within the 90° sector was measured (bottom). The data are expressed as mean ± S.D. of three independent experiments (n > 100 for each time point in each experiment). Bar, 50 μm. E, wounded monolayers were treated with neo CM or Wnt5a CM for 4 h and costained with antibodies against α-tubulin (green) and detyrosinated tubulin (Glu MTs, red) (right) merged images. The percentage of wound-edge cells positive for Glu MTs was measured (bottom). The data are expressed as mean ± S.D. of three independent experiments (n > 100 in each experiment). Bar, 50 μm.

Lysoosphatidic acid, is known to trigger MTOC reorientation after scratching the monolayers of NIH3T3 cells (32), we used CM with low serum concentration (0.5%), at which neo CM failed to induce significant MTOC reorientation after scratching (see Fig. 1D). In this condition, Wnt5a CM drastically promoted wound closure (migration of the wound edges), compared with neo CM (Fig. 1A). Labeling cells with BrdU during the course of wound-healing assay (16 h) revealed that the proportions of BrdU-positive cells were comparable between neo CM- and Wnt5a CM-treated cells (data not shown), suggesting that promoted cell migration rather than proliferation is attributable to Wnt5a-stimulated wound closure. Importantly, adding purified Wnt5a in cultured medium containing 1% FBS also significantly stimulated wound closure, albeit to a lesser extent compared with did Wnt5a CM (Fig. 1B). The difference in biological activities of Wnt5a CM and purified Wnt5a may be attributable to loss of Wnt5a activity during its purification and/or existence of a factor(s) in CM that potentiates activity of Wnt5a (33). Phalloidin staining revealed that cells treated with Wnt5a CM, but not neo CM, exhibited formation of lamellipodial membrane protrusions at the edge of the wound (Fig. 1C), indicating that Wnt5a induces actin rearrangement to form lamellipodia, producing driving force for cell migration. We next examined whether Wnt5a stimulation can induce the reorientation of the MTOC in migrating cells at the wound edge. As mentioned above, under our experimental condition, neo CM failed to induce significant MTOC reorientation due to reduced serum concentration in the CM. In contrast, treatment of cells with Wnt5a CM (Fig. 1D), which contains the same concentration of serum as neo CM, or purified Wnt5a (data not shown) resulted in a drastic increase in cells with the reoriented MTOC, indicating that Wnt5a promotes MTOC reorientation. This finding is consistent with the result reported by Schlessinger et al. (26) showing that siRNA-mediated depletion of Wnt5a inhibited reorientation of centrosome and Golgi in rat embryo fibroblasts. Effect of Wnt5a on cell polarization was then exam-
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

We have previously shown that overexpression of Wnt5a and Ror2 in NIH3T3 cells resulted in an increase in kinase activity of JNK (4). It has also been reported that Wnt5a stimulation induces activation of JNK and ERK in NIH3T3 and human umbilical vein endothelial cells, respectively (13, 25). To examine if these MAP kinases are activated during Wnt5a-stimulated wound closure, we treated monolayers of NIH3T3 cells with either neo CM or Wnt5a CM after wounding and monitored their phosphorylation status by immunoblotting with phosphorylation site-specific antibodies. As shown in Fig. 3A, Wnt5a stimulation of wounded cells resulted in a drastic increase in the level of phosphorylated-JNK, but not -ERK and -p38, without altering their total amounts (Fig. 3A). The findings indicate that among MAP kinase family members, Wnt5a primarily induces activation of JNK in wounded fibroblasts. Although wounding by itself had a little effect on JNK phosphorylation, Wnt5a-induced JNK phosphorylation was markedly augmented after wounding (Fig. 3B), suggesting that wound stimuli can potentiate Wnt5a signaling to activate JNK. To verify this, Wnt5a-induced JNK activation in wounded monolayers was monitored by immunostaining using antibody against phosphorylated c-Jun at Ser-63, a well-established phosphorylation site in c-Jun by activated JNK. Interestingly, increased phosphorylation of c-Jun was presented. We then examined the effect of Ror2 gene knockdown on lamellipodia formation and MTOC reorientation in Wnt5a-stimulated cells. Ror2-depleted cells failed to exhibit lamellipodia formation following Wnt5a stimulation (Fig. 2D). Furthermore, Wnt5a-induced MTOC reorientation was impaired in Ror2-depleted cells (Fig. 2E). Similarly, suppressed expression of Ror2 in HeLa cells resulted in impaired MTOC reorientation following Wnt5a stimulation (data not shown). The results indicate that Ror2 plays critical roles in Wnt5a-induced polarized cell migration of NIH3T3 and HeLa cells. On the other hand, apparent differences in Wnt5a-induced wound closure and MTOC reorientation were not detectable in immortalized mouse embryonic fibroblasts from the wild-type and Ror2–/– embryos (data not shown). Further study will be required to elucidate molecular and/or cellular bases explaining this discrepancy.

Wnt5a Induces JNK Activation at the Wound Edge in a Ror2-dependent Manner—We have previously shown that overexpression of Wnt5a and Ror2 in NIH3T3 cells resulted in an increase in kinase activity of JNK (4). It has also been reported that Wnt5a stimulation induces activation of JNK and ERK in NIH3T3 and human umbilical vein endothelial cells, respectively (13, 25). To examine if these MAP kinases are activated during Wnt5a-stimulated wound closure, we treated monolayers of NIH3T3 cells with either neo CM or Wnt5a CM after wounding and monitored their phosphorylation status by immunoblotting with phosphorylation site-specific antibodies. As shown in Fig. 3A, Wnt5a stimulation of wounded cells resulted in a drastic increase in the level of phosphorylated-JNK, but not -ERK and -p38, without altering their total amounts (Fig. 3A). The findings indicate that among MAP kinase family members, Wnt5a primarily induces activation of JNK in wounded fibroblasts. Although wounding by itself had a little effect on JNK phosphorylation, Wnt5a-induced JNK phosphorylation was markedly augmented after wounding (Fig. 3B), suggesting that wound stimuli can potentiate Wnt5a signaling to activate JNK. To verify this, Wnt5a-induced JNK activation in wounded monolayers was monitored by immunostaining using antibody against phosphorylated c-Jun at Ser-63, a well-established phosphorylation site in c-Jun by activated JNK. Interestingly, increased phosphorylation of c-Jun was
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

FIGURE 3. Wnt5a induces JNK activation at the wound edge. A, confluent monolayers of NIH3T3 cells were wounded and then treated with neo CM or Wnt5a CM for 30 min. The levels of total or phosphorylated forms of JNK, p38, and ERK, respectively, were determined by immunoblotting. The right panel indicates the relative levels of P-JNK, P-p38, and P-ERK, with each value of neo CM-treated cells set as 1.0. The data are expressed as mean ± S.D. of three independent experiments. B, confluent monolayers of cells were wounded or left untreated and then treated with neo CM or Wnt5a CM for 30 min. Whole cell lysates were analyzed by immunoblotting with antibodies against phospho-JNK (P-JNK) and JNK, respectively. C, wounded monolayers of cells were treated with CM as in A and costained with anti-phospho-c-Jun antibody (P-c-Jun, green) and DAPI (blue). Arrowheads indicate the wound-edge cells with enhanced P-c-Jun staining after Wnt5a treatment. Bar, 100 μm.

FIGURE 4. Ror2 is required for Wnt5a-induced JNK activation at the wound edge. A, confluent monolayers of NIH3T3 cells transfected with control siRNA or Ror2 siRNA (1) were wounded and treated with indicated CM for 30 min (left panel) or purified Wnt5a (200 ng/ml) for 20 min (right panel). The levels of Ror2, P-JNK, and total JNK were analyzed by immunoblotting, respectively. The histograms indicate the relative P-JNK levels. B, confluent monolayers of siRNA-transfected cells were wounded and treated with CM as in A. Cells were costained with anti-phospho-c-Jun antibody (P-c-Jun, green) and DAPI (blue). Arrowheads indicate the wound-edge cells with enhanced P-c-Jun staining after Wnt5a treatment. Bar, 100 μm.

detected predominantly in cells at the wound edge, following Wnt5a stimulation (Fig. 3C), indicating that Wnt5a-JNK pathway is activated predominantly in wound-edge cells. Consistent with our findings, Lallemand et al. (34) showed that although scratching the monolayers of NIH3T3 cells had little effect on c-Jun phosphorylation, stimulation of wounded monolayers with growth factors, such as EGF and PDGF, induced c-Jun phosphorylation in cells bordering the wound. Together, these results suggest that wounding the cell monolayers potentiates activation of JNK by external stimuli, including Wnt5a, in cells at the wound edge. In contrast, it has also been reported that scratching monolayers of human keratinocytes or mouse embryonic fibroblasts resulted in robust c-Jun phosphorylation in cells at the wound edge without additional external stimuli (24, 34). The discrepancy may reflect differences in cell types and/or experimental conditions used in these experiments.

While it has previously been shown that overexpression of Ror2 and Wnt5a in NIH3T3 cells resulted in an increased kinase activity of JNK (4), it remains unclear whether or not Ror2 is indeed required for Wnt5a-induced JNK activation. To clarify this issue, we examined the effect of Ror2 siRNA on Wnt5a-induced JNK activation in wounded monolayers of NIH3T3 cells. As shown in Fig. 4A, JNK phosphorylation induced by either Wnt5a CM (left panel) or purified Wnt5a (right panel) was remarkably inhibited in cells transfected with Ror2 siRNA (1). Essentially identical results were also obtained using Ror2 siRNA (2) (data not shown). On the other hand, Ror2 siRNA failed to inhibit JNK activation induced by anisomycin, an inhibitor of protein synthesis (data not shown). These results indicate an essential role of Ror2 in Wnt5a-induced JNK activation. Furthermore, Wnt5a-induced c-Jun phosphorylation in wound-edge cells was impaired in Ror2-depleted cells (Fig. 4B), confirming that Wnt5a-induced JNK activation in wound-edge cells is mediated by Ror2. Considering that Wnt5a-induced cell polarization is also seen in cells at the wound edge, these findings support a notion that Wnt5a/Ror2-mediated JNK activation may play critical roles in regulating cell polarity at the wound edge.

JNK Activity Is Required for Wnt5a-induced Polarized Cell Migration—We then examined the effect of inhibition of JNK on Wnt5a-induced polarized cell migration. To this end, we used two structurally unrelated JNK inhibitors, SP600125 and JNK inhibitor I (cell-permeable peptide consisting of HIV-TAT_{48–57} and JNK-binding domain of JIP1), that act through different mechanisms. These inhibitors efficiently suppressed Wnt5a-induced JNK activation in wounded monolayers of cells, as assessed by anti-phospho-c-Jun immunoblotting
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

Treatment of cells with SP600125 resulted in suppression of Wnt5a-induced wound closure in a dose-dependent manner (Fig. 5A). Wnt5a-induced lamellipodia formation and MTOC reorientation in wound-edge cells were also markedly suppressed by treatment with SP600125 (Fig. 5C and E). The JNK inhibitor I also inhibited these biological responses of cells to Wnt5a (Fig. 5, B, D, and F). However, it lowered the basal level of MTOC reorientation (Fig. 5F, neo CM), probably due to toxicity of this peptide inhibitor via its cell-permeable portion, HIV-TAT_{48–57}, since control peptide, which consists of the HIV-TAT_{48–57} peptide alone, also suppressed to a similar extent. Taken together, the results indicate that JNK activity is critically required for Wnt5a-induced polarized cell migration during wound closure. At present, it remains unclear about the mechanism by which JNK regulates cell polarization and migration after Wnt5a stimulation. With this respect, it should be noted that, despite increased phosphorylation of c-Jun transcription factor by JNK, gene expression may not be required at least for Wnt5a-induced MTOC reorientation, because it was unaffected by treatment with actinomycin D, an inhibitor of transcription (data not shown). It will be of interest to investigate whether or not JNK directly phosphorylates proteins that regulate MTOC reorientation.

Association of Ror2 with FLNa Is Indispensable for Wnt5a-induced JNK Activation and Polarized Cell Migration—We have recently shown that FLNa is associated with Ror2 and is required for Wnt5a-induced cell migration (3). FLNa is also known to mediate JNK activation by various stimuli, acting as a scaffold protein (35, 36). Thus, the findings prompted us to examine whether or not FLNa is involved in Wnt5a-induced JNK activation in wounded cells. To this end, we utilized the FLNa-deficient human melanoma cell line (M2) and its derivative line (A7), which stably expresses FLNa (Fig. 6A) (31). Wnt5a stimulation resulted in substantial activation of JNK in A7, but marginal in M2 cells (Fig. 6, A and C). On the other hand, anisomycin induced a marked JNK activation in both A7 and M2 cells (Fig. 6B), consistent with previous reports (35, 36). Because knockdown of mouse FLNa in NIH3T3 cells has been unsuccessful (data not shown), we examined the effect of suppressed human FLNa expression in the human osteosarcoma cell line, SaOS2. As shown, suppression of FLNa expression by...
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

FIGURE 6. Association of Ror2 with FLNa is indispensible for Wnt5a-induced JNK activation and polarized cell migration. A, confluent monolayers of M2 and A7 cells were wounded and then treated with neo CM or Wnt5a CM for 30 min. The levels of P-JNK and total JNK were analyzed by immunoblotting, respectively. Expression of FLNa in M2 and A7 cells was also shown (bottom). B, M2 and A7 cells were treated with anisomycin (12.5 μg/ml) for 30 min or left untreated. The levels of P-JNK and total JNK were analyzed by immunoblotting, respectively. C and D, JNK activities in M2 cells or M2-derived cells stably expressing Myc-tagged wild-type FLNa (WT/M2, 10) or FLNaΔ20 (Δ20/M2, 3) were determined, following treatment with Wnt5a (C) and anisomycin (D), as in A and B, respectively. Expression of Myc-tagged FLNa (WT or Δ20) was analyzed by anti-Myc immunoblotting (C). E and F, confluent monolayers of M2, WT/M2 (10), or Δ20/M2 (3) cells were wounded and treated with either neo CM or Wnt5a CM for 24 h (E) or 4 h (F). The relative migrating distance of the wound edge (E) and the percentage of wound-edge cells with reoriented MTOC (F) were determined as in Fig. 1A and Fig. 10, respectively. Each value represents the mean ± S.D. of three independent experiments. *, p < 0.01; **, p < 0.005; Student’s t test.

siRNA resulted in a substantial reduction of Wnt5a-induced JNK activation without affecting anisomycin-induced JNK activation in SaOS2 cells (supplemental Fig. S1, A and B). These results indicate that FLNa is essential for Wnt5a-induced JNK activation.

Because the FLN repeat 20 of FLNa is required for the association of FLNa with Ror2 (3), we next examined whether the FLN repeat 20 is also required for Wnt5a-induced JNK activation, by using M2 cells stably transfected with Myc-tagged wild-type FLNa (WT/M2) or the FLNa mutant lacking the FLN repeat 20, FLNaΔ20 (Δ20/M2) (3). Both WT/M2 (10) and Δ20/M2 (3) cells showed increased basal phosphorylation of JNK compared with parental M2 cells (Fig. 6C, neo), a feature that is also observed in A7 cells (see Fig. 6A). Importantly, unlike A7 and WT/M2 (10) cells, Wnt5a CM failed to augment further activation of JNK in Δ20/M2 (3) cells (Fig. 6C). It should be noted that anisomycin did activate JNK in Δ20/M2 (3) cells as did in M2 and WT/M2 (10) cells (Fig. 6D). These results further emphasize that FLNa plays an essential role in Wnt5a-induced JNK activation presumably by associating with Ror2 via the FLN repeat 20.

Our previous study indicates that Wnt5a induces cell migration of A7, but not M2 cells in transwell migration assays (3). Consistent with this observation, Wnt5a CM apparently promoted wound closure and MTOC reorientation of WT/M2 (10), but not M2 cells (Fig. 6, E and F). As shown, knockdown of FLNa expression also resulted in apparent inhibition of Wnt5a-induced MTOC reorientation in SaOS2 cells (supplemental Fig. S1C). It is also worth noting that Wnt5a CM failed to stimulate wound closure and MTOC reorientation of Δ20/M2 (3) cells (Fig. 6, E and F). Similar results were also obtained when another independent stable clones, WT/M2 (2) and Δ20/M2 (7), were examined (data not shown), excluding the possibility of clonal alterations. Taken together, these results indicate that FLNa and its association with Ror2 are critically required not only for JNK activation but also for polarized cell migration following Wnt5a stimulation.

PKCζ Is Involved in Wnt5a-induced JNK Activation and Cell Polarization—It has recently been reported that Par/aPKC pathway cooperates with Wnt5a signaling to mediate cell polarization during wound healing of rat embryo fibroblasts (26). To gain insights into the relationship between Wnt5a/Ror2/JNK and Par/aPKC pathways, we examined the effect of cell-permeable pseudo-substrate (PS) inhibitors specific for the respective PKC isoforms, including cPKC (PKCa/β), nPKC (PKCγ and PKCθ), and aPKC (PKCζ), on Wnt5a-induced MTOC reorientation. As shown in Fig. 7, PS for PKCζ, but not other PSs, suppressed Wnt5a-induced MTOC reorientation, indicating that PKCζ activity is critical for Wnt5a-induced MTOC reorientation. Furthermore, PKCζ PS inhibited Wnt5a-induced JNK phosphorylation in wounded monolayers, while it failed to exert any inhibitory effect on anisomycin-induced JNK phosphorylation (Fig. 7B). Consistent
Role of Ror2 in Wnt5a-induced Polarized Cell Migration

with the results, anti-phospho-c-Jun immunostaining also revealed that PKCζ PS treatment inhibited Wnt5a-induced c-Jun phosphorylation in wound-edge cells. Therefore, PKCζ activity is critical for Wnt5a-induced JNK activation in wound-edge cells. Together, these results suggest that PKCζ, which is activated in response to wound-induced loss of cell-cell contacts (37), cooperates with Wnt5a/Ror2 signaling to activate JNK in the wound-edge cells, leading to cell polarization.

In conclusion, we have shown that Ror2 mediates Wnt5a-induced polarized cell migration during wound healing of fibroblastic cells, via activation of JNK at the wound edge. Wnt5a-induced JNK activation and cell polarization require association of Ror2 with FLNa as well as PKCζ activity. The findings provide new insights into the role of Ror2 in the non-canonical Wnt5a/JNK pathway that regulates polarized cell migration in cooperation with Par/αPKC pathway. At present, it is unclear about the mechanisms by which FLNa and PKCζ regulate JNK activity in Wnt5a signaling and how activated JNK in turn regulates cell polarity and migration. Further study will be required to clarify these issues.

Acknowledgments—We thank E. Fuchs for helpful suggestions and comments in the research and writing of this work. We thank A. Yoda for critical reading of the manuscript. We also thank Y. Ohta for M2 and A7 cells and FLNa cDNA, T. Kitamura for Plat-A cells and pMXs-puro vector, and S. Takada for neo/L and Wnt5a/L cells.

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Role of Ror2 in Wnt5a-induced Polarized Cell Migration

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