Dissection of Wnt5a-Ror2 Signaling Leading to Matrix Metalloproteinase (MMP-13) Expression*

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Background: Constitutively active Wnt5a-Ror2 signaling in osteosarcoma cells induces MMP-13 expression.

Results: MMP-13 is expressed by binding of c-Jun and ATF2 to AP1-binding sites within its promoter during Wnt5a-Ror2 signaling.

Conclusion: MMP-13 expression by Wnt5a-Ror2 signaling is mediated by Dvl, Rac1, JNK, and AP1 transcription factors.

Significance: This provides novel molecular machineries of Wnt5a-Ror2 signaling, involved in MMP-13 expression.

It has been shown that constitutively active Wnt5a-Ror2 signaling in osteosarcoma cell lines plays crucial roles in induced expression of matrix metalloproteinase-13 (MMP-13), required for their invasiveness; however, it remains largely unclear about the molecular basis of MMP-13 gene induction by Wnt5a-Ror2 signaling. Here we show by reporter assay that the activator protein 1 (AP1) (binding site in the promoter region of MMP-13 gene is primarily responsible for its transcriptional activation by Wnt5a-Ror2 signaling in osteosarcoma cell lines SaOS-2 and U2OS. Chromatin immunoprecipitation assays revealed that c-Jun and ATF2 are crucial transcription factors recruited to the AP1-binding site in the MMP-13 gene promoter during Wnt5a-Ror2 signaling in SaOS-2 cells. Using siRNA-mediated suppression or specific inhibitors, we also show that Dishevelled2 (Dvl2) and c-Jun N-terminal kinase are required for MMP-13 gene induction presumably via phosphorylation of c-Jun and ATF2 during Wnt5a-Ror2 signaling in SaOS-2 cells. Interestingly, Dvl2 and Rac1, but not Dvl3, are required for MMP-13 expression in SaOS-2 cells, whereas Dvl3, but not Dvl2 and Rac1, is required for its expression in U2OS cells, indicating the presence of distinct intracellular signaling machineries leading to expression of the same gene, in this case MMP-13 gene in different osteosarcoma cell lines. Moreover, we provide evidence suggesting that Wnt5a-Ror2 signaling might also be required for expression of MMP-13 gene during the development of the cartilaginous tissue.

The Wnt family of proteins are secreted cysteine-rich glycoproteins with lipid modifications consisting of 19 members in human to date (1). Several lines of evidence demonstrate that Wnt proteins play important roles in developmental, physiological, and/or pathological processes (2). Wnt proteins can elicit a β-catenin-dependent (canonical) and β-catenin-independent (non-canonical) signaling pathways (3). Wnt5a is a representative of Wnt members that can activate non-canonical Wnt signaling. It has been shown that the sustained or increased expression of Wnt5a is implicated in various types of cancers (4–7) and that Wnt5a promotes cell migration and invasiveness on osteosarcoma cells or prostate carcinoma cells (8, 9).

Ror2, a member of the Ror-family of receptor-tyrosine kinase s, acts as a receptor or co-receptor for Wnt5a to mediate Wnt5a-induced various cellular functions, including cell migration, polarity, or invasion (8, 10, 11). Both Wnt5a- and Ror2-deficient mouse embryos exhibit similar abnormalities in various tissues and organs, including cardiovascular, inner ear, and skeletal systems that are caused at least partly by disrupted convergent extension movements and planar cell polarity during development (12, 13). Ror2 mediates Wnt5a signaling by inhibiting the β-catenin-T-cell factor pathway and activating Wnt/c-Jun N-terminal kinase (JNK) pathways (3, 12). The Wnt/JNK pathway can be mediated by various signaling molecules, including Dishevelled (Dvl)2, activator protein 1 (AP1), and members of the Rho-family of small GTPases in addition to JNK (12); however, it remains elusive how this pathway regulates expression of its target genes.

Recently, it has been reported that Ror2 regulates the expression of genes, encoding various matrix metalloproteinases (MMPs), which belong to a family of zinc-dependent enzymes capable of degrading extracellular matrix, including basement membrane components (8, 14, 15). Among MMPs, MMP-13 exhibits unique characteristics, i.e. it cleaves not only type II-collagen but also macromolecules of the extracellular matrix such as a fibrinogen. Spatiotemporally regulated expression of

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This article contains supplemental Figs. S1–S3.

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2 The abbreviations used are: Dvl, Dishevelled; MMP, matrix metalloproteinase; AP1, activator protein 1; OSE2, osteoblast-specific element; IHC, immunohistochemistry; qRT-PCR, quantitative RT-PCR; SFF, Src-family protein-tyrosine kinase; IB, immunoblot; WCL, whole-cell lysate.
MMP-13 is required for proper matrix remodeling under physiological conditions such as development of cartilaginous tissue (16). In contrast, sustained overexpression of MMP-13 appears to contribute to cartilage destruction in rheumatoid arthritis or to progression of various malignant tumors, including breast cancers, chondrosarcomas, melanomas, esophageal cancers, colorectal cancers, and osteosarcomas (8, 17–25). In fact it has been shown that constitutive overexpression of MMP-13 is critically required for cell invasiveness of osteosarcoma in a cell-autonomous manner and that expression of MMP-1 or MMP-2 is involved in progression of several other types of cancer cells (8, 9, 14, 15). These findings indicate that cell type-dependent expression of members of MMP-family is implicated in some if not all of cancer cells (26).

Expression of MMP-13 can be controlled by a variety of stimuli including basic fibroblast growth factor (chondrosarcoma cells), inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (human chondrocytes), IL-6 (rat fibroblast), or TGF-β1 and IL-1β (human fibroblast) (19, 27–31). Analysis of the MMP-13 gene revealed that its promoter region contains putative transcription factor binding sites containing TATA box, AP1, Polyomavirus enhancer activator-3 (PEA-3), osteoblast-specific element (OSE2), or AG-rich element (32). However, it is still unclear about the mechanism underlying MMP-13 gene expression by Wnt5a-Ror2 signaling.

In the present study we show that in addition to c-Src, Dvl2, Rac1, and JNK are required for MMP-13 gene expression by Wnt5a-Ror2 signaling via binding of c-Jun and ATF2 to AP1-binding site within the MMP-13 gene promoter in SaOS-2 cells. On the other hand, MMP-13 gene expression by Wnt5a-Ror2 signaling in U2OS cells requires Dvl3, but not Dvl2 and Rac1, indicating that distinct intracellular signaling events might play a role in regulating MMP-13 gene expression in different osteosarcoma cell lines.

**EXPERIMENTAL PROCEDURES**

Animals—The experiments using animals in this study were approved by the Institutional Animal Care and Use Committee (permission number P110307) and carried out according to the Kobe University Animal Experimentation Regulations.

Cell Culture and Transfection—Human osteosarcoma cell lines SaOS-2 and U2OS cells were maintained in DMEM (Nissui, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (BioWest, Nuaillé, France). For plasmid or siRNA transfection, we used Lipofectamine 2000 (Invitrogen) or GeneSilencer reagent (GenLantis, San Diego, CA), respectively, according to the manufacturer instructions.

Plasmids and Small Interfering RNA (siRNA)—Plasmids encoding Dvl2 wild-type (WT) and KM (K446M) mutant were prepared as described (35). The firefly luciferase reporter plasmids p253-luc, p1004-luc, and p3105-luc were constructed by inserting the PCR-amplified 253-, 1004-, and 3105-bp genomic DNA fragments from the MMP-13 genomic promoter region, respectively, into pGL3 basic reporter vector (Promega). Other reporter plasmids, p253(OSE2m)-luc or p253(AP1m)-luc, containing mutated OSE2 or AP1-binding site, respectively, in the MMP-13 gene promoter p253-luc were constructed by site-directed mutagenesis. The respective mutated element OSE2m (TACCAACTAGTACA) or AP1m (TGGTGTCA) was generated by altering five or three bases in its promoter p250, respectively. The following target sequences were used for the respective siRNAs: Wnt5a #1 (5′-TAACCCTGTTCAGATGTCA-3′); Ror2 #1 (5′-AACAGTTGCCCTTTGTAGACCTTG-3′); Ror2 #2 (5′-TAAAGGTCCTGGATCCAGAACC-3′); Dvl2 #1 (5′-TTTAGCTGACTGTGATGATTG-3′); Dvl2 #2 (5′-TAAATGTGTCATCAGGAAAGGACC-3′); Dvl2 #3 (5′-CATGGGAAAGATCAACTTCT-3′); Dvl3 #1 (5′-TTACGATGGCAAGCTATGTCAGTG-3′); Dvl3 #2 (5′-TTGAGAGAAACTATAGCTGGTGC-3′); Rac1 #1 (5′-GGTCTTTAATTTGCTTCTTTCTT-3′); Rac1 #2 (5′-GAGGAGAGAAATGCTGGTCT-3′); Control #2 (5′-TTAGCCCCAGCTCATCTCCTAT-3′).

Antibodies and Reagents—The following antibodies were purchased commercially and used for IB, chromatin immunoprecipitation (ChIP), or immunohistochemistry (IHC); mouse monoclonal antibodies against Dvl3 (sc-8027; Santa Cruz Biotechnology) for IB, β-actin (sc-1616, Santa Cruz Biotechnology) for IB, phospho-c-Jun (sc-822, Santa Cruz Biotechnology) for IB, Rac1 (23A8, Millipore) for IB; rabbit polyclonal antibodies against Dvl2 (3216, Cell Signaling Technology) for IB, c-Fos (sc-52, Santa Cruz Biotechnology) for IB, c-Jun (sc-45, Santa Cruz Biotechnology) for IB and ChIP, ATF2 (sc-6233, Santa Cruz Biotechnology) for IB and ChIP, c-Fos (sc-52, Santa Cruz Biotechnology) for ChIP, phospho-ATF2 (9255, Santa Cruz Biotechnology) for IB, MMP-13 (sc-30073, Santa Cruz Biotechnology) for IHC; an anti-Ror2 antibody for IB, and Wnt5a siRNA #2 was as described by Masck-auchán et al. (33). All other 21-bp siRNAs were synthesized by Invitrogen.

Luciferase Reporter Assay—SaOS-2 or U2OS cells were transfected with the respective siRNAs and cultured for 4 days.
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Subsequently, the cells were transfected with the firefly luciferase reporter construct containing p253, p253(OS2m), p253(AP1m), p1004, or p3105 along with the renilla luciferase reporter construct and were further cultured for 24 h. Luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and GLOMAX 96 Microplate luminometer (Promega) as previously described (8, 35, 36).

Quantitative RT-PCR (qRT-PCR)—Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) and then subjected to treatment with RNase-free DNase I (Invitrogen). For qRT-PCR, total RNA (1 μg) was reverse-transcribed using PrimerScript 1st strand cDNA synthesis kit (Takara), and real-time PCR was performed on the LightCycler 480 system (Roche Diagnostics) using LightCycler 480 SYBR Green I Master mix (Roche Diagnostics). Relative Wnt5a or MMP-13 mRNA levels were determined after normalization by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Forward and reverse primers were as follows: Wnt5a, 5′-TAA-GCCAGAGGTCCGTGTTT-3′ and 5′-CCAGAGAGCTGTGCTCCTA-3′; MMP-13, 5′-AACATCTAAAAAGCCAGAC-3′ and 5′-GGAAGTTCGCCCCAATGTA-3′; GAPDH, 5′-CAAGATCATCAGCAATGCCT-3′ and 5′-TCATGAGTTGATGCCTGGG-3′. Forward and reverse primers were as follows, respectively: Control (Ctrl) #1, Ror2 #1, or Ror2 #2 siRNA with different sequences for Ror2 gene were cultured for 4 days, and WCLs were subjected to immunoblotting with the indicated antibodies.

RESULTS

Constitutively Active Wnt5a-Ror2 Signaling Is Critical for Transcriptional Activation of MMP-13 Gene Promoter via AP1-binding Site in SaOS-2 Cells—As an attempt to elucidate the molecular basis of MMP-13 gene expression by Wnt5a-Ror2 signaling, we first performed reporter assays to identify a region within the MMP-13 gene promoter required for transcriptional activation by Wnt5a-Ror2 signaling in osteosarcoma cell line SaOS-2. Because Wnt5a-Ror2 signaling has been shown to be constitutively activated by sustained expression of both Wnt5a and Ror2 in SaOS-2 cells (8), we examined the effects of siRNA-mediated suppression of Wnt5a or Ror2 on transcriptional activation of MMP-13 gene in the cells. SaOS-2 cells transfected with Control (Ctrl) #1, Ror2 #1, or Ror2 #2 siRNA with different sequences for Ror2 gene were cultured for 4 days, and WCLs were subjected to immunoblotting with the indicated antibodies. As shown in Fig. 1A, suppressed expression of Ror2 was detected in SaOS-2 cells transfected with Ror2 #1 or Ror2 #2 siRNA compared with Ctrl #1 siRNA. SaOS-2 cells transfected with the respective siRNAs were further transfected with the indicated MMP-13 gene promoter-luciferase (luc) constructs (p3105-luc, p1004-luc, p253-luc, and basic-luc) along with the renilla luciferase construct as described under “Experimental Procedures.” Reporter assays with p3105-luc, p1004-luc, or p253-luc revealed that decreased levels of relative luciferase activities were detected in SaOS-2 cells transfected with Ror2 #1 or Ror2 #2 siRNA compared with Ctrl #1 siRNA (Fig. 1B). The basic-luc activity was not affected by suppressed expression of Ror2 (Fig. 1B). Because the identified MMP-13 promoter region (bp −253 to −1) contains two well-characterized cis-regulatory elements, the OSE2- and AP1-binding sites, we next examined which element was responsible for transcriptional activation by Ror2-mediated signaling. To this end point mutations were introduced into OSE2- and AP1-binding sites in p253-luc (253(OS2m)-luc and p253(AP1m)-luc, respectively) (Fig. 1C). SaOS-2 cells transfected with the respective siRNAs were further transfected with p253-luc, p253(OS2m)-luc, p253(AP1m)-luc, or basic-luc along with the renilla luciferase construct and cultured for 24 h. Transfection with p253(AP1m)-luc, but not p253(OS2m)-luc, resulted in a drastic decrease of luciferase activities that were unaffected by Ror2-mediated signaling (Fig. 1C). Because Ror2 is known to act as a receptor or co-receptor for Wnt5a, we also examined the effect of suppressed expression of Wnt5a on MMP-13 gene expression in SaOS-2 cells. SaOS-2 cells transfected with Ctrl #2, Wnt5a #1, or Wnt5a #2 siRNA were cultured for 4 days, and total RNAs from the respective cells were subjected to qRT-PCR to assess suppressed expression of Wnt5a by Wnt5a...
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FIGURE 1. Constitutively active Wnt5a-Ror2 signaling is critical for transcriptional activation of MMP-13 gene promoter via AP1-binding site in SaOS-2 cells. A, SaOS-2 cells transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA were cultured for 4 days, and WCLs were analyzed by immunoblotting with the indicated antibodies. B, SaOS-2 cells were transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA and cultured for 4 days. Subsequently, SaOS-2 cells were transfected with the indicated MMP-13 gene promoter-luciferase (Luc) vectors along with the renilla luciferase reporter construct and were cultured for 24 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *p < 0.01, t test. C, SaOS-2 cells were transfected with either Ctrl #1 or Ror2 #1 siRNA and cultured for 4 days. Then cells were transfected with the indicated MMP-13 gene promoter-luc vectors, whose promoters contain mutations within the OSE2- and AP1-binding sites, respectively, along with the renilla luciferase reporter construct and were cultured for 24 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *p < 0.01, t test. D, SaOS-2 cells were transfected with Ctrl #2, Wnt5a #1, or Wnt5a #2 siRNA and cultured for 4 days. Total RNAs from the respective cells were then isolated and subjected to qRT-PCR to monitor mRNA expression of either Wnt5a or GAPDH. Relative amounts of Wnt5a transcripts were determined after normalization by those of GAPDH transcripts. *p < 0.01, t test. E, SaOS-2 cells were transfected with Ctrl #2, Wnt5a #1, or Wnt5a #2 siRNA and cultured for 4 days. Subsequently, SaOS-2 cells were transfected with the indicated MMP-13 gene promoter-luciferase (Luc) vectors along with the renilla luciferase reporter construct and were cultured for 24 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *p < 0.01, t test. F, SaOS-2 cells were transfected with the respective MMP-13 gene promoter-luc vectors for 12 h. The cells were further serum-starved for 12 h and then treated with either Wnt5a (400 ng/ml) or vehicle for 12 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *p < 0.01, t test.

siRNAs (Fig. 1D). SaOS-2 cells transfected with the respective siRNAs were further transfected with p3105-luc, p1004-luc, p253-luc, and basic-luc along with the renilla luciferase construct. Like Ror2 knockdown experiments (see Fig. 1B), reporter constructs that contain the promoter region spanning bp −253 to −1, but not basal-luc, show a drastic reduction of luciferase activities after suppressed expression of Wnt5a (Fig. 1E), indicating that the proximal promoter region (bp −253 to −1), containing AP1-binding sites, is required for MMP-13 gene expression by Wnt5a-Ror2 signaling. To further assess the importance of the proximal promoter region in MMP-13 gene expression by Wnt5a-Ror2 signaling, SaOS-2 cells were serum-starved in a prolonged culture to partly reduce the basal activity of Wnt5a-Ror2 signaling (8) and subjected to luciferase reporter assay after Wnt5a stimulation. As shown in Fig. 1F, Wnt5a stimulation resulted in a significant increase of luciferase activities on p1004-luc and p253-luc, but not basal-luc, further emphasizing that the proximal promoter region of MMP-13 gene is responsive to Wnt5a-Ror2 signaling in SaOS-2 cells.

Because we have previously shown that MMP-13 gene is also induced by Wnt5a-Ror2 signaling in another osteosarcoma cell line U2OS (8), we next examined whether the same proximal promoter region is required for MMP-13 gene expression by Wnt5a-Ror2 signaling. To this end U2OS cells transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA were cultured for 4 days, and expression levels of Ror2 protein and MMP-13 transcript were assessed by anti-Ror2 immunoblotting and qRT-PCR, respectively. As shown in Fig. 2A, transfection of U2OS cells with Ror2 siRNAs resulted in a drastic inhibition of expression of Ror2 protein and MMP-13 transcript, respectively. U2OS cells transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA were further...
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**A**

| siRNA       | Relative MMP-13 level |
|-------------|-----------------------|
| Ctrl #1     | 1.0                   |
| Ctrl #2     | 0.5                   |
| Ror2 #1     | 0.5                   |
| Ror2 #2     | 0.5                   |

![Graph showing relative MMP-13 level](image)

**B**

**C**

- OSE2
- AP1
- OSE2m
- AP1m

![Graph showing relative luciferase activity](image)

**D**

- p1004-luc
- p253-luc
- p253(OSE2m)-luc
- p253(AP1m)-luc
- basic-luc

![Graph showing relative luciferase activity](image)

**Figure 2.** Constitutively active Wnt5a-Ror2 signaling is critical for transcriptional activation of MMP-13 gene promoter via AP1-binding site in U2OS cells. A. U2OS cells were transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA. After 4 days in culture, total RNAs from the respective transfected cells were isolated and subjected to qRT-PCR to monitor mRNA expression of either MMP-13 or GAPDH. WCLs were also prepared and subjected to immunoblotting with the indicated antibodies. *, *p* < 0.01, t test. B. U2OS cells were transfected with Ctrl #1, Ror2 #1, or Ror2 #2 siRNA and cultured for 4 days. Subsequently, the cells were transfected with the indicated MMP-13 gene promoter-luc vectors along with the renilla luciferase reporter construct and cultured for 24 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *, *p* < 0.01, t test. C. U2OS cells were transfected with either Ctrl #1 or Ror2 siRNA and cultured for 4 days. Then cells were transfected with the indicated MMP-13 gene promoter-luc vectors, whose promoters contain mutations within the OSE2- and AP1-binding sites, respectively, along with the renilla luciferase reporter construct and cultured for 24 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *, *p* < 0.01, t test. D. U2OS cells were transfected with the indicated MMP-13 promoter-luc vectors along with the renilla luciferase reporter construct and cultured for 12 h. The cells were further serum-starved for 12 h and then stimulated with either Wnt5a (400 ng/ml) or vehicle for 12 h. Reporter assays were performed as described under “Experimental Procedures.” The data are representative of three independent experiments. The bars represent the mean ± SD of triplicate experiments. *, *p* < 0.01, t test.

Transfected with p1004-luc, p253-luc, and basic-luc along with the renilla luciferase reporter construct as described under “Experimental Procedures.” Similar to SaOS-2 cells, reporter constructs that contain the proximal promoter region (bp −253 to −1), but not basal-luc, show a drastic reduction of luciferase activities after suppressed expression of Ror2 (Fig. 2B). We next examined which element was responsible for MMP-13 gene expression by Ror2-mediated signaling. SaOS-2 cells transfected with the respective siRNAs were further transfected with p253-luc, p253(OSE2m)-luc, p253(AP1m)-luc, or basic-luc along with the renilla luciferase construct and cultured for 24 h. Transfection with p253(AP1m)-luc, but not p253(OSE2m)-luc, resulted in a drastic decrease of luciferase activities that were by Ror2-mediated signaling (Fig. 2C). U2OS cells were also serum-starved in a prolonged culture and were subjected to luciferase reporter assay. As expected, Wnt5a stimulation of U2OS cells also resulted in a significant increase of luciferase activities that could be remarkably inhibited by deletion of the proximal promoter region (bp −253 to −1) (Fig. 2D). Taken together, the results suggest, like SaOS-2 cells, the proximal promoter region of MMP-13 gene is responsive to Wnt5a-Ror2 signaling in U2OS cells.

**API Components c-Jun and ATF2 Are Recruited to MMP-13 Gene Promoter in Wnt5a-dependent Manner**—We next examined which members of the AP1-family of transcription factors are involved in MMP-13 gene expression by Wnt5a-Ror2 signaling via their binding to the proximal promoter region containing the AP1-binding site. To this end SaOS-2 cells transfected with Ctrl #2, Wnt5a #1, or Wnt5a #2 siRNA and cultured for 4 days were subjected to a ChIP assay using the indicated antibodies as described under “Experimental Procedures.” The positions of the respective primers used in ChIP assays are shown in Fig. 3A. c-Jun, c-Fos, and ATF2 were recruited to the proximal region (bp −212 to −27) containing the AP1-binding site but not to distal region (bp −3104 to −2919) within the MMP-13 gene in cells transfected with Ctrl #2 siRNA (Fig. 3B). Interestingly, recruitment of c-Fos, but not c-Jun and ATF2, to the proximal promoter region was detected after suppressed expression of Wnt5a (Fig. 3B), suggesting that c-Jun and ATF2 were recruited to the proximal promoter region of MMP-13 gene by Wnt5a-Ror2 signaling. Although c-Fos was recruited to the proximal promoter region of MMP-13 gene, its recruitment was marginally affected by Wnt5a-Ror2 signaling. Furthermore, phosphorylation of both c-Jun and ATF2 detected in
SaOS-2 cells was inhibited by suppressed expression of Wnt5a (Fig. 3C). These results suggest that constitutively active Wnt5a-Ror2 signaling in SaOS-2 cells can induce phosphorylation of c-Jun and ATF2, thereby promoting their binding to the proximal promoter region of MMP-13 gene presumably via AP1-binding site, leading to expression of MMP-13 gene.

We further tested the effect of Wnt5a stimulation on the recruitment of AP1 components to the MMP-13 gene promoter in SaOS-2 cells after their serum starvation. For this purpose, serum-starved SaOS-2 cells were stimulated with either Wnt5a or vehicle alone for 1 h, and WCLs were prepared and subjected to immunoblotting with anti-Dvl2 antibody. As shown in Fig. 3D, Wnt5a stimulation induced phosphorylation of Dvl2, as assessed by its electrophoretic mobility shift, in serum-starved SaOS-2 cells. Serum-starved SaOS-2 cells treated with either Wnt5a or vehicle alone were also subjected to ChIP analysis with the indicated antibodies as described under “Experimental Procedures.” After Wnt5a stimulation, c-Jun and ATF2, but not c-Fos, were recruited to the proximal promoter region of MMP-13 gene (Fig. 3E). On the other hand, after Wnt5a stimulation, ATF2, but not c-Jun and c-Fos, was recruited to the distal promoter region of MMP-13 gene (Fig. 3E); however, the role of its recruitment is unclear. We also observed phosphorylation of both c-Jun and ATF2 after Wnt5a stimulation of serum-starved SaOS-2 cells (Fig. 3F), further emphasizing the critical role of c-Jun and ATF2 in MMP-13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells.

JNK Is Critical for Expression of MMP-13 Gene by Wnt5a-Ror2 Signaling in SaOS-2 Cells—To further confirm the role of c-Jun and ATF2, in particular their phosphorylation, in MMP-13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells, we examined the effect of suppressed expression of Wnt5a and Ror2 on expression of MMP-13 and phosphorylation of c-Jun and ATF2 by qRT-PCR and immunoblotting with phospho-specific antibodies, respectively. Consistent with the results of luciferase reporter assay (see Fig. 1, D and E), suppressed expression of Wnt5a in SaOS-2 cells resulted in a drastic decrease of MMP-13 transcripts as assessed by qRT-PCR (Fig. 4A). Immunoblot analysis revealed that suppressed expression of Wnt5a also resulted in a drastic inhibition of phosphorylation of c-Jun and ATF2 without affecting the total amounts of c-Jun and ATF2, respectively (Fig. 4A). Similarly, suppressed expression of Ror2 resulted in a remarkable decrease of MMP-13 transcripts and in a remarkable inhibition of phosphorylation of both c-Jun and ATF2 (Fig. 4B). Correlation between the levels of MMP-13 expression and of phosphorylation of c-Jun and ATF2 suggests that phosphorylation of c-Jun and ATF2 might be required for MMP-13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells.

It has been shown that JNK is activated by Wnt5a-Ror2 signaling and that JNK is involved in phosphorylation of c-Jun and/or ATF2 (37). Thus, we examined a possible involvement of JNK in phosphorylation of c-Jun and/or ATF2 induced by Wnt5a-Ror2 signaling in SaOS-2 cells. To this end, SaOS-2 cells were treated with either SP600125 (an inhibitor of JNK, 20 μM)...
or vehicle alone for 10 h, and expression levels of MMP-13 and phosphorylation status of c-Jun and ATF2 were monitored by qRT-PCR and immunoblotting with phospho-specific antibodies, respectively. As shown in Fig. 4C, MMP-13 expression and phosphorylation of c-Jun and ATF2 in SaOS-2 cells were remarkably inhibited by SP600125, indicating that JNK is responsible for phosphorylation of c-Jun and ATF2 and for possible subsequent induction of MMP-13 gene by Wnt5a-Ror2 signaling. Consistent with the results, ChIP analysis revealed that treatment of SaOS-2 cells with SP600125 resulted in failure to recruit c-Jun and ATF2, but not c-Fos, to the proximal promoter region, but not to the distal region, within the MMP-13 gene promoter (supplemental Fig. S1). To further clarify the role of JNK in MMP-13 gene expression, SaOS-2 cells transfected with the respective MMP-13 gene promoter-luc vectors (p1004-luc, p253-luc, and basic-luc) were cultured for 12 h. Subsequently, the cells were serum-starved for 12 h and further treated with either SP600125 (20 μM) or vehicle for 12 h. As shown in supplemental Fig. S2, inhibition of JNK resulted in a significant decrease in relative luciferase activities in SaOS-2 cells. Taken together, these results indicate that JNK plays a critical role in the expression of MMP-13 gene by Wnt5a-Ror2 signaling via phosphorylation and subsequent recruitment of c-Jun and ATF2 to its proximal promoter region in SaOS-2 cells.

Dvl2, but Not Dvl3, Is Required for Expression of MMP-13 Gene via Phosphorylation of Both c-Jun and ATF2 in SaOS-2 Cells—Because we recently showed that Dvl2 and Rac1 are involved in Wnt5a-induced AP1-luc activation in L cells expressing Ror2 (35), we next addressed a question of whether Dvl proteins (Dvl2 and Dvl3) and Rac1 are also involved in MMP-13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells. To this end, SaOS-2 cells transfected with Ctrl #1, Dvl2 #1, Dvl2 #2, Dvl3 #1, or Dvl3 #2 siRNA were cultured for 4 days, and WCLs and total RNAs prepared from the respective transfectants were subjected to immunoblotting with anti-Dvl2 and Dvl3 antibodies and qRT-PCR, respectively. Interestingly, suppressed expression of Dvl2, but not Dvl3, resulted in a remarkable inhibition of MMP-13 gene expression in SaOS-2 cells (Fig. 5A). We also performed a similar experiment to examine whether Dvl2 is indeed required for phosphorylation of c-Jun and ATF2 presumably via JNK by Wnt5a-Ror2 signaling in SaOS-2 cells. As expected, suppressed expression of Dvl2, but not Dvl3, resulted in a significant inhibition of phosphorylation of both c-Jun and ATF2 (Fig. 5, B and C), indicating that Dvl2, but not Dvl3, plays a critical role in JNK-mediated phosphorylation of c-Jun and ATF2, leading to expression of MMP-13 gene in SaOS-2 cells. It has been shown that the Dvl2 mutant, Dvl2(KM), bearing an amino acid substitution (K446M) within the DEP domain (corresponding to K417M mutant of Drosophila Dsh that is defective in activating the Wnt/planar cell polarity pathway but functional for the β-catenin-dependent signaling pathway) fails to mediate the Wnt5a-induced AP1 activation in the Dvl2-knockdown L cells expressing Ror2 (35). Thus, we examined whether Dvl2(KM) can restore the failure of Dvl2-knockdown SaOS-2 cells to induce MMP-13 gene expression. As shown in Fig. 5D, siRNA-resistant Dvl2(WT), but not Dvl2(KM), could restore MMP-13 gene expression by Wnt5a-Ror2 signaling in the Dvl2-knockdown SaOS-2 cells, suggesting that Dvl2 function, mediating the Wnt/planar cell polarity pathway, is required for MMP-13 gene expression by Wnt5a-Ror2 signaling.

Because Dvl2 is co-localized with Rac1, a member of the Rho-family of small GTPases, after stimulation of serum-
starved SaOS-2 cells with Wnt5a (data not shown) (35), we next addressed the question of whether Rac1 is also involved in 
MMP-13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells. Suppressed expression of Rac1 resulted in significant inhibition of 
MMP-13 gene expression in SaOS-2 cells as assessed by qRT-PCR analysis (Fig. 5E). It should be noted that Dvl2 phosphorylation by Wnt5a-Ror2 signaling in SaOS-2 cells was unaffected by suppressed expression of 
Rac1 (Fig. 5E), suggesting that Rac1 might act downstream of Dvl2 in Wnt5a-Ror2 signaling. For this purpose SaOS-2 cells were serum-starved in the presence of either PP2 (an inhibitor of SFKs, 2 μM) or PP3 (a relevant control compound, 2 μM) for 12 h and then treated with either Wnt5a or vehicle alone for 1 h. WCLs and total RNAs from the respective transfected cells were subjected to immunoblotting with anti-Dvl2 antibody and qRT-PCR, respectively. As expected, treatment with PP2, but not PP3, significantly inhibited Wnt5a-induced expression of 
MMP-13 gene (supplemental Fig. S3). Importantly, Dvl2 phosphorylation induced by Wnt5a stimulation was abrogated by treatment with PP2, but not PP3, suggesting that a SFK might act upstream of Dvl2. Collectively, these results indicate that Dvl2, but not Dvl3, is required for 
MMP-13 gene expression in SaOS-2 cells and that a SFK-Dvl2-Rac1-JNK pathway might play an important role in MMP-
**Wnt5a-Ror2 Signaling in MMP-13 Expression**

13 gene expression by Wnt5a-Ror2 signaling in SaOS-2 cells.

Because, like SaOS-2 cells, the same proximal promoter region of MMP-13 gene is required for MMP-13 gene expression by Wnt5a-Ror2 signaling in another osteosarcoma cell line U2OS (see Fig. 2, A–D), we next examined possible roles of Dvl2, Dvl3, and Rac1 in MMP-13 gene expression by Wnt5a-Ror2 signaling in U2OS cells. To this end, U2OS transfectected with Ctrl #1, Dvl2 #1, Dvl3 #1, or Dvl3 #2 siRNA were cultured for 4 days, and WCLs and total RNAs from the respective transfected cells were subjected to immunoblotting with anti-Dvl2 and Dvl3 antibodies and qRT-PCR, respectively. Surprisingly, suppressed expression of Dvl3, but not Dvl2, resulted in a drastic inhibition of MMP-13 gene expression in U2OS cells (Fig. 6A), indicating that Dvl3, but not Dvl2, is required for MMP-13 gene expression by Wnt5a-Ror2 signaling in U2OS cells. In addition, unlike SaOS-2 cells, suppressed expression of Rac1 failed to affect MMP-13 gene expression by Wnt5a-Ror2 signaling in U2OS cells (Fig. 6B). Taken together, these results indicate that different signaling molecules (Dvl2 and Rac1 in SaOS-2 and Dvl3 in U2OS cells) are utilized in different osteosarcoma cell lines, i.e. SaOS-2 and U2OS cells, to mediate Wnt5a-Ror2 signaling, leading to expression of the same gene, MMP-13 gene.

**Wnt5a-Ror2 Signaling Is Involved in Endochondral Ossification of Cartilaginous Tissues**—We next addressed a question of whether Wnt5a-Ror2 signaling is also involved in physiological conditions. For this purpose the paraffin specimens, prepared from the cartilaginous tissues from both male mouse embryos at E15.5, were fixed and stained with antibodies against Ror2 and MMP-13 as described under “Experimental Procedures.” IHC analysis revealed that endochondral ossification was detected in cartilaginous tissues from male Ror2+/−, but not Ror2−/− mouse embryos at E15.5 (Fig. 7). Expression of MMP-13 was clearly detected at the ossification centers of the cartilaginous tissues from Ror2+/−, but not Ror2−/− mouse embryos (Fig. 7). Furthermore, expression of Ror2 was detectable at the ossification centers from Ror2+/−, but not Ror2−/− mouse embryos (Fig. 7). These results suggest that Wnt5a-Ror2 signaling might be required for expression of MMP-13 during the development of the cartilaginous tissues.

**DISCUSSION**

We have previously shown that both Wnt5a and Ror2 are expressed in osteosarcoma SaOS-2 cells at least partly by endogenous expression of Snail in the cells (15) and that as a result Wnt5a-Ror2 signaling is constitutively activated in SaOS-2 cells, leading to the induction of MMP-13, which plays an important role in their invasiveness via activation of a SFK (8). However, it remains largely unknown about the molecular mechanism underlying MMP-13 gene expression by Wnt5a-
Ror2 signaling. Here we show by using reporter assays that the proximal promoter region of *MMP-13* gene containing both the OSE2 and AP1-binding site is primarily required for expression of *MMP-13* gene in SaOS-2 cells (Fig. 1). In addition, mutagenesis within either the OSE2- or AP1-binding site revealed that the AP1-binding site, but not the OSE2-binding site, is critically required for expression of *MMP-13* gene by Wnt5a-Ror2 signaling in SaOS-2 cells (Fig. 1). As reported previously, *MMP-13* gene is also expressed by constitutively active Wnt5a-Ror2 signaling in another osteosarcoma cell line U2OS cells (8). Importantly, we also identified the AP1-binding site within the proximal promoter region of *MMP-13* gene as the critical responsive element to Wnt5a-Ror2 signaling in U2OS cells (8). Importantly, we also identified the AP1-binding site within the proximal promoter region of *MMP-13* gene as the critical responsive element to Wnt5a-Ror2 signaling in U2OS cells. Considering that both SaOS-2 and U2OS cells are osteoblast-like cells, one can assume that Wnt5a-Ror2 signaling is also involved in the development of the skeletal system. In fact, we show that endochondral ossification was clearly detected at the ossification centers of the cartilaginous tissues from *Ror2*+/-, but not *Ror2*-/-- mouse embryos at E15.5 (Fig. 7). Expression of Ror2 was detectable at the ossification centers at E15.5 (Fig. 7). The results suggest that Wnt5a-Ror2 signaling might play a role in endochondral ossification. It has been shown that MMP-13 is also involved in endochondral ossification via extracellular matrix degradation at the ossification centers (16, 42). In this study we show that expression of MMP-13 was clearly detected at the ossification centers of the cartilaginous tissues from *Ror2*+/-, but not *Ror2*-/-- mouse embryos at E15.5 (Fig. 7). Distribution of MMP-13 expression was overlapped if not identical with that of Ror2 expression in the cartilaginous tissues (Fig. 7). Based on these findings, it is conceivable that MMP-13 might be one of target genes of Wnt5a-Ror2 signaling under a physiological condition, i.e. this case the development of the cartilaginous tissues. It should be noted that Runx2, which is known to bind to the OSE2 within the proximal promoter region of *MMP-13* gene (43), also plays a crucial role in the development of the cartilaginous tissue (44). Therefore, it is of interest to examine how Wnt5a-Ror2 signaling and Runx2 function might be integrated to achieve the proper development of the cartilaginous tissues.

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