Orphan receptor tyrosine kinase ROR2 as a potential therapeutic target for osteosarcoma

Kazuhito Morioka,[1,2] Chizu Tanikawa,[1] Kensuke Ochi,[1] Yataro Daigo,[1] Toyomasa Katagiri,[1] Hirotaka Kawano,[2] Hiroshi Kawaguchi,[2] Akira Myoui,[3] Hideki Yoshikawa,[3] Norifumi Naka,[4] Nobuto Araki,[4] Ikuo Kudawara,[5] Makoto Ieguchi,[5] Kozo Nakamura,[2] Yusuke Nakamura[1] and Koichi Matsuda[1,6]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo; [2]Sensory and Motor System Medicine, Faculty of Medicine, University of Tokyo, Tokyo; Department of Orthopaedic Surgery, [3]Osaka University, Osaka, [4]Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; [5]Osaka National Hospital, Osaka, Japan

(Received December 16, 2008/Revised February 28, 2009/Accepted March 12, 2009/Online publication April 15, 2009)

Osteosarcoma is the most prevalent bone malignant tumor in children and adolescents, and displays heterogeneous histology and high propensity for distant metastasis. Although adjuvant chemotherapy remarkably improved treatment outcome over the past few decades, prognosis for osteosarcoma patients with pulmonary metastasis is still unsatisfactory. To identify novel therapeutic targets for osteosarcoma, we investigated the gene expression profile of osteosarcomas by cDNA microarray analysis and found transactivation of receptor tyrosine kinase-like orphan receptor 2 (*ROR2*) expression in the majority of osteosarcoma samples. Treatment of osteosarcoma cell lines with siRNA against *ROR2* significantly inhibited cell proliferation and migration. We also identified wingless-type MMTV integration site family, member 5B (*WNT5B*) as a putative ROR2 ligand and that the physiological interaction of WNT5B and ROR2 could enhance cell migration, indicating the possible roles of ROR2 and WNT5B in the metastatic property of osteosarcoma cells. Taken together, our findings revealed that the WNT5B/ROR2 signaling pathway is a promising therapeutic target for osteosarcoma. (*Cancer Sci* 2009; 100: 1227–1233)

Osteosarcoma is the most prevalent bone malignant tumor characterized by the direct formation of osteoid or bone by the tumor cells. Since osteosarcoma generally indicates very heterogeneous histological properties and a complex etiology, its underlying pathogenesis has not been well clarified thus far. Osteosarcoma prominently affects the extremities of children and adolescents, and indicates aggressive growth in the primary lesion and high propensity for lung metastasis. As a result, the long-term survival rate was as low as about 10–20% before the 1970s. During the 1980s, the introduction of neoadjuvant chemotherapy coupled with definitive surgery drastically improved the long-term survival rate up to approximately 60–70% for typical osteosarcoma patients.[1–3] Thus, osteosarcoma is no more a non-curable disease for patients with localized lesions. However, 10–20% of patients with metastases at the time of diagnosis still show a poor 5-year survival rate of less than 30%.[4,5] Since no effective treatment modalities have been developed for patients with pulmonary metastasis, the overall prognosis has remained relatively unchanged for the past two decades.[6,7] Moreover, systemic chemotherapy is often accompanied by damage to reproductive organs and increases the risk for second malignancies.[2] Therefore, development of novel therapeutics that could control metastatic tumors with minimal adverse effects is eagerly awaited.

Genes frequently and specifically up-regulated in human cancer tissues have been considered to be promising targets for drug development. For example, human EGFR-related 2 (HER2) in breast cancer cells is a valuable target for antibody-based therapy. Moreover, small molecular compounds that could inhibit the kinase activity of the tumor-specific Bcr–Abl protein have been used for treatment of chronic myelogenous leukemia. Many oncogenes are shown to be overexpressed in human osteosarcoma tissues, such as c-*myc*,[8] c-*ras*,[9] c-*fos*,[10] v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (erbB2),[11] beta-catenin,[12] and Mdm2 p53 binding protein homolog (MDM2).[13] Additionally, some prognosis factors or putative therapeutic targets have been reported so far,[14] such as reversion-inducing-cysteine-rich protein with kazal motifs (RECK),[15] urokinase plasminogen activator (uPA),[16] P-glycoprotein,[17] C-X-C chemokine receptor type 4 (CXCR4),[18] and Ezrin.[19,20] However, some of them were expressed in vital organs, therapeutics targeting these molecules might cause adverse effects.

To identify molecular targets applicable for development of anti-cancer treatment, we have analyzed more than 1000 cancer tissues using cDNA microarray and identified a number of therapeutic targets or cancer biomarkers.[21–26] Here we report the identification of receptor tyrosine kinase-like orphan receptor 2 (ROR2) as a novel therapeutic target for osteosarcoma through the gene expression profile analyses of 27 osteosarcoma tissues and 29 normal tissues. We also found wingless-type MMTV integration site family, member 5B (WNT5B) to be a putative ROR2 ligand in osteosarcoma cells. In this study, we demonstrate the novel role of the WNT5B/ROR2 signaling in the pathogenesis of osteosarcoma.

Materials and Methods

cDNA microarray. cDNA microarray analysis was performed as described previously.[21] Twenty-seven patients were diagnosed and treated by the Osaka University sarcoma group (Department of Orthopaedic Surgery, Osaka University School of Medicine; Department of Orthopaedic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases; and Department of Orthopaedic Surgery, Osaka National Hospital, Osaka, Japan). Biopsy samples prior to any treatment were snap-frozen in liquid nitrogen immediately after resection. All patients were diagnosed as conventional central osteosarcoma, in which >90% of cells in samples were considered to be sarcoma cells by histological examination.[21] We used human mesenchymal stem cells (hMSCs) as a control for competitive hybridization.[21–23] We purified total RNAs using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. After two cycles of T7-based amplification using the Ampliscribe T7-Transcription Kit (Epicentre Technologies, Madison, MI, USA), aliquots of aRNA from each sample were reverse-transcribed in the presence of Cy5-dCTP or Cy3-dCTP (Amersham Biosciences, Buckinghamshire, UK), and

[6]To whom correspondence should be addressed. E-mail: koichima@ims.u-tokyo.ac.jp.

doi: 10.1111/j.1349-7006.2009.01165.x

hybridized on microarray slides. All clinical samples were obtained with informed consent from patients undergoing biopsy prior to any treatment.

Cell lines. Cells from human osteosarcoma cell lines HuO 3 N1, HuO 9 N2, and MG-63 were purchased from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. Cells from human osteosarcoma cell lines Saos-2 and U-2 OS, HEK293 human embryonic kidney cells, NIH 3T3 mouse fibroblasts, and COS-7 monkey kidney cells were purchased from ATCC (Manassas, VA, USA). Cells from human osteosarcoma cell lines NOS-1 and 143B were purchased from RIKEN Gene Bank (Ibaraki, Japan). hMSCs were purchased from BioWhittaker (Walkersville, MD, USA). All cells were cultured under conditions recommended by their respective depositors.

Semi-quantitative and real-time quantitative RT-PCR. Total RNAs from primary osteosarcoma samples, normal tissues, and cultured cells were extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). mRNA from the heart, lung, liver, kidney, and bone marrow were purchased from Takara Clontech (Kyoto, Japan). RNA from adult bone tissues were prepared as described previously.[26] RNAs purified from osteosarcoma samples and normal tissues were amplified by T7-based RNA amplification methods and were reversely-transcribed using random hexamer and SuperScript II reverse transciptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative RT-PCR was performed with the ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). We prepared appropriate dilutions of each single-stranded cDNA by monitoring an amount of β2-microgrobulin (*B2 M*) as a quantitative control. The primer sets for semi-quantitative RT-PCR were as follows: forward, 5′-CCCGATTCCAACTCTGAAAG-3′ and reverse, 5′-CTTTAGCCACCGCACGTTAG-3′ (*ROR2*); and forward, 5′-CACCCCCACTGAAAAAGATGA-3′ and reverse, 5′-TACCTGTGGAGCAACCTGC-3′ (*B2 M*). The primer sets for quantitative real-time PCR were as follows: forward, 5′-CCCGAT TCCAACTCTGAAAG-3′ and reverse, 5′-CTTTAGCCACCGCAC GTTAG-3′ (*ROR2*); and forward, 5′-CACCCCCACTGAAAAAG ATGA-3′ and reverse, 5′-TACCTGTGGAGCAACCTGC-3′ (*B2 M*).

Northern blotting. Human multiple-tissue blots (Takara Clontech) were hybridized with α-^{32}P-dCTP-labeled *ROR2* cDNA (GeneBank accession no. NM_004560) according to the supplier's recommendations. The *ROR2* cDNA fragment was prepared by RT-PCR using the following primers: forward, 5′-ATGGGAGTTCCTGAGATA CAACA-3′ and reverse, 5′-CCAACGTGGGTAACATAAACAGT-3′. Blots were autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY, USA) at –80°C for 14 days.

Gene silencing effect by RNA interference. Plasmids designed to express siRNA were prepared by cloning double-stranded oligonucleotides into the Bbs I site of psiU6BX vectors. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: siROR2, 5′-AACAATATCACCATTGTCCAA-3′; siMock, 5′-ATGTCTTCCGATCGGAAGACCA-3′. U-2 OS or Saos-2 cells (0.5×10^6 per six-well dish) were transfected with 2.5 μg each of siRNA expression vectors using Lipofectamine 2000 (Invitrogen) or FuGENE6 transfection reagent (Roche, Mannheim, Germany) according to the suppliers' protocol. We selected siRNA-introduced cells with the medium containing 0.4 mg/mL of neomycin (Invitrogen). To evaluate the knockdown effect of siRNAs by semi-quantitative RT-PCR, total RNAs were extracted from the transfected cells at 4 days after neomycin treatment. Cell viability was measured by MTT assays using cell counting kit-8 (Dojindo, Kumamoto, Japan). siRNA-introduced cells were fixed with 4% paraformaldehyde and stained with giemsa solutions at 14 days after the treatment with neomycin. These experiments were performed in triplicate.

Plasmid constructions. Entire coding cDNA fragments of *ROR2* or *WNT5B* (GeneBank accession nos. NM_032642 and NM_030775) were amplified by RT-PCR and subcloned into the multi-cloning site of pcDNA3.1/myc-His A MCS (Invitrogen) (pcDNA3.1/myc-ROR2), pcDNA3.1 (Invitrogen) (pcDNA3.1-ROR2), or p3xFLAG-CMV10 expression vector (Promega, Madison, CA, USA) (pCMV/FLAG-WNT5B). pcDNA3.1/Fc-ROR2 expression plasmid was constructed as follows: a signal sequence from human Trypsinogen A (amino acids 1–20 of GenBank accession no. NM_002769) and a DNA fragment encoding the Fc region of human IgG1 (amino acids 100–329 of GenBank accession no. AJ294730) were inserted at the N-terminal of the extracellualr regions of ROR2 (amino acids 1–403). The primer sets for amplification were as follows: forward, 5′-AAAGGTACCGCCTGCGGCATGGCCCGGGGC-3′ and reverse, 5′-TTTCTCGAGTCAAGCTTCCAGCTGGACTTG-3′ (pcDNA3.1/myc-ROR2); forward, 5′-AAAGGTACCGCCTGCG GCATGGCCCGGGGC-3′ and reverse, 5′-TTTCTCGAGAGCTT CCAGCTGGACTTGGG-3′ (pcDNA3.1-ROR2); forward, 5′-AAA GAATTCGGCCGACCATGCCCAGCCTG-3′ and reverse, 5′-TT TCTCGAGTTTACAGATGTACTGGTCCAC-3′ (pCMV/FLAG-WNT5B); and forward, 5′- AAAAAGCTTGCCTGCGGCATGG CCCGGGGCTC-3′ and reverse, 5′-TTTGGTACCCCCCATCTTG CTGCTGTCTC-3′ (pcDNA3.1/Fc-ROR2).

Immunoprecipitation and western blotting. HEK293 cells were transiently transfected with myc-tagged ROR2 (pcDNA3.1/myc-ROR2), FLAG-tagged WNT5B (pCMV/FLAG-WNT5B), and/or Fc-ROR2 (pcDNA3.1/Fc-ROR2) and incubated for 48 h. Then cells were lysed in the lysis buffer (for immunoprecipitation) including 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 20 mM NaF, 2 mM Na_3VO_4 and Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA, USA) or the lysis buffer (for western blotting) including 0.5% TritonX-100, 150 mM NaCl, 10 mM HEPES pH 7.4, 20 mM NaF, 1 mM Na_3VO_4, and Protease Inhibitor Cocktail Set III. For immunoprecipitation analysis, the cell extracts were incubated with mouse anti-myc antibody, mouse anti-FLAG M2 antibody (Sigma, St Louis, MO, USA), or Protein G agarose (Zymed Lab., South San Francisco, CA, USA), respectively. Immunoprecipitated proteins were eluted with SDS-sample buffer after washing several times with the lysis buffer and were subjected to western blotting with either rabbit anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-FLAG antibody (Sigma), or goat anti-ROR2 antibody from (Abnova, Taipei City, Taiwan). β-Actin (clone AC-15, Sigma) served as a loading control.

Cell invasion assay. An invasion assay was performed using a Matrigel invasion chamber (BD Biocoat Cellware, San Jose, CA, USA) according to the manufacturer's instructions. NIH 3T3 or COS-7 cells that were transiently transfected with ROR2 expression plasmid were allowed to invade for 24 h, and then the invaded cells were fixed and counted. Forty-eight hours after transfection with siRNA oligonucleotide designed to inhibit ROR2 expression, U-2 OS or Saos-2 cells were also subjected to the invasion assay. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: siROR2, 5′-AACAATATCACCATTG TCCAA-3′; siLuci, 5′-GTGCGCTGCTGGTGCCAAC-3′; and siEGFP, 5′-GCAGCACGACTTCTTCAAG-3′. To evaluate the effect of WNT5B, the conditioned medium from WNT5B- or mock-transfected HEK293 cells was added to the lower chamber and cell migration was assessed. Invaded cells were counted using ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/). These experiments were performed in triplicate.

Statistical analysis. Statistical significance was determined by Student's *t*-test. Statistical significance is denoted by $P < 0.05$.

Results

Identification of ROR2 up-regulation in osteosarcoma. We have previously investigated the gene expression profiles of osteosarcoma samples using cDNA microarray analysis to identify the prognostic factors that could predict the response to neoadjuvant chemotherapy.[21] We have identified a number of therapeutic targets or

doi: 10.1111/j.1349-7006.2009.01165.x

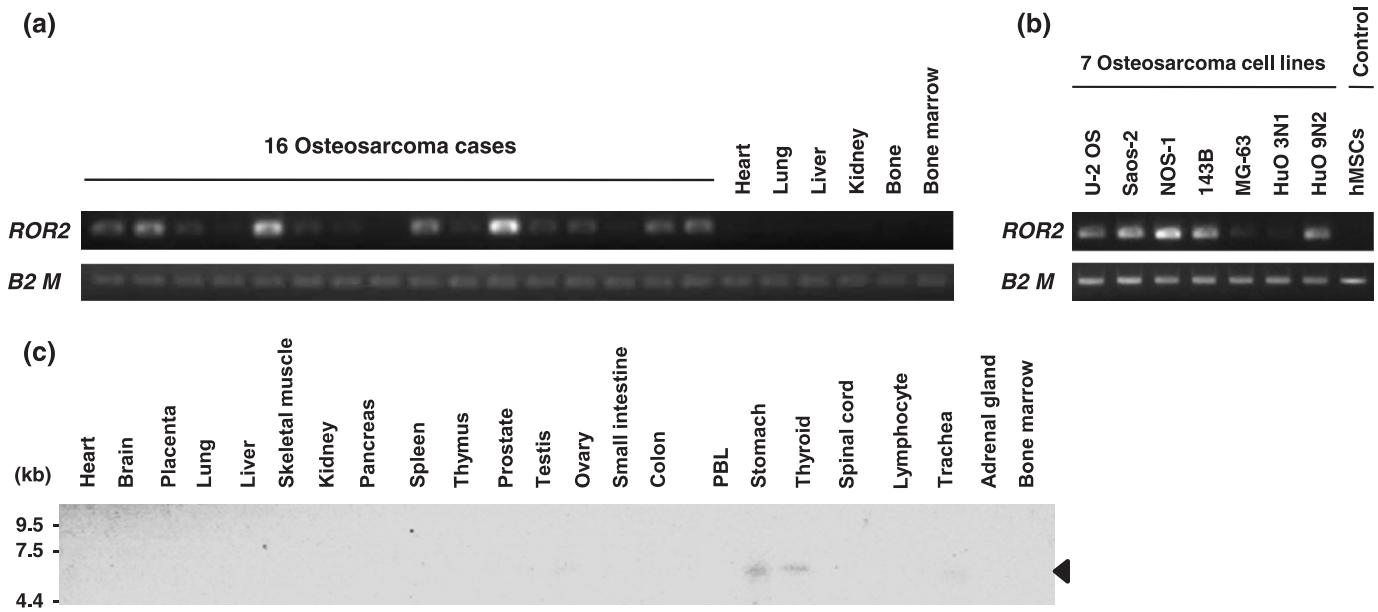

Fig. 1. Expression of *ROR2* in osteosarcoma and normal tissues. (a) Expression of *ROR2* in 16 osteosarcoma tissues and normal adult human tissues (heart, lung, liver, kidney, bone, bone marrow). Expression of *B2 M* served as a quantity control. (b) Expression of *ROR2* in seven osteosarcoma cell lines and human mesenchymal stem cells (hMSCs) examined by semi-quantitative RT-PCR. Expression of *B2 M* served as a quantity control. (c) Northern blot analysis of the *ROR2* transcript in 23 normal human tissues. *ROR2* was weakly expressed in thyroid and stomach. PBL, peripheral blood leukocytes. Arrowhead, *ROR2* transcript.

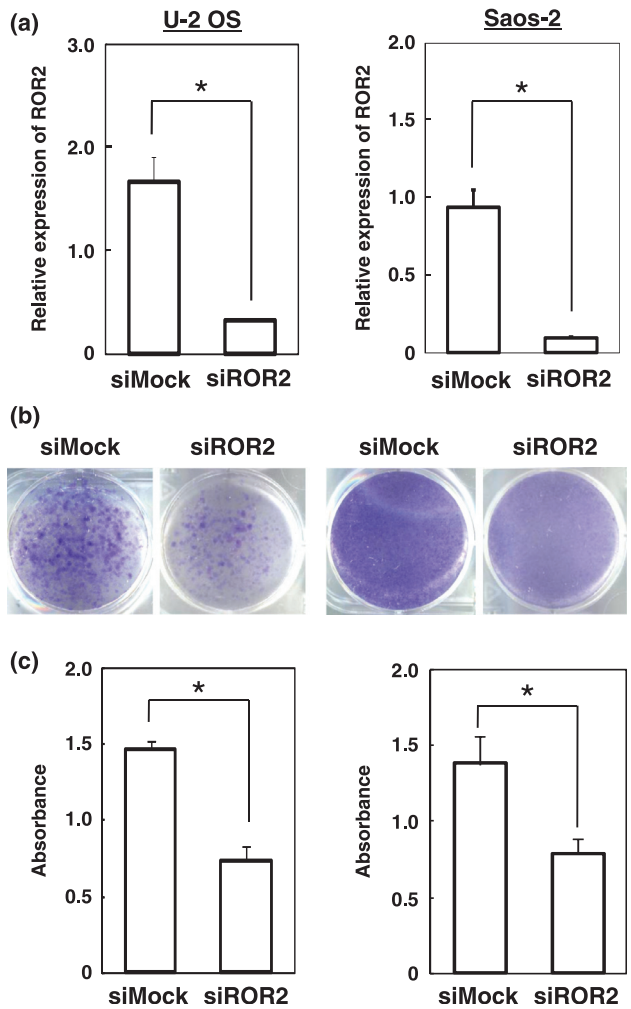

Fig. 2. Cell proliferation inhibitory effects on cells from the osteosarcoma cell lines U-2 OS and Saos-2 by ROR2 siRNA. (a) Reduction of ROR2 expression by siRNA-targeting *ROR2* (siROR2) was confirmed by quantitative real-time PCR. The non-specific siRNA (siMock) gene served as a negative control. Total RNAs were extracted from the transfected cells at 4 days after neomycin treatment. Expressions of ROR2 were normalized against that of *B2 M*. (b) Colony formation assays of U-2 OS or Saos-2 cells that were transfected with plasmids expressing siROR2 or siMock. Cells were stained at 14 days after the treatment with neomycin. (c) Viability of U-2 OS and Saos-2 cells evaluated by MTT assays in cells treated with siROR2 compared with cells treated with siMock. $*P < 0.01$ (unpaired *t*-test).

biomarkers for various cancers through the gene expression profile analysis of cancer tissues as well as normal tissues.[27] By using the same strategy, we identified *ROR2* as a putative therapeutic target that was upregulated in 56% (15 in 27 samples) of osteosarcoma tissues. We further evaluated the expression of *ROR2* by semi-quantitative RT-PCR in clinical osteosarcoma samples and seven osteosarcoma cell lines, and found its high level of expression in most of the samples, although no expression was observed in five vital organs (heart, lung, liver, kidney, and bone marrow) or bone tissue or hMSCs (Fig. 1a,b). Multi-tissue northern blot analysis of 23 normal adult human tissues also confirmed the absent expression of *ROR2* in vital organs, but its very weak expression was observed in the stomach and thyroid (Fig. 1c).

ROR2 involvement in tumor cell growth and invasion. To investigate the biological role of ROR2 up-regulation on tumor cell growth, we constructed a siRNA expression vector under the control of the U6 promoter (psiU6BX-siROR2). Transfection of psiU6BX-siROR2 into U-2 OS or Saos-2 cells remarkably reduced the amount of *ROR2* mRNA (Fig. 2a). Concordant with the knockdown effect by siROR2, MTT and colony formation assays revealed the decreased number of viable cells in both cell lines (Fig. 2b,c). These findings indicated that knockdown of *ROR2* expression had a suppressive effect on the proliferation of osteosarcoma cells.

Osteosarcoma cells generally indicate a high propensity for metastatic spreading to the lung. CAM-1, the *Caenorhabditis*

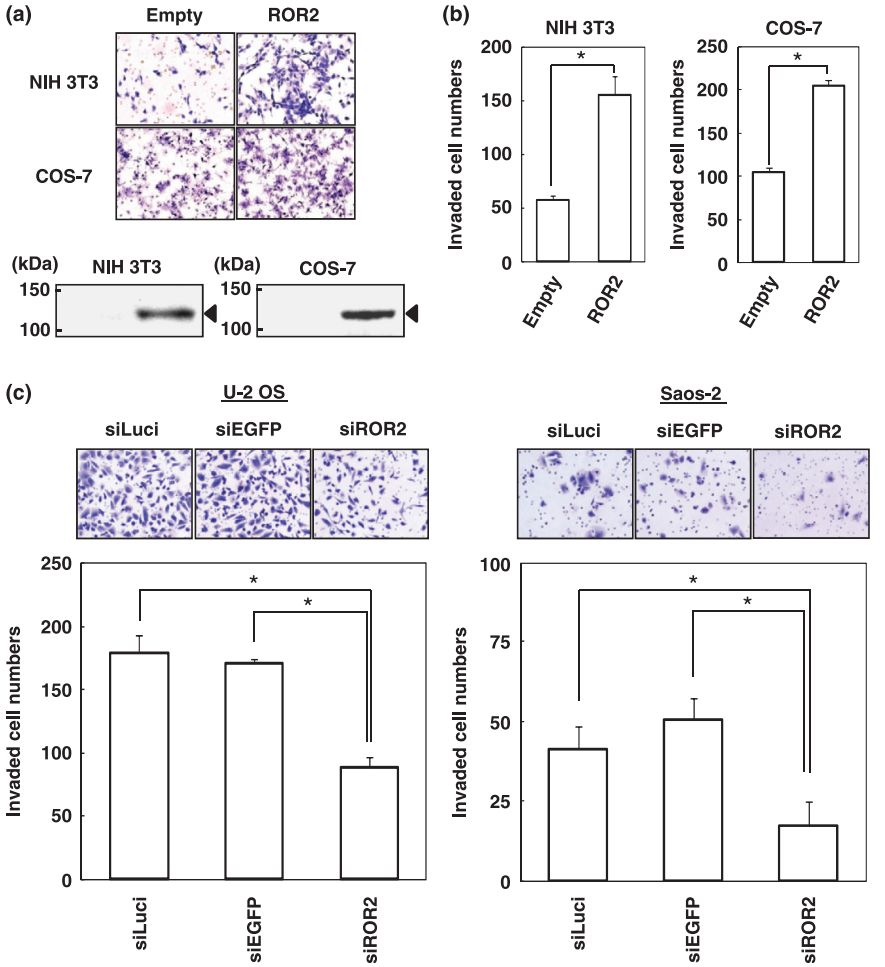

Fig. 3. Role of ROR2 on cell invasion. (a) NIH 3T3 or COS-7 cells were transiently transfected with ROR2 expression vector (pcDNA3.1-ROR2) or empty vector (pcDNA3.1). Representative image of invaded cells stained with Giemsa solution after 24 h incubation (upper). Western blot analysis of ROR2-expression with mouse anti-ROR2 monoclonal antibody (lower). (b) The numbers of invaded cells were counted by ImageJ. $*P < 0.01$ (unpaired *t*-test). (c) U-2 OS and Saos-2 cells were transfected with siRNA oligonucleotide against *ROR2* (siROR2). siRNA oligonucleotides against Luciferase (siLuci) or enchanced green fluorescent protein (siEGFP) were used as control siRNAs. Representative image of invaded cells that were stained with Giemsa solution after 24 h incubation (upper). The number of invaded cells was counted by ImageJ (lower). $*P < 0.01$ (unpaired *t*-test).

elegans ortholog of ROR2, was shown to regulate neuronal cell migration during embryonic development.[28] Therefore, we examined the role of ROR2 in cell migration. We transfected NIH 3T3 and COS-7 cells with plasmid designed to express ROR2 and performed invasion assays using Matrigel-coated filter. As shown in Fig. 3(a,b), the introduction of ROR2 enhanced the cell invasive activity of the two cell lines. We subsequently suppressed ROR2 expression in U-2 OS and Saos-2 cells, and found that the number of invaded cells was reduced to less than half by the knocking down of ROR2 (Fig. 3c,d). Though cell viabilities that were assessed by MTT assay were marginally affected after 24 h of siRNA treatment (about 90% of control cells, data not shown), the effects on cell migration were more obvious. These findings indicated that ROR2 might be involved in the cell proliferation and migration that are two major biological characteristics of osteosarcoma cells.

Identification of WNT5B as a putative ROR2 ligand in osteosarcoma cells. Xenopus Wnt proteins were shown to bind to the extracellular frizzled-like cysteine-rich domain of Xenopus Ror2,[29] and this domain was conserved in human ROR2. Since WNT family members are shown to play key roles in developmental morphogenesis, embryogenesis, and tumorigenesis,[30,31] we examined a possible interaction between ROR2 and WNT family members. We investigated the expressions of 19 *WNT* members in human osteosarcoma tissues and normal tissues by semi-quantitative RT-PCR analysis and found that eight of them were expressed in osteosarcoma tissues as indicated in Fig. 4(a). We noticed that *WNT5B* were up-regulated in the majority of osteosarcoma tissues. Moreover, the up-regulation pattern of *WNT5B* was very similar to that of ROR2 in the osteosarcoma tissues examined (Fig. 4a), indicating their possible interaction. We then performed a co-immunoprecipitation experiment by introducing myc-ROR2 and/or FLAG-WNT5B expression plasmids into HEK293 cells. Immunoprecipitation of the cell extracts and subsequent western blotting with anti-myc or anti-FLAG antibody clearly demonstrated the interaction of ROR2 and WNT5B (Fig. 4b). To determine the interacting domain of ROR2, we generated plasmid that the express extracellular domain of ROR2 fused with the Fc region of human IgG1. As shown in Fig. 4(c), Fc-ROR2 was co-immunoprecipitated with WNT5B. Thus our data clearly indicated that WNT5B could bind to the extracellular domain of ROR2.

Then we evaluated the effect of WNT5B on cell migration. COS-7 cells that were transiently transfected with ROR2 expression plasmid were seeded on Matrigel-coated filters with conditioned medium from WNT5B- or mock-transfected cells. As shown in Fig. 4(d), the number of invaded cells was significantly increased in ROR2 transfected cells and this effect was enhanced by the conditioned medium from WNT5B-transfected cells. These findings clearly implicated that WNT5B functions as a possible ROR2 ligand in osteosarcoma cells that could activate cell motility.

Discussion

We have previously reported a set of genes that could predict the clinical outcome of osteosarcoma patients after the treatment of neoadjuvant chemotherapy containing doxorubicin, *cis*-platinum, and ifosfamide by means of cDNA microarray analysis.[21] By analyzing the same dataset, we found ROR2 to be up-regulated in the majority of osteosarcomas and subsequent experiments described

doi: 10.1111/j.1349-7006.2009.01165.x

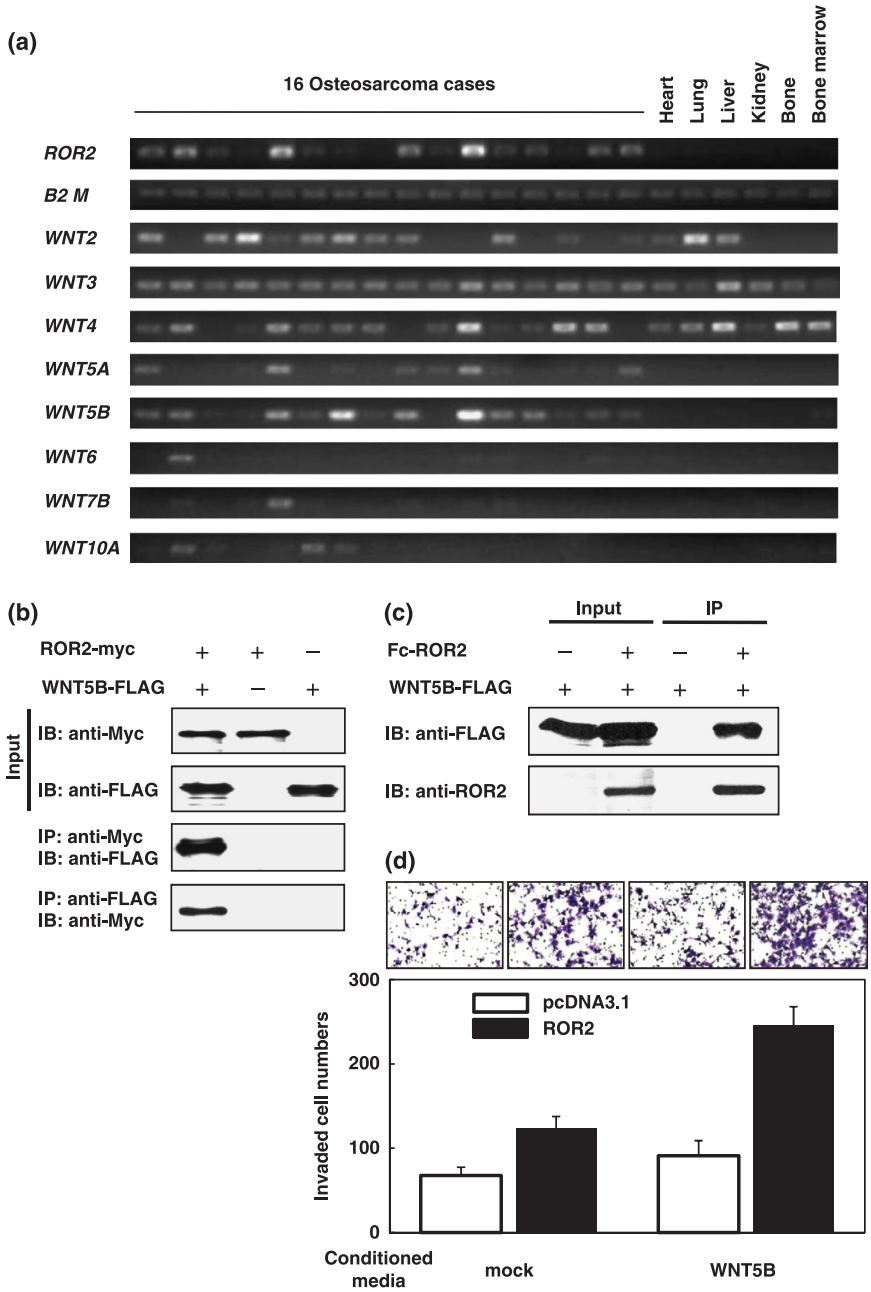

Fig. 4. Interaction between ROR2 and WNT5B. (a) Expression of *ROR2* and *WNT* family members in 16 osteosarcoma cases and normal adult human tissues (heart, lung, liver, kidney, bone, bone marrow). Results of eight *WNT* genes among 19 *WNT* family members that were expressed in osteosarcoma tissues. (b) Co-immunoprecipitation assay of ROR2 and WNT5B. Cell extracts from HEK293 cells cotransfected with myc-tagged ROR2 (pcDNA3.1/myc-ROR2) and/or FLAG-tagged WNT5B (pCMV/FLAG-WNT5B) were immunoprecipitated with anti-myc or anti-FLAG antibodies. Immunoprecipitates were immunoblotted with monoclonal anti-myc or anti-FLAG antibodies. (c) Co-immunoprecipitation assay with the extracellular domain of ROR2 and WNT5B. Cell extracts from HEK293 cells cotransfected with Fc-ROR2 (pcDNA3.1/Fc-ROR2) and/or FLAG-tagged WNT5B (pCMV/FLAG-WNT5B) were immunoprecipitated with protein-G agarose. Immunoprecipitates were immunoblotted with anti-FLAG antibody or anti-ROR2 antibody. (d) COS-7 cells that were transiently transfected with pcDNA3.1-ROR2 (closed bar) or pcDNA3.1 (open bar) were incubated with conditioned medium from WNT5B- or mock-transfected HEK293 cells. Representative images of invaded cells stained with Giemsa solution (upper panel). The numbers of invaded cells were counted by ImageJ (lower panel).

here indicated ROR2 and WNT5B (and also their interaction) to be novel therapeutic targets for osteosarcoma.

Osteosarcoma commonly arose in the metaphysis of long bones adjacent to the epiphysial growth plate that include a large number of hMSCs.[32–36] hMSCs have multi-differentiation potency for various tissues such as bone, cartilage, muscle, fat, and other connective tissues,[37] and osteosarcoma cells were considered to be generated from hMSCs. Thus we used hMSCs as a control for competitive hybridization. Using the same strategy, we analyzed the gene expression profiles of soft tissue sarcomas and successfully identified frizzled homolog 10 (FZD10) as a therapeutic target of synovial sarcoma.[22,23]

Receptor tyrosine kinase (RTK) families are crucial components of the cellular signaling apparatus that plays significant roles in developmental processes as well as oncogenic pathways by regulating cellular proliferation, differentiation, and motility.[38] Recently, RTK family members have been considered as good therapeutic targets for various cancers,[39,40] and pharmacological inhibitors of RTK, including c-Kit, epidermal growth factor receptor, insulin-like growth factor-I receptor, and vascular endothelial growth factor receptor, have been developed as potential anti-tumor agents.[39,40] In addition, their downstream signaling molecules, such as phosphoinositide 3-kinase (PI3 K), Akt, and mTOR have also been considered as candidates for molecular-targeting drugs. Since ROR2 belongs to the RTK superfamily with an intracellular tyrosine-kinase domain, we consider ROR2 to be a good target for drug development using a neutralizing antibody or a small molecule(s) that could inhibit the kinase activity.

The involvement of ROR2 in human carcinogenesis has not been reported previously, but homozygous mutations in the *ROR2* gene were shown to be responsible for Robinow syndrome (MIM:268310), which exhibits a severe skeletal dysplasia with generalized limb shortening, segmental defects of the spine, and a dysmorphic facial appearance.[41,42] Furthermore, heterozygous mutations in the *ROR2* gene were found in patients with brachydactly B1 (MIM:113000) characterized by terminal deficiency of

fingers and toes.[41,42] ROR2 was indicated its involvement in the bone morphogenetic protein (BMP)-mediated[43,44] and GDF5-mediated signaling pathways.[45] Thus, ROR2 is suggested to play important roles in the chondrogenesis of developing cartilage anlagen[46] as well as osteoblast differentiation.[43] Therefore, inhibition of ROR2 function is not likely to cause severe adverse effects, and we expected that ROR2 would be a promising target for drug development.

We also found that WNT5B was highly expressed in osteosarcoma tissues and that its expression seemed to be concordant with that of ROR2 in osteosarcoma tissues. Since the introduction of WNT5B into the ROR2-expressing cells significantly enhanced cell motility, we considered the WNT5B/ROR2 signaling to play a crucial role in the pathogenesis of osteosarcoma. On the other hand, WNT5B showed minor effect on cell proliferation (data not shown). Therefore ROR2 was expected to regulate cell proliferation through the WNT5B-independent pathway. WNT1 activates the canonical WNT-signaling pathway through ROR2;[47] however, WNT5A/ROR2 signaling inhibits the canonical WNT pathway.[48] Thus, ROR2 exerts diverse effects depending on the type of ligands. Considering that many of the WNT family members were increased in osteosarcoma tissues, the role of other WNTs in the development and progression of osteosarcoma should be analyzed in future studies.

Taken together, our findings suggesting that ROR2 and WNT5B may be promising therapeutic targets for osteosarcoma should contribute to further improving the overall prognosis of patients through the better management of lung metastasis.

Acknowledgments

We thank Dr T. Kidokoro, Dr C. Fukukawa, Dr T. Motoi (University of Tokyo), Dr T. Goto, and Dr T. Okuma (Tokyo Metropolitan Hospital) for helpful discussion and A. Takahashi for technical assistance. This work was supported partly by a grant from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 18687012 to K.M).

References

1 Rosen G, Caparros B, Huvos AG *et al*. Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. *Cancer* 1982; **49**: 1221–30.
2 Ferrari S, Palmerini E. Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma. *Curr Opin Oncol* 2007; **19**: 341–6.
3 Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A, Picci P. Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy: 15-year experience in 789 patients treated at a single institution. *Cancer* 2006; **106**: 1154–61.
4 Mankin HJ, Hornicek FJ, Rosenberg AE, Harmon DC, Gebhardt MC. Survival data for 648 patients with osteosarcoma treated at one institution. *Clin Orthop Relat Res* 2004; **429**: 286–91.
5 Daw NC, Billups CA, Rodriguez-Galindo C *et al*. Metastatic osteosarcoma. *Cancer* 2006; **106**: 403–12.
6 Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. *Expert Rev Anticancer Ther* 2006; **6**: 1075–85.
7 Gorlick R, Anderson P, Andrulis I *et al*. Biology of childhood osteogenic sarcoma and potential targets for therapeutic development: meeting summary. *Clin Cancer Res* 2003; **9**: 5442–53.
8 Bogenmann E, Moghadam H, DeClerck YA, Mock A. c-myc amplification and expression in newly established human osteosarcoma cell lines. *Cancer Res* 1987; **47**: 3808–14.
9 Nardeux PC, Daya-Grosjean L, Landin RM, Andeol Y, Suarez HG. A c-ras-Ki oncogene is activated, amplified and overexpressed in a human osteosarcoma cell line. *Biochem Biophys Res Commun* 1987; **146**: 395–402.
10 Wu JX, Carpenter PM, Gresens C *et al*. The proto-oncogene c-fos is over-expressed in the majority of human osteosarcomas. *Oncogene* 1990; **5**: 989–1000.
11 Onda M, Matsuda S, Higaki S *et al*. ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. *Cancer* 1996; **77**: 71–8.
12 Iwao K, Miyoshi Y, Nawa G, Yoshikawa H, Ochi T, Nakamura Y. Frequent beta-catenin abnormalities in bone and soft-tissue tumors. *Jpn J Cancer Res* 1999; **90**: 205–9.
13 Ladanyi M, Cha C, Lewis R *et al*. MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res* 1993; **53**: 16–8.
14 Clark JC, Dass CR, Choong PF. A review of clinical and molecular prognostic factors in osteosarcoma. *J Cancer Res Clin Oncol* 2008; **134**: 281–97.
15 Kang HG, Kim HS, Kim KJ. RECK expression in osteosarcoma: correlation with matrix metalloproteinases activation and tumor invasiveness. *J Orthop Res* 2007; **25**: 696–702.
16 Dass CR, Nadesapillai AP, Robin D *et al*. Downregulation of uPAR confirms link in growth and metastasis of osteosarcoma. *Clin Exp Metastasis* 2005; **22**: 643–52.
17 Ferrari S, Bertoni F, Zanella L *et al*. Evaluation of P-glycoprotein, HER-2/ErbB-2, p53, and Bcl-2 in primary tumor and metachronous lung metastases in patients with high-grade osteosarcoma. *Cancer* 2004; **100**: 1936–42.
18 von Luettichau I, Segerer S, Wechselberger A *et al.* A complex pattern of chemokine receptor expression is seen in osteosarcoma. *BMC Cancer* 2008; **8**: 23.
19 Khanna C, Wang X, Bose S *et al*. The membrane-cyto-skelton linker ezrin is necessary for osteosarcoma metastasis. *Nat Med* 2004; **10**: 182–6.
20 Park HR, Jung WW, Bacchini P, Bertoni F, Kim YW, Park YK. Ezrin in osteosarcoma: comaparison between conventional high-grade and central low-grade osteosarcoma. *Pathol Res Pract* 2006; **202**: 509–15.
21 Ochi K, Daigo Y, Katagiri T *et al*. Prediction of response to neoadjuvant chemotherapy for osteosarcoma by gene-expression profiles. *Int J Oncol* 2004; **24**: 647–55.
22 Nagayama S, Katagiri T, Tsunoda T *et al*. Genome-wide analysis gene expression in synovial sarcomas using a cDNA microarray. *Cacer Res* 2002; **62**: 5859–66.
23 Nagayama S, Fukukawa C, Katagiri T *et al*. Therapeutic potential of antibodies against FZD10, a cell-surface protein, for synovial sarcomas. *Oncogene* 2005; **24**: 6201–12.
24 Ono K, Tanaka T, Tsunoda T *et al*. Identification by cDNA microarray of genes involved in ovarian carcinogenesis. *Cancer Res* 2000; **60**: 5007–11.
25 Kidokoro T, Tanikawa C, Furukawa Y, Katagiri T, Nakamura Y, Matsuda K. CDC20, a potential cancer therapeutic target, is negatively regulated by p53. *Oncogene* 2008; **27**: 1562–71.
26 Tamura K, Furihata M, Tsunoda T *et al*. Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. *Cancer Res* 2007; **67**: 5117–25.
27 Saito-Hisaminato A, Katagiri T, Kakiuchi S *et al*. Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. *DNA Res* 2002; **9**: 35–45.
28 Forrester WC. The Ror receptor tyrosine kinase family. *Cell Mol Life Sci* 2002; **59**: 83–96.
29 Hikasa H, Shibata M, Hiratani I, Taira M. The xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. *Development* 2002; **129**: 5227–39.
30 Nusse R. The Wnt gene family in tumorgenesis and in normal development. *J Steroid Biochem Mol Biol* 1992; **43**: 9–12.
31 Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* 1998; **14**: 59–88.
32 Gibbs CP, Kukekov VG, Reith JD *et al*. Stem-like cells in bone sarcomas: Implications for tumorigenesis. *Neoplasia* 2005; **7**: 967–76.
33 Tataria M, Quarto N, Longaker MT, Sylvester KG. Absence of the p53 tumor suppressor gene promotes osteogenesis in mesenchymal stem cells. *J Pediatr Surg* 2006; **41**: 624–32.
34 Miura M, Miura Y, Padilla-Nash HM *et al*. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells* 2006; **24**: 1095–103.
35 Tolar J, Nauta AJ, Osborn MJ *et al*. Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 2007; **25**: 371–9.
36 Wu C, Wei Q, Utomo V *et al*. Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. *Cancer Res* 2007; **67**: 8216–22.
37 Pittenger MF, Mackay AM, Beck SC *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143–7.
38 Schlessinger J. Cell signaling by receptor tyrosine kinase. *Cell* 2000; **103**: 211–25.
39 Fabbro D, Ruetz S, Buchdunger E. Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharmacol Ther* 2002; **93**: 79–98.
40 Bianco R, Melisi D, Ciardiello F, Tortora G. Key cancer cell signal transduction pathways as therapeutic targets. *Eur J Cancer* 2006; **42**: 290–4.
41 Afzral AR, Jeffery S. One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinow syndrome and autosomal dominant brachdactyly type B. *Hum Mutat* 2003; **22**: 1–11.
42 Afzral AR, Rajab A, Fenske CD *et al*. Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. *Nat Genet* 2000; **25**: 419–22.
43 Liu Y, Bhat RA, Seestaller-Wehr LM *et al*. The orphan receptor tyrosine

doi: 10.1111/j.1349-7006.2009.01165.x

kinase Ror2 promotes osteoblast differentiation and enhances ex vivo bone formation. *Mol Endocrinol* 2007; **21**: 376–87.
44 Lehmann K, Seemann P, Silan F *et al*. A new subtype of brachydactyly type B caused by point mutations in the bone morphogenetic protein antagonist NOGGIN. *Am J Hum Genet* 2007; **81**: 388–96.
45 Sammar M, Stricker S, Schwabe GC *et al*. Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. *Genes Cells* 2004; **9**: 1227–38.
46 DeChiara TM, Kimble RB, Poueymirou WT *et al*. Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. *Nat Genet* 2000; **24**: 271–4.
47 Billiard J, Way DS, Seestaller-Wehr LM *et al*. The orphan receptor tyrosine kinase Ror2 modulates canonical Wnt signaling in osteoblastic cells. *Mol Endocrinol* 2005; **19**: 90–101.
48 Mikels AJ, Nusse R. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol* 2006; **4**: e115.