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DACH1 Negatively Regulates the Human RANK Ligand Gene Expression in Stromal/Preosteoblast Cells

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Abstract Receptor activator of NF-κB ligand (RANKL) is a critical osteoclastogenic factor that is expressed on bone marrow stromal/preosteoblast cells. Most bone resorption stimuli induce osteoclast formation by modulating RANKL expression in these cells. However, little is known about the mechanisms regulating RANKL gene expression. We recently reported that heat shock factor-2 (HSF-2) is a downstream target for FGF-2 signaling to enhance RANKL gene transcription in marrow stromal/preosteoblast cells. In this study, we show that DACH1 (human homologue of *Drosophila dachshund* gene) negatively regulates RANKL gene expression and suppresses FGF-2-enhanced RANKL gene expression in these cells. DACH1 contains a conserved dachshund domain (DS) in the N-terminal region, which interacts with the nuclear co-repressor (NCoR) to repress gene expression. Co-expression of DACH1 with hRANKL promoter-luciferase reporter plasmid in normal human bone marrow-derived stromal cells significantly decreased (3.3-fold) FGF-2-stimulated hRANKL gene promoter activity. Deletion of DS domain abolished DACH1 inhibition of FGF-2-enhanced RANKL gene promoter activity. Western blot analysis confirmed that DACH1 suppressed FGF-2-stimulated RANKL expression in marrow stromal/preosteoblast cells. We show HSF-2 co-immune precipitated with DACH1 and that FGF-2 stimulation significantly increased (2.7-fold) HSF-2 binding to DACH1. Confocal microscopy analysis further demonstrated that FGF-2 promotes HSF-2 nuclear transport and co-localization with DACH1 in marrow stromal cells. Co-expression of NCoR with DACH1 significantly decreased (5.3-fold) and siRNA suppression of NCoR in DACH1 co-transfected cells increased (3.6-fold) RANKL promoter activity. Furthermore, DACH1 co-expression with NCoR significantly decreased (7.5-fold) RANKL mRNA expression in marrow stromal cells. Collectively, these studies indicate that NCoR participates in DACH1 repression of RANKL gene expression in marrow stromal/preosteoblast cells. Thus, DACH1 plays an important role in negative regulation of RANKL gene expression in marrow stromal/preosteoblast cells in the bone microenvironment. J. Cell. Biochem. 103: 1747–1759, 2008.

Key words: RANK ligand; DACH1; FGF-2; HSF-2; NCoR

Tumor necrosis factor (TNF) family member, the receptor activator of NF-κB ligand (RANKL) is expressed on marrow stromal/osteoblast cells in response to several osteotropic factors and is critical for osteoclast precursor differentiation to form multinucleated osteoclasts, which resorb bone [Lacey et al., 1998; Hsu et al., 1999; Kong et al., 1999]. Enhanced levels of RANKL are associated with pathologic conditions such as Paget's disease of bone [Menaa et al., 2000; Neale et al., 2000] and multiple myeloma [Roux et al., 2002]. Several osteotropic factors such as 1,25-$(OH)_2D_3$, parathyroid hormone (PTH), interleukin 1β (IL-1β), interleukin-11, and prostaglandin E2 (PGE2) induce osteoclast differentiation through enhanced

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expression of RANKL in marrow stromal/osteoblast cells [Nakashima et al., 2000; Lee et al., 2002], but the molecular mechanisms which regulate RANKL gene expression are unclear. It has been reported that IL-1β and TNF-α stimulate RANKL expression in human bone marrow stromal cells through activation of p38 MAP kinase pathway [Rossa et al., 2006]. In addition, fibroblast growth factor-2 (FGF-2) has been shown to induce RANKL production through COX-2-mediated prostaglandin synthesis and by suppressing osteoclastogenesis inhibitory factor in mouse osteoblastic cells [Nakagawa et al., 1999]. Similarly, lipopolysaccharide treatment increased the levels of RANKL expression through activation of Toll-like receptors in primary murine osteoblast cells [Kikuchi et al., 2001]. Furthermore, transforming growth factor β (TGF-β) has been shown to increase RANKL expression in activated T-cells by increasing anti-CD3 [Wang et al., 2002]. Recently, it has also been reported that PTH stimulates RANKL expression through cAMP/protein kinase A/CREB cascade [Fu et al., 2006].

We recently demonstrated that heat shock factor-2 (HSF-2) is a downstream target of FGF-2 signaling to induce RANKL expression in bone marrow stromal/preosteoblast cells [Roccisana et al., 2004]. Heat shock proteins (HSP) are molecular chaperones expressed in cells in response to a variety of stimuli such as temperature and stimulation of membrane-bound receptors by hormones/cytokines and other chemical factors. Thus, HSP are an integral part of mammalian development [Christians et al., 2003]. Heat shock transcription factors (HSF), which binds to the heat shock responsive element (HSE), modulate expression of HSP and several other genes including TNF-α family [Mathew et al., 1998; Snoeckx et al., 2001]. Although multiple osteotropic factors including FGF-2 are known to modulate RANKL gene expression in the bone microenvironment, the transcriptional regulatory mechanisms operative in marrow stromal/preosteoblast cells are not well established.

DACH1 is a human homolog of the *Drosophila dachshund* gene, which is a key regulator for organ development and is considered a cell fate determination factor. DACH1 contains a conserved domain (dachshund domain (DS)) in the N-terminal region that is structurally homologous with the *Ski* and *Sno* proto-oncogenes and interacts with the nuclear co-repressor NCoR to modulate transcription factor activity. We have previously reported that DACH1 represses TGF-β signaling through binding to Smad4 [Wu et al., 2003]. Two vertebrate homologues, Dach1 and Dach2 are partially functionally redundant, since *Dach1*$^{-/-}$ mice survive to birth but exhibit postnatal lethality associated with a failure to suckle, cyanosis, and respiratory distress [Davis et al., 2001]. DACH1 expression is lost in a subset of human breast cancers associated with poor prognosis. Reintroduction of DACH1 inhibits breast tumor cell proliferation and tumor growth through suppression of cyclin D1 [Wu et al., 2006]. DACH1 inhibits TGF-β signaling in ovarian cancer cells [Sunde et al., 2006]. These studies are part of a growing body of evidence that DACH1 may function as a tumor suppressor. Recent evidence indicates that DACH1 is associated with FGF signaling during limb and skeletal development and may regulate cell proliferation or differentiation [Horner et al., 2002]. Dach1 binds with Runx2/Cbfa1 and co-localizes with cyclin-dependent kinase inhibitor p27 (Kip1) and p57 (Kip2) at the growth plate region in chondrocytes [Horner et al., 2002]. In this study, we show that DACH1 negatively regulate RANKL gene expression in human bone marrow-derived stromal/preosteoblast cells and that FGF-2 signaling promotes HSF-2 binding to DACH1 to modulate RANKL gene expression in these cells.

MATERIALS AND METHODS

Reagents and Antibodies

The cell culture and DNA transfection reagents were purchased from Invitrogen (Carlsbad, CA). FGF-2 and anti-human RANKL antibody were purchased from R&D systems, Inc. (Minneapolis, MN). Anti-goat-HSF-2, anti-goat NCoR, anti-rabbit-HA, siRNAs and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Super signal-enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Piscataway, NJ), and nitrocellulose membranes were purchased from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). HSF-2 cDNA was a generous gift

from Dr. Robert E. Kingston, Harvard Medical School, Boston.

hRANKL Promoter-Luciferase Reporter Gene Assay

Normal human bone marrow-derived SAKA-T stromal cells [Roccisana et al., 2004] were cultured in α-Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. DNA transfections were performed using Lipofectamine-Plus transfection reagent (Invitrogen) according to the manufacturer's protocol. We have previously developed hRANKL promoter (from +1 to −2 kb relative to the transcription start site as per Genbank Accession no. AF 333234)-luciferase reporter plasmid construct (hRANKL P#3) as described [Roccisana et al., 2004] and used for all the studies as indicated. The cells were transiently transfected with an appropriate combination of the hRANKL gene promoter-luciferase reporter plasmid with DACH1 and DS domain deletion mutant ΔDS expression vectors. The transfection efficiency was normalized by co-transfection with 0.2 μg of pRSV β-gal plasmid and measuring the β-galactosidase activity in the cells (Promega, Madison, WI). LacZ cytochemical activity staining (Invitrogen, Inc., San Diego, CA) indicated the DNA transfection efficiency of 80% and 70% in SAKA-T and primary human bone marrow-derived stromal/preosteoblast cells, respectively. The cells were cultured in the presence or absence of FGF-2 (4 ng/ml) for 48 h. The concentration of FGF-2 and treatment period were found to be optimal for FGF-2 signaling and RANKL promoter-luciferase reporter gene assays in vitro as reported previously [Nakano et al., 2004; Roccisana et al., 2004]. The cell monolayer was washed twice with phosphate buffered saline and incubated at room temperature for 15 min with 0.3 ml cell lysis reagent. The monolayer was scraped and spun briefly in a microfuge to pellet the debris. A 20 μl aliquot of each sample was mixed with 100 μl of the luciferase assay reagent. The light emission was measured for 10 s of integrated time using Sirius Luminometer following the manufacturer's instructions (Promega, Madison, WI).

Western Blot Analysis

SAKA-T-cells and a homogeneous population of normal human bone marrow-derived primary stromal/preosteoblast cells isolated, as described by Roccisana et al. [2004], were seeded (5×10^5 cells/well) in 6-well plates and supplemented with α-MEM containing 10% fetal calf serum. A day after seeding, cells were transfected with expression plasmids encoding DACH1 and the DS domain deletion mutant ΔDS [Wu et al., 2003, 2006] and stimulated with FGF-2 (4 ng/ml) for 48 h. The cells were lysed in a buffer containing 20 mM Tris-HCl at pH 7.4, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$ 10% glycerol, 150 mM NaCl, 0.1 mM Na_3VO_4 and 1× protease inhibitor cocktail. The protein content of the samples was measured using the BCA protein assay reagent (Pierce, Rockford, IL). Protein (20 μg) samples were then subjected to SDS–PAGE using 12% Tris-HCl gels and blot transferred on to a nitrocellulose membrane, immunoblotted with anti-RANKL, anti-NCoR antibody. The bands were detected using the enhanced chemiluminescence detection system. The band intensity was quantified by densitometric analysis using the NIH Image J Program.

Co-Immunoprecipitation Assay

Human bone marrow-derived stromal cells were transfected with expression vectors containing hemagglutinin (HA)-tagged DACH1 or ΔDS cDNA [Wu et al., 2003]. Transfected cells were treated with FGF-2 (4 ng/ml) for 48 h, and then lysed in a lysis buffer (50 mM HEPES (pH 7.5), 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, 0.5% NP-40). Lysates were immunoprecipitated using anti-HA antibody as described in the reference [Reddy et al., 1998]. Immunoprecipitants were subjected to SDS–PAGE and Western blot analysis was performed using anti-goat-HSF-2 antibody as described above.

Nuclear Co-Localization of DACH1 and HSF-2

SAKA-T human bone marrow-derived stromal cells were cultured (1×10^3/well) in a Lab-Tek 4 well chamber slides (Nunc International, Rochester, NY) and transfected with DACH1 expression plasmid. After 24 h transfection cells were cultured in the presence of FGF-2 (4 ng/ml) for 48 h and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked for 1 h with PBS containing 2% horse serum. Cells were immunostained with anti-rabbit-HA and anti-goat HSF-2 (10 μg/ml) antibodies in PBS

containing 2% horse serum and incubated for 3 h at room temperature. After extensive washing with PBS, cells were treated with Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-goat IgG in PBS containing 2% horse serum for 1 h at room temperature. Nuclear staining was performed with DRAQ5 and co-localization of DACH1 and HSF-2 was visualized by confocal microscopy (LSM 510; Carl Zeiss, Inc., Thornwood, NY).

siRNA Interference

Bone marrow stromal/preosteoblast cells were seeded (5×10^5 cells/well) in 6-well plates and supplemented with α-MEM containing 10% fetal calf serum. A day after seeding, cells were co-transfected with hRANKL promoter-luciferase reporter plasmid (2 μg) and DACH1, ΔDS in the presence or absence of double-stranded siRNA (10 μM) against NCoR and HSF-2 (Santa Cruz Biotechnology, Inc., CA) by Lipofectamine method (Invitrogen, Carlsbad, CA). The cells were cultured with or without FGF-2 (4 ng/ml) for 48 h period. Luciferase activity levels were measured in the total cell lysates and transfection efficiency was normalized by measuring β-galactosidase activity co-expressed in these cells. siRNA suppression of NCoR and HSF-2 expression was confirmed by Western blot analysis using anti-goat-NCoR and anti-goat HSF-2 antibodies.

Quantitative Real-Time RT-PCR

RANKL mRNA expression levels were determined by real-time reverse transcription-PCR as described previously [Sundaram et al., 2007]. Briefly, total RNA was isolated from normal human bone marrow-derived stromal/preosteoblast cells transfected with DACH1, ΔDS, and stimulated with and without FGF-2 (4 ng/ml) for 48 h, using Ultra Spec reagent (Biotecx labs, TX) according to the manufacturer's protocol. Reverse transcription reaction was performed using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystem) in 25 μl reaction volume containing total RNA (2 μg), 1× PCR buffer and 2 mM $MgCl_2$, at 42°C for 15 min followed by 95°C for 5 min. The quantitative real-time PCR was performed using IQ™ SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real Time-PCR detection system; Bio-Rad, Hercules, CA). The primer sequences used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5′ CCTACCCCCAATGTATCCGTTGTG-3′ (sense) and 5′-GGAGGAATGGGAGTTGCTGTTGAA-3′ (anti-sense) and for hRANKL mRNA were 5′-ACCAGCATCAAAATCCCAAG-3′ (sense) and 5′-TAAGGAGTTGGAGACCT-3′ (anti-sense). Thermal cycling parameters were 94°C for 3 min, followed by 40 cycles of amplifications at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min as the final elongation step. Relative levels of RANKL mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification.

Statistical Analysis

Results are presented as mean ± SD for three independent experiments and were compared by Student *t*-test. Results were considered significantly different for $P < 0.05$.

RESULTS

DACH1 Inhibit FGF-2-Stimulated hRANKL Expression

Recently, we have reported that FGF-2 enhances hRANKL gene expression through activation of HSF-2 in human bone marrow-derived SAKA-T stromal cell line as well as in human bone marrow-derived primary stromal/preosteoblast cells [Roccisana et al., 2004]. Evidence also indicates that DACH1 is a target gene of FGF signaling which may function as an intermediary in FGF modulation of cell proliferation and differentiation during limb skeletal development [Horner et al., 2002]. Therefore, in this study, we examine the role that DACH1 may play in FGF-stimulated hRANKL gene expression in stromal/preosteoblast cells. DACH1 contains a dachshund domain (DS) in the N-terminal region that interacts with nuclear co-repressor NCoR to modulate gene expression [Wu et al., 2003]. SAKA-T-cells were transiently transfected with HA-epitope-tagged DACH1 and a DS domain deletion mutant, ΔDS containing expression vectors. The cells were stimulated with FGF-2 (4 ng/ml) for 48 h and total cell lysates obtained were subjected to Western blot analysis. As shown in Figure 1A, RANKL expression was significantly increased (3-fold) in FGF-2-stimulated cells. Interestingly, DACH1 expression resulted in suppression (4.2-fold) of RANKL expression in FGF-2-stimulated SAKA-T stromal cells compared to mock-transfected cells. In contrast, there was

no significant change in the level of RANKL expression in ΔDS-transfected cells. FGF-2 stimulation did not significantly affect the level of DACH1 expression in these cells. Also, DACH1 did not affect the basal level expression of RANKL in these cells (data not shown). The expression of DACH1 and ΔDS protein in SAKA-T-cells was confirmed by Western blot analysis by anti-HA tag antibody (Fig. 1B).

We previously characterized the functional role for HSF-2 in the transcriptional regulation of hRANKL gene promoter activity in FGF-2-stimulated normal human bone marrow-derived stromal cell line (SAKA-T) and homogeneous population of primary human bone marrow-derived stromal/preosteoblast cells [Roccisana et al., 2004]. We further examined the role of DACH1 in transcriptional regulation of hRANKL gene promoter activation in SAKA-T stromal cells. hRANKL promoter-luciferase reporter plasmid was transiently co-transfected with DACH1 and ΔDS into SAKA-T-cells using Lipofectamine method. The cells were cultured in the presence or absence of FGF-2 (4 ng/ml) for 48 h. Total cell lysates obtained from these cells were analyzed for luciferase activity as described in the section "Materials and Methods". As shown in Figure 2, FGF-2 stimulation resulted in significant increase (4-fold) in hRANKL gene promoter activity compared to unstimulated cells as reported earlier. However, co-expression of DACH1 significantly decreased (3.3-fold) FGF-2-stimulated hRANKL promoter activity in these cells. In contrast, there was no significant change in RANKL promoter activity in ΔDS-transfected cells compared to control cells in response to FGF-2 stimulation. Transfection efficiency was normalized by co-expression of pRSV β-galactosidase plasmid and measuring the β-galactosidase activity in these cells. Taken together, these results suggest that DACH1 negatively regulates FGF-2-stimulated RANKL gene expression and that DS domain of the DACH1 is essential for transcriptional regulation of hRANKL gene promoter activity in marrow stromal/preosteoblast cells.

DACH1 Interaction With HSF-2 Suppress RANKL Gene Expression

HSF-2 is a downstream target molecule for FGF-2 signaling to enhance RANKL expression in marrow stromal/preosteoblast cells. FGF-2 promotes nuclear translocation of HSF-2 which binds to HSE present in the RANKL gene promoter to modulate RANKL gene expression in these cells [Roccisana et al., 2004]. To

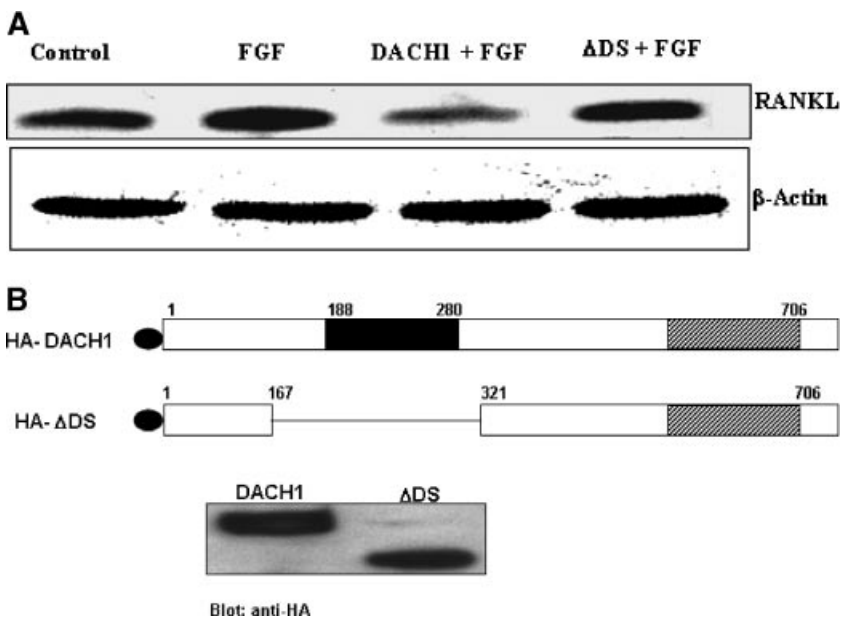

Fig. 1. DACH1 overexpression suppresses FGF-2-induced RANKL expression in SAKA-T-cells. **A**: The cells were transfected with expression plasmids containing HA-DACH1 and ΔDS. Transfected cells were treated with and without FGF-2 (4 ng/ml) for 48 h. Cell lysates were subjected to Western blot analysis using anti-RANKL antibody. **B**: Western blot analysis of DACH1 and DS domain deletion mutant (ΔDS) expression in SAKA-T-cells. The cells were transfected with HA-DACH1 and ΔDS. Total cell lysates obtained after 48 h and subjected to Western blot analysis using anti-HA antibody.

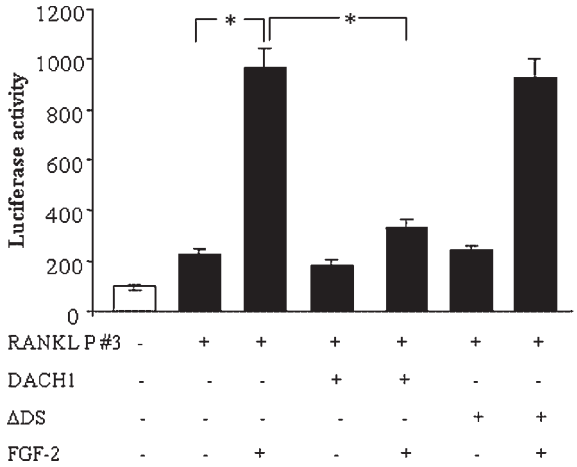

Fig. 2. DACH1 inhibition of FGF-2-stimulated hRANKL gene promoter activity. SAKA-T-stromal cells transfected with hRANKL promoter-luciferase reporter plasmid (hRANKL P#3) were co-expressed with DACH1 and ΔDS. The cells were stimulated with and without FGF-2 (4 ng/ml) for 48 h. Total cell lysates prepared were assayed for luciferase activity. The transfection efficiency was normalized by β-galactosidase activity co-expressed in these cells.

determine if DACH1 regulates RANKL expression through interaction with HSF-2, SAKA-T stromal cells were co-transfected with hRANKL promoter-luciferase reporter plasmid with HSF-2, DACH1, and ΔDS expression vectors and transfected with HSF-2 siRNA. The cells were stimulated with and without FGF-2 (4 ng/ml) for 48 h and total cell lysates obtained were analyzed for luciferase activity. As shown in Figure 3A, the RANKL promoter activity was (4.3-fold) increased in HSF-2-transfected SAKA-T-cells. In contrast, siRNA suppression of HSF-2 resulted in significant decrease in RANKL promoter activity compared to control cells stimulated with FGF-2. Recently, we showed that FGF-2 treatment to HSF-2 null mice bone marrow-derived stromal cells fail to induce RANKL expression [Kajiya et al., 2006]. Therefore, we further determined if DACH1 over-expression in HSF-2 deficient human bone marrow-derived stromal/preosteoblast cells affect RANKL gene transcription. As shown in Figure 3A, DACH1 over-expression in human bone marrow-derived stromal cells transfected with siRNA against HSF-2 did not significantly affect RANKL gene expression in these cells. In contrast, there was no change in the RANKL promoter activity in SAKA-T-cells co-expressed with ΔDS and HSF-2 deficient stromal cells. Also, FGF-2 treatment to cells transfected with or without control siRNA did not show significance difference in luciferase activity. Western blot analysis further confirmed that siRNA suppression of HSF-2 resulted in significant decrease (4.3-fold) in RANKL expression in human bone marrow-derived stromal/preosteoblast cells (Fig. 3B). These results suggested a role for HSF-2 in the DACH1 inhibition of RANKL expression.

We further confirmed the binding of DACH1 with HSF-2 by co-immuneprecipitation assay. Normal human bone marrow-derived stromal/preosteoblast cells were transfected with HA-DACH1 and HA-ΔDS expression plasmids. These cells were stimulated with FGF-2 for 48 h. The total cell lysates obtained were subjected to immunoprecipitation of DACH1 and ΔDS using anti-HA agarose beads and the co-immune precipitation of HSF-2 were identified by Western blot analysis using anti-HSF-2 antibody. As shown in Figure 4, FGF-2 enhanced binding of HSF-2 with both DACH1 (2.7-fold) and ΔDS (1.4-fold). These results suggest that the DS domain present at the N-terminus of DACH1 is not involved in its interaction with HSF-2.

The co-immunoprecipitation study suggested that DACH1 binds with HSF-2 in response to FGF-2. In naive cells, cellular localization of DACH1 and HSF-2 was confined to the nucleus and cytoplasm, respectively [Roccisana et al., 2004; Wu et al., 2006]. Therefore, we further confirmed DACH1 nuclear co-localization with HSF-2 in response to FGF-2 stimulation by confocal microscopy. SAKA-T-cells were transfected with an expression vector encoding HA-DACH1 and stimulated with and without FGF-2 (4 ng/ml). After, 48 h incubation, the cells were fixed and stained with anti-HA and anti-HSF-2 antibody. Confocal microscopy demonstrated nuclear co-localization of DACH1 and HSF-2 upon FGF-2 stimulation (Fig. 5). In contrast, HSF-2 expression is confined to cytosol in untreated control cells as we have reported earlier [Roccisana et al., 2004]. These results suggest that FGF-2 promotes nuclear translocation of HSF-2 and co-localization with DACH1 to modulate the RANKL gene expression.

NCoR Is Essential for DACH1 Inhibition of FGF-2-Induced RANKL Gene Expression

We previously demonstrated that DS domain of DACH1 interacts with the nuclear co-repressor NCoR and suppresses TGF-β signal-

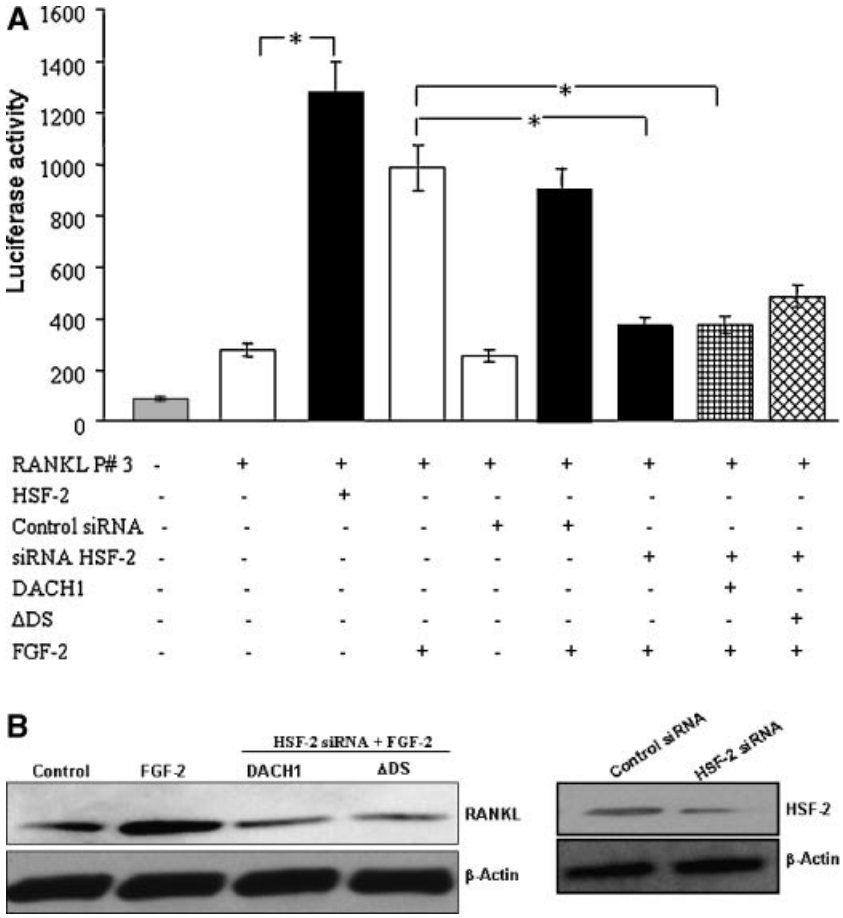

Fig. 3. DACH1 over-expression inhibits HSF-2-enhanced RANKL expression. **A**: SAKA-T-cells were transiently co-transfected with hRANKL promoter luciferase reporter plasmid (hRANKL P#3), DACH1, ΔDS expression plasmids, and HSF2-siRNA by lipofectamine method and cultured in the presence or absence of FGF-2 (4 ng/ml). Total cell lysates prepared after 48 h period were assayed for luciferase activity. Transfection efficiency was normalized by measuring β-galactosidase activity co-expressed in these cells. **B**: The cells were transfected with HSF2 siRNA and over-expressed with DACH1 and ΔDS. The cells were stimulated with FGF-2 (4 ng/ml) and total cell lysates obtained after 48 h were subjected to Western blot analysis as described in the section ''Materials and Methods''.

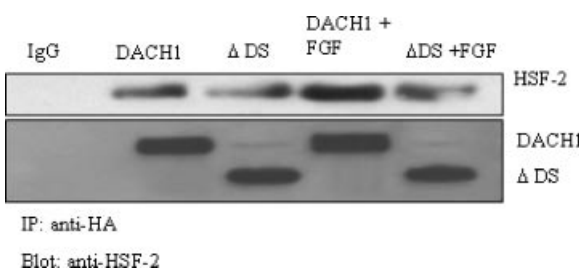

Fig. 4. Co-immunoprecipitation of HSF-2 with DACH1. Normal human bone marrow-derived primary stromal/preosteoblast cells were transfected with HA-DACH1 and ΔDS expression plasmids. The cells were stimulated with and without FGF-2 (4 ng/ml) for 48 h and cell lysates were immunoprecipitated using an anti-HA antibody. Immunoprecipitants were subjected to Western blot analysis using anti-HSF-2 antibody. Protein content was normalized with respect to total levels of DACH1 and ΔDS expression in these cells.

ing through binding to Smad4 [Wu et al., 2003]. To examine a potential role for NCoR in DACH1-mediated inhibition of FGF-2 signaling, the hRANKL gene promoter was used. SAKA-T stromal cells were co-transfected with hRANKL promoter-luciferase reporter plasmid and expression vectors for DACH1 and ΔDS in the presence or absence of NCoR siRNA. siRNA suppression of NCoR expression was further confirmed by Western blot analysis in these cells. As shown in Figure 6, siRNA suppression of NCoR expression abolished DACH1 inhibition of RANKL expression in FGF-2-stimulated cells. The levels of RANKL promoter activity were increased in ΔDS-transfected cells with and without NCoR siRNA. Also, FGF-2 treatment to cells transfected with or without control

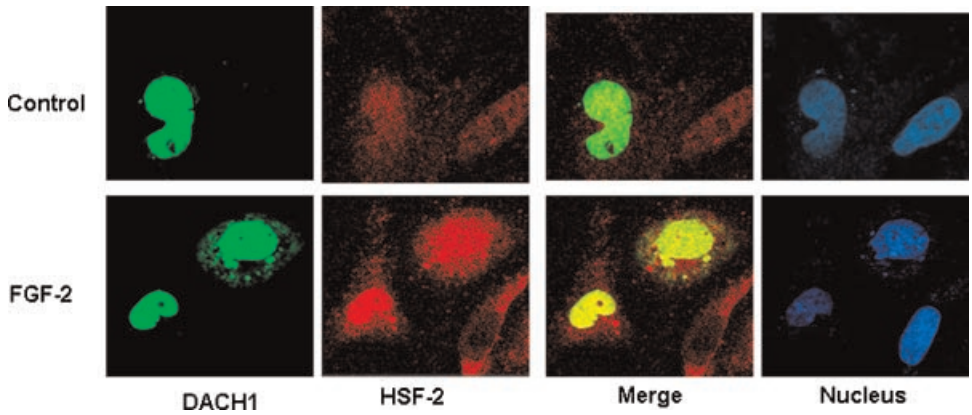

Fig. 5. Confocal microscopy analysis of DACH1 and HSF-2 nuclear co-localization. SAKA-T-cells were transfected with expression plasmid HA-DACH1 and treated with FGF-2 (4 ng/ml) for 48 h and untreated control cells were subjected to confocal microscopy analysis. Immunostaining for DACH1 and HSF-2 is shown detected by Alexa 488 conjugated anti-rabbit IgG and Alexa 568 conjugated anti-goat IgG and nuclear staining by DRAQ5. The merged image demonstrates co-localization of DACH1 and HSF-2 in FGF-2-stimulated cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

siRNA did not show significant difference in RANKL promoter activity.

We further determined the effect of NCoR expression on DACH1 suppression of RANKL expression. SAKA-T stromal cells were co-transfected with a hRANKL promoter-luciferase reporter plasmid and different concentrations (0.5–2 μg) of expression vectors for DACH1 and ΔDS together with a FLAG epitope-tagged NCoR expression plasmid. The cells were stimulated with FGF-2 for 48 h and total cell lysates were assayed for RANKL gene promoter activity as described. As shown in Figure 7, DACH1 and NCoR co-expression decreased (5.3-fold) RANKL promoter activity in FGF-2-stimulated cells. In contrast, NCoR

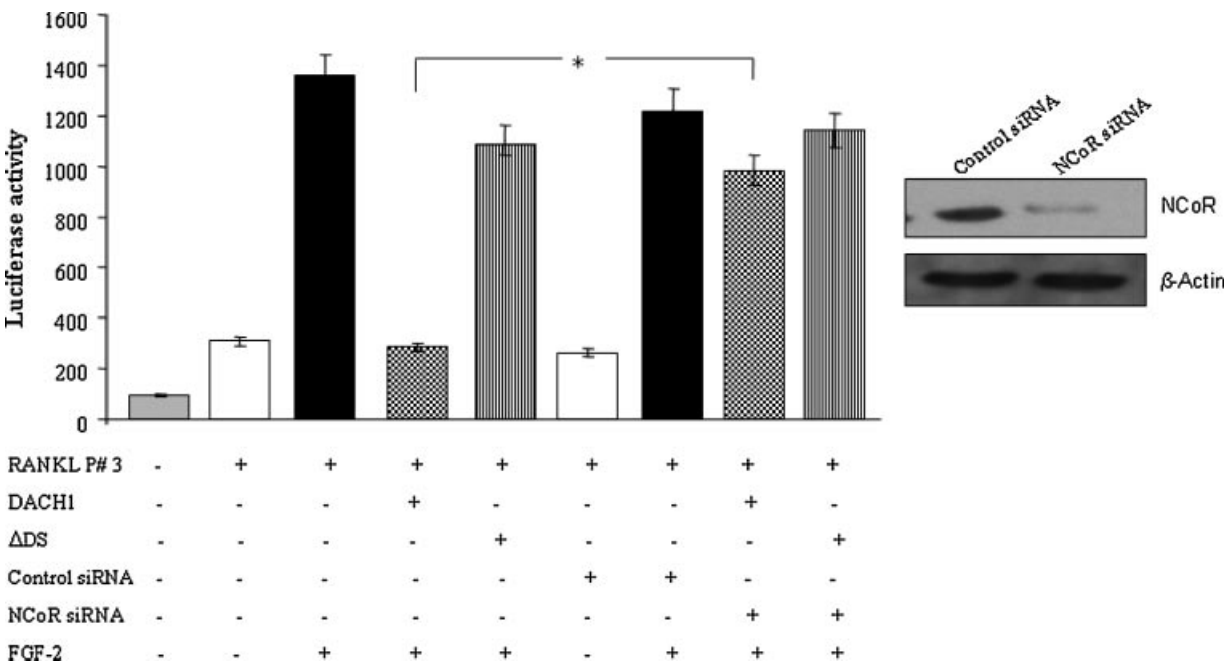

Fig. 6. DACH1 interacts with NCoR to suppress RANKL promoter activity. SAKA-T-cells were co-transfected with hRANKL promoter luciferase reporter plasmid (hRANKL P#3) and DACH1, ΔDS in the presence or absence of NCoR siRNA. The cells were stimulated with FGF-2 (4 ng/ml) for 48 h and total cell lysates prepared were assayed for luciferase activity as described in ''Materials and Methods''. The transfection efficiency was normalized by β-galactosidase activity co-expressed in these cells.

expression with ΔDS did not affect RANKL gene promoter activity. These results indicate that NCoR is an essential co-repressor for DACH1 inhibition of RANKL gene expression in bone marrow stromal/preosteoblast cells.

We next determined the effect of NCoR on DACH1 inhibition of hRANKL mRNA expression by real-time PCR. SAKA-T-cells were transfected with DACH1 and ΔDS expression vectors at different concentrations (0.5–2 μg) in the presence or absence of NCoR (0.5–2 μg) expression plasmid and stimulated with FGF-2 for 48 h. Total RNA isolated from these cells was subjected to real-time PCR analysis of RANKL mRNA expression. RANKL mRNA expression was significantly increased (6-fold) in FGF-2-stimulated cells (Fig. 8). Furthermore, DACH1 co-expression with NCoR significantly decreased (7.5-fold) RANKL mRNA expression in these cells. However, ΔDS alone or co-expression with NCoR did not affect the FGF-2-enhanced levels of RANKL mRNA expression in these cells. Taken together, our results suggest that DACH1 negatively regulates, and that NCoR is an essential co-repressor of FGF-2 signaling to modulate RANKL gene expression in marrow stromal/preosteoblast cells.

DISCUSSION

High level RANKL expression in the bone microenvironment plays an important role in osteoclastogenesis and high bone turnover. Pathological conditions such as Paget's disease of bone demonstrated increased levels of FGF-2 expression in osteoclasts compared with normal bone [Mills and Frausto, 1997]. However, FGF-2 has been shown to affect multiple stages during osteoblast differentiation. FGF-2 promotes human bone marrow stromal cell proliferation and maintenance of osteogenic precursors [Martin et al., 1997]. Disruption of FGF-2 gene decreased bone mass and bone formation in mice [Montero et al., 2000]. Although multiple osteotropic factors including b-FGF to modulate RANKL expression, little is known about the control of the human RANKL gene expression in stromal/preosteoblast cells. Immature osteoblasts are the major responders to RANKL inducing cytokines, suggesting that the relative proportions of immature and mature osteoblasts in the local microenvironment may control the degree of bone resorption at specific site [Thomas et al., 2001]. HSF-2 is a downstream target for FGF-2 enhancing

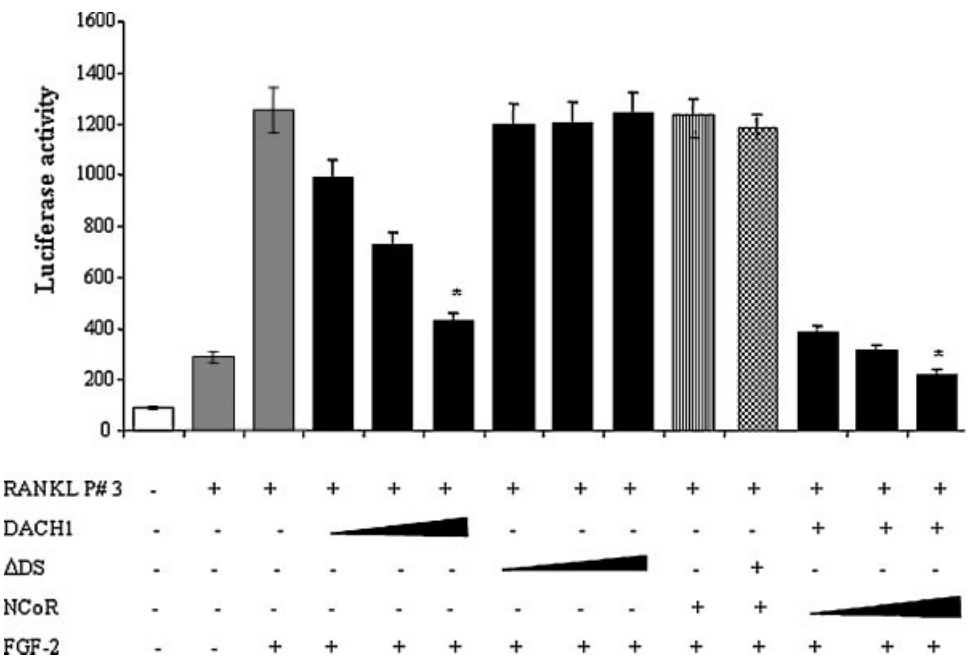

Fig. 7. DACH1 DS domain plays an essential role in NCoR interaction and suppression of RANKL promoter activity. SAKA-T-cells were co-transfected with hRANKL promoter-luciferase reporter plasmid (hRANKL P#3) with different concentrations (0.5–2 μg) of DACH1, ΔDS, and NCoR expression constructs. The cells were stimulated with FGF-2 (4 ng/ml) for 48 h and total cell lysates prepared were assayed for luciferase activity. The transfection efficiency was normalized by β-galactosidase activity co-expressed in these cells.

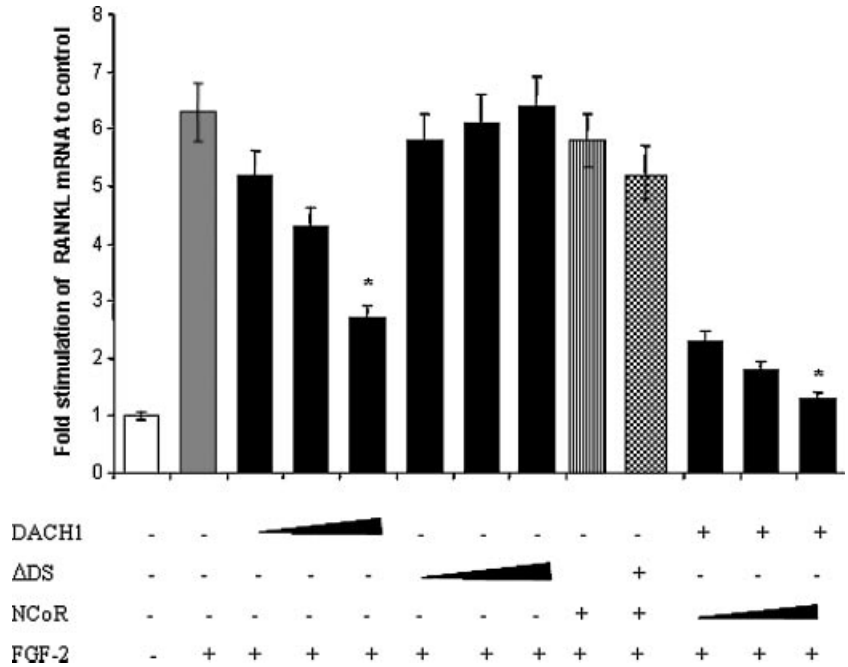

Fig. 8. DACH1 inhibition of RANKL mRNA expression in normal human bone marrow-derived stromal/preosteoblast cells. The cells were transfected with different concentrations (0.5–2 μg) of DACH1 and ΔDS expression plasmids in combination with NCoR expression construct and stimulated with and without FGF-2 (4 ng/ml) for 48 h. Total RNA was isolated and the level of RANKL mRNA expression was analyzed by real-time PCR as described in the ''Materials and Methods''.

RANKL expression in marrow-derived stromal/preosteoblast cells [Roccisana et al., 2004]. Extracellular and intracellular factors associated with several pathological conditions activate HSF-mediated gene transcription [Snoeckx et al., 2001]. FGF signaling regulates DACH1 nuclear protein expression during skeletal development [Horner et al., 2002]. We therefore investigated the role of DACH1 in FGF-2 regulation of RANKL gene expression in marrow stromal/preosteoblast cells. DACH1 repress gene expression through a conserved DS domain, which interacts with the nuclear co-repressor NCoR [Wu et al., 2003]. We hypothesized that DACH1 may negatively regulate RANKL gene expression in marrow stromal/preosteoblast cells. Herein, co-expression of DACH1 inhibited FGF-2-stimulated RANKL expression and hRANKL promoter activity. The DACH1 DS domain was required to negatively regulate RANKL gene expression and suppress FGF-2-enhanced RANKL gene expression. Co-immune precipitation-Western blot analysis identified HSF-2 binding to DACH1 and that was increased by FGF-2 stimulation. Confocal microscopy confirmed that FGF-2 signaling promoted HSF-2 nuclear transport and co-localization with DACH1 in marrow stromal/preosteoblast cells. FGF-2 signaling promotes DACH1 and HSF-2 interaction to enhance RANKL expression in marrow stromal/preosteoblast cells. Since the DS domain plays a critical role in DACH1 negative regulation of RANKL expression, we considered the potential role of nuclear co-repressor NCoR which interacts with DACH1 DS domain. siRNA suppression of NCoR and over-expression of NCoR further suggested NCoR participation in DACH1 negative regulation of RANKL gene expression in marrow stromal/preosteoblast cells.

More recently, we have shown that FGF-2 treatment did not induce RANKL expression in HSF2−/− stromal/preosteoblast cells. Furthermore, HSF-2 deficiency resulted in a rapid induction of alkaline phosphatase and osteocalcin expression in stromal/preosteoblast cells [Kajiya et al., 2006]. Therefore, it is possible that HSF-2 may have pleotropic effects on gene expression during osteoblast differentiation. FGF-2 has been shown to stimulate osteoclast formation in mouse bone marrow cultures by mechanisms that require prostaglandin synthesis [Hurley et al., 1998]. However, our

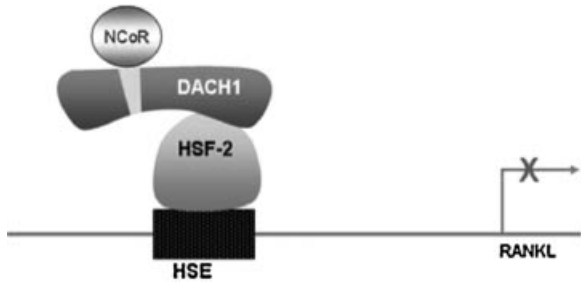

Fig. 9. DACH1 and HSF-2 modulation of hRANKL gene expression. FGF-2 signaling promotes HSF-2 binding to HSE present in the hRANKL promoter region and HSF-2 interaction with DACH1. Nuclear co-repressor NCoR binding to DS domain is essential for DACH1 inhibition of hRANKL gene expression in marrow stromal/preosteoblast cells.

results do not delineate if a prostaglandin pathway is involved in HSF-2 activation in response to FGF-2 treatment to stromal/preosteoblastic cells. Recent evidence further indicates that FGF signaling controls osteoblast differentiation through induction of Sox2 and the Wnt β-catenin pathway [Mansukhani et al., 2005]. Thus, DACH1 and HSFs may regulate maturation of osteoblastic and osteoclastic cells to remodel bone. The physical interactions between protein phosphotase-5 (PP5), HSF-1, and HSP-90 complexes diminished HSF-1 binding to DNA and transcriptional activity. Thus, PP5 is a negative modulator of HSF-1 activity [Conde et al., 2005]. Although our results implicate DACH1 and HSF-2 plays an important role in FGF-2-stimulated RANKL expression in marrow stromal/osteoblastic cells, it is possible that other osteotropic factors induce RANKL gene expression in these cells through complex regulatory mechanisms. Reactive oxygen species, for example, promote HSF-2 binding to a heat shock element (HSE) in the hRANKL promoter stimulating RANKL gene expression [Bai et al., 2005]. In addition, Sp1 and Sp3 regulate RANKL gene transcription in stromal/osteoblast cells [Liu et al., 2005].

The mouse RANKL gene 5′-flanking region contains CCAAT box, Cbfa1, and vitamin D responsive element (VDRE) motifs [Kitazawa et al., 1999; O'Brien et al., 2002]. Cbfa1 binds to the consensus motifs in the murine RANKL promoter region [Kitazawa et al., 1999]. Interestingly, mouse RANKL expression has also been shown to be upregulated by calcemic hormones such as 1,25-dihydroxyvitamin D3 and PTH; however transcriptional regulatory regions which are confined long-range (76 kb) relative to transcription site [Fu et al., 2006; Kim et al., 2006]. Transgenic mice over-expressing Cbfa1/Runx2 in cells of the osteoblastic lineage and have showed that RANKL levels were markedly increased in transgenic cells and enhanced bone resorption in vivo [Geoffroy et al., 2002]. DACH1 has been shown to co-localize with Runx2/Cbfa1, cyclin-dependent kinase inhibitors such as Kip1 and Kip2 in chondrocytes of the growth plate and in the epiphysis but not in the primary spongiosa region [Horner et al., 2002]. We have previously reported that DACH1 represses TGF-β signaling through binding to Smad4 [Wu et al., 2003]. TGF-β has been shown to inhibit RANKL mRNA expression in cultured osteoblasts [Quinn et al., 2001]. Therefore, DACH1 protein interactions may coordinate temporal and spatial expression during skeletal development and in response to osteotropic factors/inflammatory cytokine stimuli.

In summary, we show that FGF-2 signaling promotes nuclear translocation of HSF-2 and interaction with DACH1. The nuclear co-repressor NCoR is essential for DACH1 repression of RANKL gene expression in human bone marrow stromal/preosteoblast cells (Fig. 9). These data suggest DACH1 and HSF may regulate normal and pathologic bone remodeling. Novel therapeutic agents that modulate DACH1 and HSF-2 activation/interaction may have therapeutic utility against FGF-2-stimulated RANKL gene expression associated with rheumatoid arthritis and other bone diseases such as Paget's disease.

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