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RANK Ligand Expression in Heat Shock Factor-2 Deficient Mouse Bone Marrow Stromal/Preosteoblast Cells

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Abstract Heat Shock Proteins (HSP) are molecular chaperones activated upon cellular stress/stimuli. HSP gene expression is regulated by Heat Shock Factors (HSF). We have recently demonstrated a functional role for heat shock factor-2 (HSF-2) in fibroblast growth factor-2 (FGF-2)-induced RANK ligand (RANKL), a critical osteoclastogenic factor expression on stromal/preosteoblast cells. In the present study, we show that FGF-2 treatment did not induce RANKL expression in HSF-2 −/− stromal/preosteoblast cells. Interestingly, HSF-2 deficiency resulted in rapid induction of alkaline phosphatase (ALP) activity and osteocalcin mRNA expression in these cells. Furthermore, FGF-2 did not induce osteoclast formation in co-culture of normal mouse spleen cells and HSF-2 −/− stromal/preosteoblast cells. Electron microscopy analysis demonstrated that osteoclasts from HSF-2 −/− mice have poorly developed ruffled borders. These data further confirm that HSF-2 plays an important role in FGF-2-induced RANKL expression in stromal/preosteoblast cells. HSF-2 deficiency has pleotropic effects on gene expression during osteoblast differentiation and osteoclastogenesis in the bone microenvironment. Novel therapeutic agents that modulate HSF-2 activation may have therapeutic utility against increased levels of FGF-2 and bone destruction associated with pathologic conditions. J. Cell. Biochem. 97: 1362–1369, 2006.

Key words: RANK ligand (RANKL); alkaline phosphatase (ALP); heat shock transcription factor-2 (HSF-2); osteoclast; stromal/preosteoblast cells

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., 2001; Snoeckx et al., 2001]. Recently it has been reported that physical interactions between protein phosphotase-5 (PP5), HSF-1, and HSP-90 complexes diminished HSF-1 binding to DNA and transcriptional activity. This indicated PP5 as a negative modulator of HSF-1 activity [Conde et al., 2005]. Heat Shock Factor-2 (HSF-2) has been shown to be a transcriptional regulator of HSP gene expression during differentiation and development of eukaryotic cells in a tissue dependent manner [Schuetz et al., 1991; Goodson et al., 1995].

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Furthermore, strong correlation between expression of HSPs and CXC chemokines in vivo and in vitro model systems suggests that putative HSEs present in the CXC chemokine genes are functionally active [Nagarsekar et al., 2005].

The osteoclast is a primary bone resorbing cell; RANK ligand (RANKL) is a critical osteoclastogenic factor that is expressed on marrow stromal/preosteoblastic cells. Most osteotropic factors including 1,25-dihydroxy vitamin D3 ($1,25(OH)_2D_3$), IL-1, IL-11, and parathyroid hormone (PTH), and PGE2 induce RANKL expression on marrow stromal/preosteoblastic cells [Boyle et al., 2003; Teitelbaum and Ross, 2003]. In contrast, mechanical strain inhibits RANKL expression in osteoprogenitor stromal cells [Rubin et al., 2003]. Furthermore, it has also been reported that RANKL expression is upregulated in pathologic conditions such as Paget's disease of bone, osteoclastoma, and multiple myeloma resulting in enhanced osteoclastic activity and bone destruction [Menaa et al., 2000; Mundy, 2002]. Fibroblast growth factor-2 (FGF-2) binding to heparin sulfite proteoglycan is reported to induce RANKL expression and osteoclast maturation in rheumatoid arthritis conditions [Nakano et al., 2004]. Furthermore, non-targeted overexpression of FGF-2 results in decreased endochondral and intramembranous bone formation in transgenic mice suggesting that FGF-2 functions as a negative regulator of postnatal bone growth and remodeling [Sobue et al., 2005]. We have recently cloned the human RANKL gene promoter sequence and identified the presence of functional HSF-2 responsive HSE. We further demonstrated that HSF-2 is a downstream target of FGF-2 to induce RANKL expression in stromal/preosteoblast cells. These results suggested that HSP/HSF may play an important role in bone remodeling [Roccisana et al., 2004]. More recently, it has been shown that reactive oxygen species induce RANKL expression via extracellular signal-regulated kinases (ERKs) and promote HSF-2 binding to HRE present in the human RANKL promoter region in osteoblastic cells [Bai et al., 2005]. Therefore, in this study, we seek to determine whether HSF-2 deficiency in mice affects RANKL expression in stromal/preosteoblastic cells and test the capacity of HSF-2 −/− stromal/preosteoblast cells to support osteoclastogenesis in response to osteotropic factors.

MATERIALS AND METHODS

Western Blot Analysis of RANKL Expression

Wild type and HSF-2 −/− mice derived bone marrow stromal/preosteoblastic cells adherent to polystyrene culture dishes were collected as described previously [Roccisana et al., 2004]. To measure RANKL expression in mouse, stromal/preosteoblast cells (wild type or HSF-2 −/−) were stimulated with FGF-2 (4 ng/ml) for 48 h and total cell lysates were subjected to Western blot analysis. The cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1% triton-X100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 μM sodium vanadate, 1 mM PMSF, and 1 mg/ml aprotinin. The protein content of the samples was measured using a BioRad reagent following the manufacturer protocol (BioRad, Hercules, CA). Protein (20 μg) samples were subjected to SDS–polyacrylamide gel electrophoresis, using 12% Tris-HCl gel. The proteins were transferred from the gel onto nitrocellulose membrane for immunoblot analysis. After blocking with 5% non-fat dry milk in 150 mM NaCl, 50 mM Tris (pH 7.2), 0.05% Tween-20 (TBST) buffer, the membrane was incubated for 1 h with anti-RANKL and β-actin antibodies diluted 1:1,000 in 5% non-fat dry milk-TBST buffer. Then the blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG, diluted 1:2,500 in 5% non-fat dry milk-TBST buffer and developed using an ECL system (Amersham, Arlington Heights, IL). Band density was quantified by NIH Image software (version 1.67, NIH, Bethesda).

Alkaline Phosphatase (ALP) Activity Staining

Wild type and HSF-2 −/− mice bone marrow-derived stromal cells were cultured with an osteogenic medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid for an indicated period and stained for ALP activity (Sigma Chemical Co., St. Louis).

RT-PCR Analysis of ALP and Osteocalcin mRNA Expression

Total RNA (2 μg) isolated from stromal/preosteoblast cells using RNAzol reagent was reverse transcribed with random hexamers and AMV reverse transcriptase. The resulting cDNA products were subjected to PCR analysis using gene specific primers for osteoblastic

lineage markers, ALP, and osteocalcin mRNA expression as described [Roccisana et al., 2004]. The PCR mixture contained 0.15 μM concentrations of sense and anti-sense primers, 2 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3) in a 100 μl volume. The PCR amplification was performed by incubating the samples at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, and 60°C for 1 min, with a final extension for 5 min at 60°C. The amplified products were electrophoresed on a 1.2% agarose gel with a DNA size marker. The bands were visualized by ethidium bromide staining.

HSF-2 −/− Preosteoblast and Wild-Type Spleen Cell Co-Culture Assay

Normal mouse spleen cells and wild type or HSF-2 −/− stromal/preosteoblast cells were co-cultured with FGF-2 (4 ng/ml) or $1,25(OH)_2D_3$ (10^{-8} M) in the presence or absence of dexamethasone (DEX, 10^{-7} M) for 10 days. Spleens from 2-month-old mice were macerated with a sterile needle. Disaggregated cells were collected by allowing the splenic connective tissue to settle in a sterile plastic tube and washed three times with phosphate buffered saline by centrifugation. Wild type and HSF-2 −/− mice derived bone marrow stromal/preosteoblastic cells adherent to polystyrene culture dishes were collected as described previously [Roccisana et al., 2004]. The spleen cells (2×10^6 cells/well of a 24-well culture plate) were co-cultured with wild or HSF-2 −/− mouse stromal cells (2×10^4 cell/well) for 10 days. At the end of the culture period, osteoclast-like multinucleated cells formed were identified by positive staining for tartrate-resistant acid phosphatase (TRAP) activity.

Electron Microscopic Analysis of the Osteoclast Phenotype

Wild type and HSF-2 −/− mouse (C57BL/6J; 8 weeks old) tibiae were fixed in situ for 60 min at 4°C in 4% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer. Following decalcification in a 10% ethylenediaminetetra acetic acid disodium salt (EDTA-2Na) solution, the tissue blocks were postfixed in 1% OsO_4 reduced with 1.5% potassium ferrocyanide, dehydrated in an ascending series of ethanol, and finally embedded in Epon 812 (Taab, Berkshire, UK). Ultrathin sections (70 nm thickness) were double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope [Reddy et al., 2001].

RESULTS

RANKL Expression in HSF-2 −/− Preosteoblast Cells

RANKL is a critical osteoclastogenic factor expressed in stromal/preosteoblast cells in response to several osteotropic factors [Roodman, 1999]. We have recently identified that HSF-2 plays an important role in FGF-2-induced RANKL expression in stromal/preosteoblast cells [Roccisana et al., 2004]. Therefore, we further examined HSF-2 null mouse bone marrow-derived stromal/preosteoblast cells for RANKL expression in response to FGF-2 stimulation. Western blot analysis demonstrated that FGF-2 (4 ng/ml) treatment (48 h) did not induce RANKL expression in HSF-2 −/− stromal/preosteoblast cells. In contrast, FGF-2-induced a 3.8-fold increase in RANKL expression in cells derived from the wild-type mouse. In addition, the basal level of RANKL expression was decreased threefold in HSF-2 −/− stromal/preosteoblast cells compared to wild-type littermates. However, $1,25(OH)_2D_3$ and DEX treatment induced RANKL expression in both HSF-2 −/− and wild-type mouse-derived cells (Fig. 1). These data further confirm our previous results which show that HSF-2 plays an important role in FGF-2 stimulation of RANKL expression in stromal/preosteoblast cells [Roccisana et al., 2004].

ALP Expression

Evidence indicates that HSPs and HSF have pleotropic effects on gene expression in mammalian cells. For example, HSF-1 has been shown to repress TNF-α gene transcription through redundant mechanisms in murine macrophage cells [Singh et al., 2002]. We examined the ALP gene expression in HSF-2 −/− stromal/preosteoblastic cells. HSF-2 −/− stromal/preosteoblastic cells cultured with osteogenic medium showed rapid induction of ALP activity compared to wild-type mouse derived cells, suggesting HSF-2 is an important regulator of osteoblast differentiation (Fig. 2A).

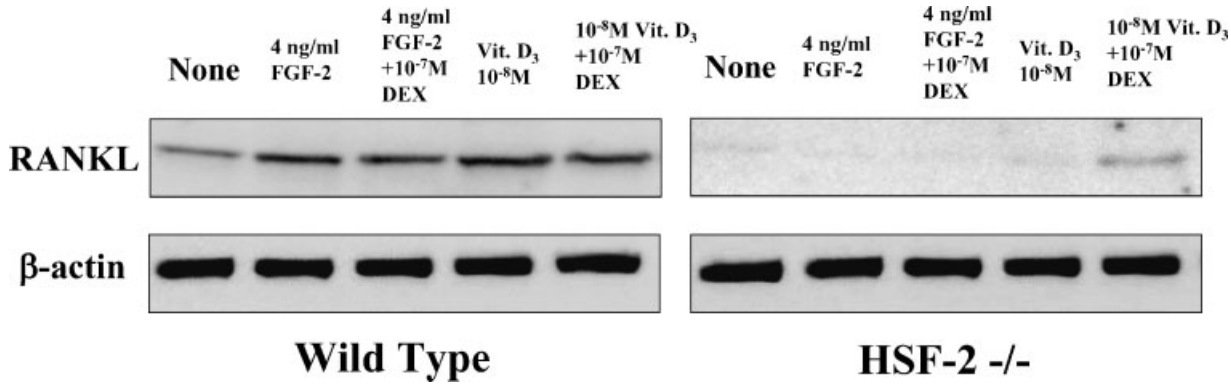

Fig. 1. RANKL expression in wild type and HSF-2 −/− mouse derived stromal/ preosteoblast cells.

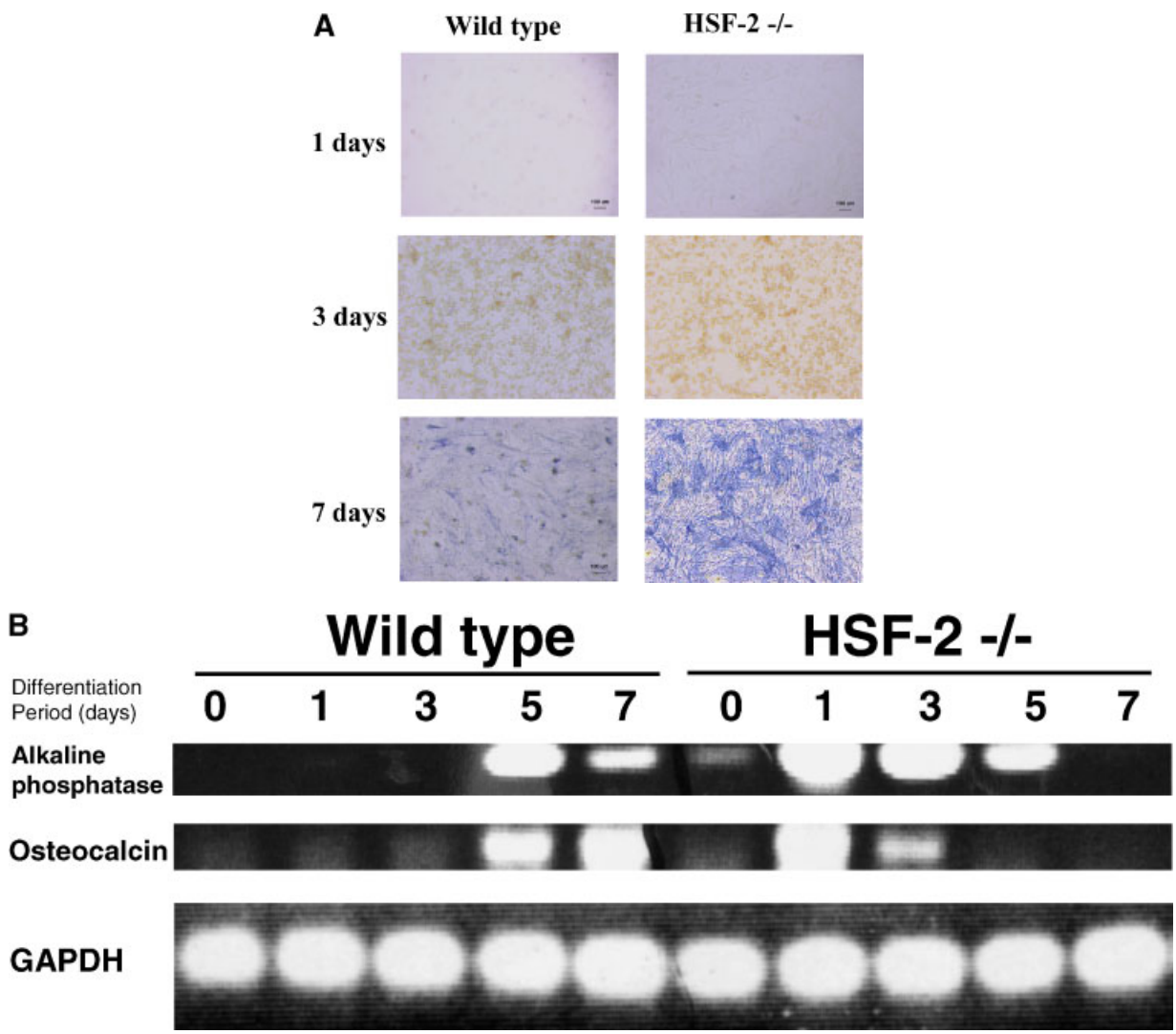

Fig. 2. HSF-2 deficiency modulates gene expression in stromal/ preosteoblast cells. **A**: Alkaline phosphatase (ALP) activity staining of wild type and HSF-2 −/− stromal/preosteoblastic cells. HSF-2 −/− cells showed rapid induction of ALP activity in response to osteogenic medium compared to wild-type mouse derived stromal/preosteoblast cells. **B**: RT-PCR analysis of ALP and osteocalcin mRNA expression during osteoblastic differentiation of wild type and HSF-2 −/− stromal/preosteoblastic cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RT-PCR analysis for ALP and osteocalcin mRNA expression in wild type and HSF-2 −/− stromal/preosteoblastic cells further indicated a rapid induction of ALP and osteocalcin mRNA expression during osteoblastic differentiation of HSF-2 −/− cells compared to wild type (Fig. 2B). Therefore, it is also possible that HSF-2 may have pleotropic effects on gene expression associated with osteoblast differentiation.

HSF-2 −/− Stromal/Preosteoblastic Cells Support Osteoclastogenesis

Recent studies with HSF-2 gene knockout mice indicated that it is dispensable for normal development, fertility, and postnatal psychomotor function [McMillan et al., 2002]. However, our data, which indicate that HSF-2 is a downstream target of FGF-2 to induce RANKL expression in stromal/preosteoblast cells, suggest that HSF-2 deficiency may affect osteoclast development [Roccisana et al., 2004]. We therefore, examined the ability of HSF-2 −/− stromal/preosteoblast cells to support osteoclastogenesis in vitro. Wild-type mouse-derived spleen cells co-cultured with HSF-2 −/− mouse stromal/preosteoblast cells in the presence of DEX (10^{-7} M) and FGF-2 (4 ng/ml) did not form osteoclasts. In contrast, spleen cells co-cultured with wild-type stromal/preosteoblast cells formed significant numbers of osteoclasts (Fig. 3). However, wild-type spleen cells co-cultured with HSF-2 −/− mice stromal/preosteoblast cells in the presence of DEX and $1,25(OH)_2D_3$ did not affect osteoclast formation in these cultures. These results indicate that HSF-2 −/− cells does not support osteoclastogenesis in response to FGF-2 stimulation. However, the HSF-2 −/− stromal/preosteoblast cells are responsive to other osteotropic factors such as $1,25(OH)_2D_3$ in supporting osteoclastogenesis.

To further determine if HSF-2 deficiency affected osteoclast phenotype, we have performed electron microscopic analysis of the ultrastructure of osteoclast in vivo. As shown in Figure 4, electron microscopic analysis demonstrated that osteoclasts derived from HSF-2 −/− mice have underdeveloped ruffled borders compared to the wild-type littermates. However, osteoclasts derived from the HSF-2 −/− mouse appeared to be in direct contact with the bone surface. Therefore, our data further suggests that HSF-2 deficiency affects osteoclastogenesis in the bone microenvironment and thus plays an important role in bone remodeling.

DISCUSSION

Several members of the HSP gene family have been reported to exhibit differential expression during osteoblast differentiation. The differences in HSP expression are consistent with involvement in mediating a series of regulatory events functionally related to the physiologic control of cell growth and differentiation [Shakoori et al., 1992]. Therefore, the steady state levels of HSP may be associated

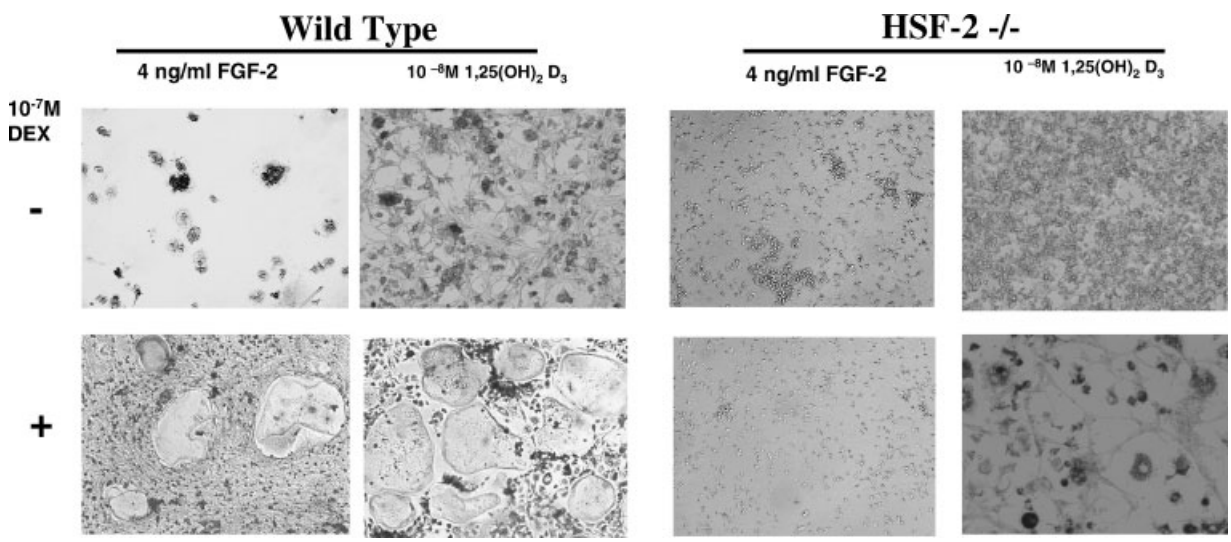

Fig. 3. Osteolast formation in HSF-2 −/− stromal/preosteoblast and wild-type spleen cell co-culture system. Osteoclast-like multinucleated cells were identified by positive staining for tartrate-resistant acid phosphatase (TRAP) activity.

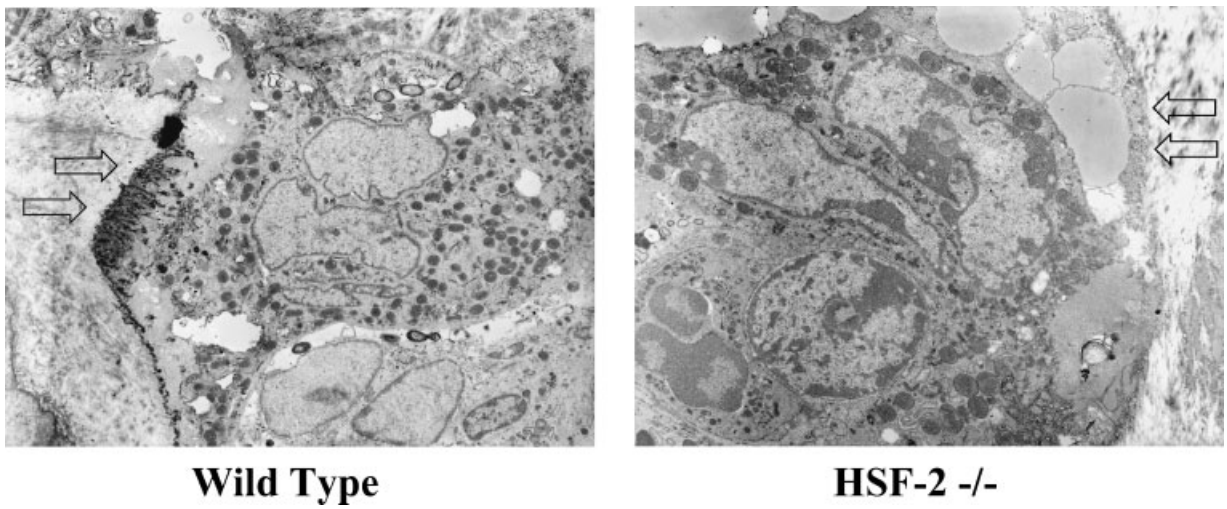

Fig. 4. Ultra-structure of the osteoclast. Electron microscopic analysis demonstrated HSF-2 −/− mice derived osteoclasts are in direct contact with bone; however ruffled borders were poorly developed compared to wild-type littermates. Data shown is representative of three individual mice analyzed. Arrows indicate ruffled border regions of the osteoclasts.

with modifications in gene expression that occur during osteoblast differentiation. These events may influence osteoblastic/osteoclastic maturation and coupling mechanisms of action required for normal bone development in response to osteotropic factors. Thus, local concentrations of osteotropic factors including FGF-2 may have significant effects on HSP and HSF activation in both physiologic and pathologic conditions that modulate RANKL gene expression in stromal/preosteoblastic cells present in the bone microenvironment. We have previously shown that addition of $1,25(OH)_2D_3$ to human bone marrow-derived stromal cells did not affect HSF-2 binding to HSE present in the RANKL gene promoter [Roccisana et al., 2004]. Although our results further confirm that HSF-2 plays an important role in FGF-2-induced RANKL expression in marrow stromal/osteoblastic cells, it is possible that several osteotropic factors induce RANKL gene expression in these cells through complex regulatory mechanisms. This is because recent evidence suggests that transcription factors such as Sp1 and Sp3 regulate the basal RANKL gene transcription in stromal/osteoblast cells [Liu et al., 2005]. It has also been shown that RANKL gene expression in marrow stromal/osteoblast cells is regulated by chromatin remodeling [Fan et al., 2004]. In contrast to RANKL expression, HSF-2 deficiency results in a rapid induction of ALP and osteocalcin expression in stromal/preosteoblast cells. These results suggest that HSF-2 deficiency 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]. Disruption of the *FGF-2* gene resulted in decreased bone mass and bone formation in mice [Montero et al., 2000]. In addition, FGF −/− mouse bone marrow cultures demonstrated impaired osteoclast formation in response to PTH further suggesting that endogenous FGF-2 is necessary for maximal osteoclast formation by multiple bone resorbing factors [Okada et al., 2003]. Recent evidence further indicates that FGF signaling could control many aspects of osteoblast differentiation through induction of Sox2 and regulation of the Wnt beta-catenin pathway [Mansukhani et al., 2005]. Therefore, it is possible that HSFs may have specific regulatory mechanisms associated with maturation of osteoblastic and osteoclastic cells modulating bone remodeling in response to osteotropic factors. FGF-2 has been shown to activate avian osteoclastic bone resorption activity via mechanisms inhibited by prostaglandin inhibitor or MAPK activation inhibitor suggesting that FGF-2 regulate bone remodeling through direct and indirect mechanisms that promote osteoclast differentiation and activation [Collin-Osdoby et al., 2002]. It also has been shown that a stress signal for HSF-2 activation occurs when the ubiquitin-proteasome pathway is inhibited

[Mathew et al., 1998]. However, our results do not delineate if prostaglandin pathway is involved in HSF-2 activation in response to FGF-2 treatment to stromal/preosteoblastic cells. Our results with co-culture experiments indicated that HSF-2 −/− cells does not support osteoclastogenesis in response to FGF-2 stimulation, however, HSF-2 −/− stromal/preosteoblast cells are responsive to other osteotropic factors such as $1,25(OH)_2D_3$ in supporting osteoclastogenesis. Evident from the electron microscopic analysis, HSF-2 −/− derived osteoclasts have underdeveloped ruffled borders, however in contact with the bone surface. It has been shown that grey-lethal mouse mutant which demonstrated osteopetrotic bone phenotype due to defective cytoskeletal reorganization and ruffled border development in osteoclasts [Rajapurohitam et al., 2001]. Therefore, it is possible that HSF-2 −/− mice may have mild osteopetrosis, however a thorough bone histomorphometric analysis is necessary to delineate spatial and temporal effects of HSF-2 expression in normal and pathologic bone remodeling. Recent evidence indicates that overexpression of FGF-2 causes defective bone mineralization and osteopenia in transgenic mice [Sobue et al., 2005]. These data are consistent with the concept that FGF-2 functions as a negative regulator of bone remodeling. Our results indicating that HSF-2 deficiency results in rapid ALP and osteocalcin induction in stromal/preosteoblast cells further support FGF-2 actions modulating osteoblast function and coupling mechanisms associated with osteoclastogenesis. However, it is unknown if HSF-2 may have any significance in FGF-2 stimulated osteoblastic proliferation. Alternatively, local concentrations of FGF-2 may have differential effects on signaling pathways associated with osteoblastic proliferation and differentiation/function.

Recent developments in the field of osteoimmunology clearly indicate the profound influence of immune cells on osteoclastogenesis and bone destruction [Takayanagi, 2005]. HSPs have been shown to prevent inflammatory damage through production of anti-inflammatory cytokines, indicating that HSPs have immunoregulatory potential [van Eden et al., 2005]. Our results suggest that HSF-2 plays an important role in FGF-2 stimulated osteoclast formation. Therefore, novel therapeutic agents that modulate HSF-2 activation may have therapeutic utility against increased levels of FGF-2 associated with rheumatoid arthritis conditions and other bone diseases such as Paget's disease. In conclusion, our data further suggests that HSF-2 deficiency modulates gene expression in stromal/preosteoblast cells and affect osteoclastogenesis in the bone microenvironment. These data implicate HSP and HSFs play an important role in normal and pathologic bone remodeling.

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