Functional Role for Heat Shock Factors in the Transcriptional Regulation of Human RANK Ligand Gene Expression in Stromal/Osteoblast Cells*

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RANK Ligand (RANKL) is a critical osteoclastogenic factor that is expressed on stromal cells and osteoblasts. Most resorption stimuli induce osteoclast formation by modulating RANKL gene expression in marrow stromal/osteoblast cells. However, it is unclear how these stimuli modulate RANKL gene expression in the bone microenvironment. To characterize the transcriptional control of human RANKL gene expression in stromal/osteoblast cells, we PCR-amplified and cloned a 2-kb 5′-flanking sequence of the RANKL gene, using normal human osteoblast derived genomic DNA as a template. Sequence analysis identified the presence of several potential Heat Shock Factor (HSF) responsive elements (HSE) in the human RANKL gene promoter region. Co-expression of HSF-1 or HSF-2 with the RANKL gene promoter-luciferase reporter plasmid in human osteoblastic cells (NOBC) demonstrated a 2-fold and 4.5-fold increase in promoter activity, respectively. RT-PCR analysis for HSF-1 and 2 mRNA expression in human bone marrow-derived stromal cells (SAKA-T) and osteoblast cells detected only HSF-2 expression. As evident from EMSA analysis, in contrast to 1,25(OH)2D3 SAKA-T cells treated with b-FGF demonstrated increased levels of HSF-2 binding to the HSE present in the RANKL gene promoter region. Immunocytochemical staining further confirmed nuclear localization of HSF-2 in both SAKA-T transformed stromal cells and human bone marrow derived primary stromal/preosteoblastic cells in response to b-FGF treatment. Furthermore, b-FGF treatment of SAKA-T cells transfected with the luciferase reporter plasmid containing the hRANKL HSE region (−2 kb to −1275 bp) upstream to a heterologous promoter showed increased levels of transactivation. Western blot analysis further demonstrated enhanced levels of RANKL expression and HSP-27 phosphorylation in SAKA-T cells treated with b-FGF. In addition, overexpression of HSF-2 in SAKA-T cells resulted in a 5-fold increase in the levels of RANKL expression in these cells. These data further suggest that HSF-2 is a downstream target of b-FGF to induce RANKL expression in stromal/osteoblast cells, and that HSF may play an important role in modulating RANKL gene expression in the bone microenvironment.

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1 The abbreviations used are: RANKL, receptor activator of nuclear factor (NF-κB) ligand; RANK(L) is a member of the tumor necrosis factor (TNF) family and is a critical osteoclastogenic factor that is expressed on bone marrow stromal/osteoblast cells (1). RANKL is also expressed on cells of the immune system including T cells, dendritic cells, and fibroblastic stromal cells (2, 3). There have been three isoforms of RANKL identified in the murine bone marrow stromal cell line ST2 and the pre-osteoblastic cell line MC3T3-E1 (4). Interestingly, overexpression of RANKL in bone marrow stromal/osteoblast cells was also reported in pathologic conditions such as Paget’s disease and Multiple Myeloma (5, 6). Several osteotropic factors/resorption stimuli including 1,25(OH)2D3, parathyroid hormone (PTH), interleukin 1β (IL-1β), interleukin-11, and prostaglandin E2 (PGE2) induce osteoclast differentiation through up-regulation of RANKL expression on marrow stromal/osteoblast cells (7, 8) but the regulation of RANKL expression appears to be complex. Hofbauer et al. (9) have previously reported that IL-1β and TNF-α but not IL-6 stimulate RANKL expression in human osteoblastic cells. In addition, b-FGF has been shown to stimulate RANKL production through COX-2 mediated prostaglandin synthesis in mouse osteoblastic cell (10). However, b-FGF has also been shown to induce RANKL expression in both human bone marrow stromal cells and vascular endothelial cells via non-prostaglandin-mediated mechanisms (11). Therefore, the downstream targets for b-FGF actions that modulate RANKL expression are unclear. Similarly, lipopolysaccharide treatment of primary murine osteoblasts results in increased levels of RANKL expression through Toll-like receptors (12). Furthermore, transforming growth factor β (TGF-β) has been shown to dramatically increase the expression of anti-CD3-induced RANKL expression in activated T cells (13). In addition, cellular stress has been implicated in osteoclastogenesis (14). However, it is unclear how these stimuli modulate RANKL gene expression in the bone microenvironment. Although multiple osteotropic factors including b-FGF are known to modulate RANKL expression, but not common regulatory mechanisms known. Even less is known about control of the human RANKL gene expression in stromal/osteoblast cells.

Recently, the mouse RANKL gene 5′-flanking regions were isolated and shown to have a TATA box, CCAAT boxes, and putative Cbfα1 and vitamin D responsive element (VDRE) motifs (15, 16). Interestingly, Cbfα1 did not regulate the murine...
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RANKL gene promoter activity, however it demonstrated binding to the consensus motifs present in the promoter region, suggesting a novel function for Cbfal in RANKL gene expression (16). More recently, Geoffroy et al. (17) have developed transgenic mice overexpressing Cbfal/Runx2 in cells of the osteoblastic lineage and showed that RANKL levels were markedly increased in transgenic cells and enhanced bone resorption in vivo. Similarly, functional VDRE were identified in the murine RANKL promoter region by transient expression of luciferase reporter gene constructs in murine ST-2 stromal osteoblastic cells (18). TGF-β has been shown to induce RANKL expression in endothelial cells by stimulating transcription factors that bind to the CRE-like domains in the murine RANKL gene promoter (19). Although immediate upstream sequences from the transcription start site (215 bp) of the mouse and human RANKL gene appear to be conserved (74% identity), there is no significant homology of sequences further upstream (15).

To determine if common regulatory elements in the hRANKL promoter region that may be involved in transcriptional control, we have cloned and sequence confirmed the hRANKL promoter region. We have further identified the presence of several HSE in the hRANKL gene promoter region. Two members of the heat shock transcription factor family, HSF-1 and HSF-2 both function as transcriptional activators of heat shock protein (HSP) gene expression (20). HSP are molecular chaperones that catalyze protein folding and modulate HSF activation (21). HSF-1 gene knockout mice have been shown to exaggerate production of the pro-inflammatory cytokine TNF-α (22). In contrast, HSF-1 has been shown to repress interleukin 1β gene transcription through physical interaction with the nuclear factor of IL-6 (23). HSF play important roles in inflammatory cytokine expression. HSF-1 has been shown to be a negative regulator of TNF-α expression in murine macrophage cells (24). It has been reported that various extracellular and intracellular factors associated with several pathological conditions resulted in activation of HSP and HSF modulating the gene transcription (21). High level expression of RANKL in the bone microenvironment play important role in osteoclastogenesis and high bone turnover. Furthermore, transgenic mice overexpressing soluble RANKL demonstrated severe osteopetrosis (25). However, the effect of osteotropic stimuli on HSP/HSF activation and the role that HSF may play in the transcriptional control of hRANKL gene expression are unknown. Therefore, we examined the functional role of HSF in the transcriptional regulation of the RANKL gene expression in stromal/osteoblast cells present in the bone microenvironment.

EXPERIMENTAL PROCEDURES

Reagents—bFGF (FGF-2) was purchased from R&D systems Inc (Minneapolis, MN). Restriction endonucleases used were obtained from New England Biolabs (Beverly, MA) and all other reagents were obtained from Sigma. HSF-1 and HSF-2 cDNA containing plasmids were a generous gift from Dr. Robert E. Kingston, Harvard Medical School, Boston.

Cloning and Identification of HSE in the hRANKL Gene Promoter—To characterize the transcriptional control of human RANKL gene expression in stromal/osteoblast cells, the RANKL gene promoter region (1 to 2 kb relative to the transcription start site) was PCR-amplified using the template genomic DNA isolated from normal human osteoblast cells and RANKL gene-specific primers, Sense: 5′-CTC CCC GGG AGG AAC TCT GTA GGA AGT TAA CAT A-3′ and antisense 5′-GCC CCC GTC GCT CCG GGC CCG GAG CCG G-3′ (Genbank™ accession no. AF 333234). Sequences underlined are the SmaI restriction enzyme site added for subcloning purposes. The PCR-amplified RANKL promoter fragment was subcloned into the PCl 2.1 TA vector, and the resulting plasmid termed TA-hRANKLP 8 was sequence analyzed using the TF data base search. The presence of potential HSE, NGAA contigs (26) in the hRANKL gene promoter region were thus identified.

hRANKL Gene Promoter Activity Assays—The human RANKL gene promoter fragment (2 kb) was excised from the TA-hRANKLP 8 plasmid by SmaI restriction enzyme digestion and subcloned into a pGL2 Basic vector at the SmaI site. The resulting plasmid was termed, hRANKLP 3 and contained the full-length RANKL promoter capable of driving luciferase reporter gene expression. RANKL promoter deletion mutants were also generated by digesting the TA-hRANKLP 8 plasmid by HindIII and EcoRV restriction enzymes. The hRANKL promoter fragment (~1 bp to ~1.27 kb) thus obtained was gel-purified and was subcloned into the pGL2 Basic vector at the SmaI enzyme site. The resulting hRANKLP promoter deletion plasmid was termed hRANKLP Δ1.2. Similarly a 0.72-kb EcoRV fragment was obtained into the pGL2 promoter plasmid at the SmaI site and the resulting plasmid termed hRANKLP 5.8 kb.

The hRANKL-luciferase reporter plasmid constructs (2 µg) were transiently transfected into stromal/osteoblast cells, SAKA-T, and the normal human osteoblast cell line (NOBC) respectively by the Lipofect-AMINE method following the manufacturer’s protocol (Invitrogen Life Technologies, Inc.). Human bone marrow-derived stromal cells, SAKA-T, and normal human bone-derived osteoblast cells (NOBC) were immortalized using Adeno-SV40 recombinant virus expressing SV40 virus large T-antigen as described previously (27, 28). SAKA-T cells were further transformed by telomerase expression enhancing the growth of these cells (29). DNA transfection in these cells was normalized by co-transfection with the pCMV β-galactosidase reporter plasmid and measuring β-galactosidase activity in the total cellular lysate. RT-PCR Analysis—Approximately 2 µg of total RNA was isolated from stromal/osteoblast cells using RNAzol reagent (Bioket, Houston, TX) (31) and reverse transcribed with random hexamers and AMV reverse transcriptase. The resulting cDNA products were subjected to PCR analysis using gene-specific primers for HSF-1: Sense, 5′-TAC AGC AGC TCC ACC CTG TAC G-3′; Antisense, 5′-TGG CCG TGG TGA GGG CTT GGA ATG TAA CAT A-3′. The PCR mixture contained 0.15 µM of sense and antisense primers, 2 units of TaqDNA polymerase (PerkinElmer Cetus, Norwalk, CT), 2 mM MgCl2, 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 123-bp DNA ladder (Invitrogen Life Technologies) as a size marker. The bands were visualized by ethidium bromide staining.

Western Blot Analysis—SAKA-T cells were seeded in 10-cm plates at a density of 10° cells in 10 µl of α-MEM media containing 10% fetal calf serum and cultured for 48 h in the presence of TF. The cells were lysed in a buffer containing 1% sodium dodecyl sulfate, 50 mM Tris, pH 7.4, NaCl 150 mM, Triton X-100, 10% glycerol, 1.5 µM MgCl2, 1 mM EGTA, 200 µM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 mg/ml). The protein content of the samples was measured using the BioRad reagent as per the manufacturer’s protocol (BioRad, Hercules, CA). Protein (20 µg) samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE), using 12% Tris-HCl gels. The proteins were transferred from SDS gels onto a NitroBind nitrocellulose membrane (Micron Separations Inc., Westboro, MA) for immunoblot analysis. Blocking was performed with 5% nonfat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween 20 (TBST) buffer. The membrane was then incubated for 1 h with the anti-RANKL, HSF-2, and anti-phospho-Santa Cruz monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:500 in 5% nonfat dry milk-TBST. The blots then were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG, diluted 1:2500 in 5% nonfat dry milk-TBST, and developed using an ECL system (Amersham Biosciences).

Electrophoretic Gel Mobility Shift Assay (EMSA)—The human RANKL gene promoter region (~170 bp relative to transcription start site) was gel-purified from a pGL2 Basic vector, and the resulting plasmid termed TA-hRANKLP 8 was sequence analyzed using the TF data base search. The presence of potential HSE, NGAA contigs (26) in the hRANKL gene promoter region were thus identified.

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native polyacrylamide gel with 10 mM Tris-glycine (pH 8.6) buffer followed by autoradiography.

Immunostaining of HSF-2—Human bone marrow derived primary stromal cells/preosteoblastic cells were isolated as described (33).

Briefly, bone marrow aspirates obtained from three normal subjects were suspended in 10 ml of α-MEM containing 20 units/ml heparin. Marrow mononuclear cells were separated by density gradient centrifugation on Histopaque 1077 (Sigma Chemical Co.) and allowed to

**Fig. 1.** Nucleotide sequence analysis of hRANKL promoter region. HSE identified were underlined in **bold**. Other putative transcription factors binding motifs with significant homology (>85%) were **underlined**. TATA box and a putative Cbfa 1 motif as identified by O’Brien et al. (15) were marked in **bold font**.
In the presence of 0.1% hydrogen peroxide.

-diaminobenzidine (Pierce) with peroxidase-conjugated bovine anti-goat IgG (1:1000 dilution) for 1% bovine serum albumin. The cells were washed and then incubated activated by 0.3% H2O2 in methanol. Blocking was performed in 1% bovine serum albumin in saline. The cells were incubated overnight at 4 °C in the presence of goat anti-human HSF-2 polyclonal antibody (Santa Cruz Biotechnology) at 1:500 dilution in phosphate-buffered saline and mRNA isolated from normal human bone marrow-derived stromal cells (SAKA-T) and Pagetic marrow-derived stromal cells (PSV10), normal human bone and Pagetic bone-derived osteoblast-like cells, NOBC and POBC, respectively. RT-PCR analysis was performed using HSF-1 and HSF-2 gene-specific primers as described under “Experimental Procedures.”

As shown in the Fig. 4, nuclear extracts derived from the control SAKA-T cells demonstrated HSF-2 binding to the hRANKL HSE (32P-radiolabeled probe) and that b-FGF treatment of these cells significantly enhanced HSF-2 binding to the HSE. In contrast 1,25(OH)2D3 (10−8 M) treatment to these cells did not significantly affect HSF-2 binding. Furthermore, addition of 100× cold HSE probe to the EMSA reaction mixture completely blocked HSF-2 binding to the HSE further confirming the specificity of DNA binding.

To further confirm the capacity of b-FGF to stimulate hRANKL promoter activity, we subcloned the hRANKL promoter region (−2 kb to −1275 bp), which contains the HSE, into the pGL2 promoter plasmid upstream to the SV40 minimal promoter driving the luciferase reporter gene expression. The resulting plasmid, hRANKLPΔ0.8 was transiently transfected into SAKA-T cells using the LipofectAMINE method and the cells cultured in the presence and absence of b-FGF. As shown in Fig. 5, total cell lysates obtained from the b-FGF-treated SAKA-T cells showed a significant increase in luciferase activity compared with untreated cells. However, b-FGF did not affect luciferase activity in cells that were transfected with the empty vector. Transfection efficiency was normalized by co-expression of pCMV β-galactosidase plasmid and measuring the β-galactosidase activity in these cells. Taken together, our data suggest that HSF-2 is the downstream target for b-FGF induction of RANKL expression in stromal cells.

b-FGF Enhances RANKL Expression and HSP-27 Phosphorylation in SAKA-T Cells—Previously it has been reported that...
small HSPs such as HSP27 are inducible and phosphorylated in response to cellular stress (21). Therefore, we have further determined the effects of b-FGF on RANKL expression, the status of HSP27 and HSF-2 in SAKA-T cells. As shown in Fig. 6, A and B, Western blot analysis of total cell lysates obtained from the SAKA-T cells treated with b-FGF (4 ng/ml) for a period of 24 h demonstrated significantly enhanced (2.5-fold) levels of RANKL expression. In contrast b-FGF treatment to these cells did not affect the levels of HSP27, but moderately (1.5–2-fold) increased HSF-2 expression. However, total cell lysates derived from the b-FGF treated cells demonstrated a significant increase (3-fold) in HSP27 phosphorylation compared with untreated SAKA-T cells. There was no significant change in the levels of HSP70 and HSP90 expression in these cells (data not shown). We have further confirmed these results in normal human bone marrow-derived primary stromal/preosteoblastic cells by immunoblot analysis. Similar to SAKA-T cells, primary stromal cells also demonstrated enhanced levels of RANKL expression in response to b-FGF treatment. Also, we have detected high levels of HSF-2 expression in these cells. Furthermore, b-FGF treatment moderately enhanced HSF-2 expression in primary stromal cells compared with SAKA-T transformed stromal cells. However, we have observed a 2.5-fold increase in HSP27 phosphorylation upon b-FGF treatment to these cells analogous to SAKA-T cells (Fig. 6C).

Nuclear Localization of HSF-2 in Human Bone Marrow-derived Primary Stromal Cells—We have performed immunocytochemical staining of human bone marrow-derived primary stromal/preosteoblastic cells and SAKA-T cells to determine the nuclear localization of HSF-2 in response to b-FGF treatment. Primary stromal/preosteoblastic cells and SAKA-T cells were stimulated with b-FGF (4 ng/ml) for a 24-h period and immunocytochemical staining was performed using anti-human HSF-2 specific polyclonal antibody as described in methods. As shown in Fig. 7A, both SV40 T-antigen-immortalized human bone marrow derived stromal cells (SAKA-T) and primary stromal/preosteoblastic cells demonstrated nuclear localization of HSF-2 in response to b-FGF treatment. In contrast, in the absence of b-FGF, these cells demonstrated cytosolic distribution of HSF-2. RT-PCR analysis for osteoblast lineage markers further demonstrated high level expression of type I collagen and low levels of osteocalcin and alkaline phosphatase mRNA expression in the human bone marrow derived primary cells used in these experiments (Fig. 7B). We have confirmed these data using marrow aspirates obtained from three different normal donors (data not shown). These data suggest that b-FGF induces activation of HSF-2 in primary stromal/preosteoblastic cells and that the transformed phenotype of SAKA-T cells is associated with increased levels of HSF-2 activity.

Fig. 5. Effect of b-FGF on hRANKL promoter activity in SAKA-T cells. hRANKLPΔ0.8 plasmid DNA (2 μg) was transiently transfected into SAKA-T cells by the LipofectAMINE method and cultured in the presence and absence of b-FGF (4 ng/ml). SAKA-T cells that were mock-transfected with the empty vector (EV) served as controls. Total cell lysates prepared after a 48-h period were assayed for luciferase activity. Transfection efficiency was normalized by measuring β-galactosidase activity co-expressed in these cells. p < 0.05.
cells did not significantly affect the associated mechanisms.

**HSF-2 Enhances RANKL Expression in SAKA-T Cells**—As shown above, HSF-2 but not HSF-1 were expressed in SAKA-T cells. Therefore, to further confirm the functional role of HSF-2 in RANKL gene expression, SAKA-T cells were stably transfected with pCMV-HSF-2 plasmid, and a clonal cell line SAKA-T/HSF-2 was established. Western blot analysis demonstrated significantly enhanced levels (5-fold) of RANKL expression in total cell lysates derived from the SAKA-T/HSF-2 cells compared with mock-transfected SAKA-T cells (Fig. 8). These data further support the role of HSF-2 in RANKL expression since constitutive overexpression of HSF-2 enhances RANKL expression in marrow stromal/osteoblast cells.

**DISCUSSION**

We have identified the presence of several HSE in the hRANKL promoter region. Our results showing HSF-2 but not HSF-1 is expressed in stromal/osteoblast cells suggest that HSF-2 is a prime target for heat shock proteins and cytokines, which affect the status of these molecular chaperones. HSPs are molecular chaperones with diverse cellular functions. HSP70 has been shown to autoregulate through negative regulation of HSF-1 transcriptional activity (34). HSP90 has also been shown to maintain steroid receptors in an inactive form in the absence of hormone (35). In the present study, b-FGF enhanced HSP27 phosphorylation suggesting that activation of HSP27 may be in part responsible for the significantly increased levels of HSF-2 binding to the hRANKL promoter in human stromal/osteoblast cells. Previously, b-FGF has been shown to stimulate HSP27 expression through protein kinase C (PKC) activation in murine osteoblast cells (36). Similarly, prostaglandin D2 increased HSP27 expression through p44/p42 MAP kinase activation and p38 MAP kinase activation in murine osteoblast-like cells (MC3T3-E1) (37). However, b-FGF treatment did not result in significant change in the levels of HSP27 in SAKA-T cells. Recently, it has been shown that telomerase overexpression in human bone marrow stromal cells modulates gene expression (38). Therefore, it is possible that SAKA-T cells, which contain telomerase (29), may have differential effects on HSP/HSF expression and activation in response to cytokine stimulation. Furthermore, the lack of a
significant effect of 1,25(OH)2D3 on hRANKL promoter suggests that vitamin D3 up-regulates RANKL expression through functional VDRE present in the RANKL gene promoter region, analogous to the mouse RANKL gene promoter (18).

Hurley et al. (39) have previously reported that b-FGF stimulates osteoclast formation in mouse bone marrow cultures by mechanisms that require prostaglandin synthesis. Furthermore, it has been shown that parathyroid hormone (PTH) modulates expression of FGF-2 and its receptor in osteoblastic cells (40). More recently, Okada et al. (41) have reported impaired osteoclast formation in bone marrow cultures of FGF-2–/– mice in response to PTH further suggesting endogenous FGF-2 is necessary for maximal osteoclast formation by multiple bone resorbing factors. Therefore, it is possible that HSFs may have a functional role to modulate RANKL expression in response to multiple osteotropic factors aside from b-FGF. Furthermore, at low concentrations (10–12 M), FGF-2 acts directly on mature osteoclasts to osteoclasts to moderately resorb bone, whereas at high concentrations (>10–9 M), it acts on osteoblastic cells to induce COX-2 and stimulates bone resorption efficiently (42). b-FGF has also been shown to induce RANKL gene expression in primary osteoblastic cells through a COX-2 mediated prostaglandin synthesis (10). Okada et al. (43) have reported that COX-2 is not necessary for normal bone development but plays a critical role in bone resorption stimulated by osteotropic factors. More recently, b-FGF has been shown to stimulate RANKL and inhibit osteoprotegerin (OPG) expression in human bone marrow stromal cells and endothelial cells via prostaglandin-independent MAPK activation (11). Therefore, our results showing that b-FGF enhanced HSF-2 binding to the HSE present in the hRANKL promoter region in SAKA-T cells further suggests that HSF-2 is a downstream target for b-FGF to enhance RANKL expression in marrow stromal/osteoblast cells.

We have noticed moderate levels in up-regulation of hRANKL promoter activity upon transient co-expression with HSF-1 or HSF-2 in NOBC cells. This is because of moderate levels (<50%) of DNA transfection efficiency obtained in these cells. However, constitutive overexpression of HSF-2 significantly enhanced RANKL expression in SAKA-T cells. Therefore, it is possible that the overexpression of HSF-2 results in autoactivation and function as transcriptional activator enhancing the RANKL expression in stromal/osteoblast cells. Previously, in addition to its role as a transcription factor, HSF-2 has been shown to interact with the protein phosphatase 2A and stimulates its activity (44). Both HSF-1 and HSF-2 have been shown to be up-regulated by estrogen and their transcriptional activity is repressed by constitutive phosphorylation (45). Furthermore, proteosome inhibition results in activation of all members of HSF (46). However, activation of HSF and DNA binding activity are known to be specific for cell type and stimuli (20).

Shakoori et al. (47) have previously identified that several members of the heat shock gene family exhibit differential expression throughout the developmental stages of rat osteoblasts. They have observed significant increase in the levels of HSP27 during osteoblast proliferation but declined upon differentiation. Our results further confirm the expression of HSP27 in human stromal/preosteoblastic cells. Therefore, the steady state levels of HSP may be associated with modifications in gene expression and cellular architecture that occur during osteoblast differentiation. The differences in HSP expression are consistent with involvement in mediating a series of regulatory events functionally related to the control of cell growth and differentiation. Enhanced levels of RANKL expression and nuclear localization of HSF-2 in b-FGF treated SAKA-T cells and primary stromal/preosteoblastic cells further suggests that the transformed phenotype of the SAKA-T cells did not significantly affect the status of HSF-2 activation in these cells. Furthermore, it has also been shown that 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 (48). Disruption of b-FGF (FGF-2) gene resulted in decreased bone mass and bone formation in mice (49). Pathological conditions such as Paget’s disease of bone demonstrated increased levels of b-FGF expression in osteoclasts compared with normal bone (50). Therefore, local concentrations of osteotropic factors including b-FGF may have significant effects on HSF and HSF activation in both physiological and pathological conditions in modulating RANKL gene expression in stromal/osteoblastic cells present in the bone microenvironment. However, b-FGF has been shown to affect multiple stages during osteoblast differentiation. It has also been reported that b-FGF promote human bone marrow stromal cells proliferation and maintenance of osteogenic precursors (51). Therefore, HSF-2+/– mice (22) would be an excellent model to further assess the role of HSF-2 in response to various osteotropic stimuli and to delineate the effects on different stages of osteoblast differentiation in modulating the RANKL gene expression and osteoclastogenesis. Taken together, our results indicate that HSF-2 is a downstream target of b-FGF to induce RANKL expression in stromal/preosteoblastic cells and that HSF may play an important role in modulating RANKL gene expression in the bone microenvironment.

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