Induction of Osteoclast Differentiation by Runx2 through Receptor Activator of Nuclear Factor-κB Ligand (RANKL) and Osteoprotegerin Regulation and Partial Rescue of Osteoclastogenesis in Runx2−/− Mice by RANKL Transgene*

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Hirayuki Enomoto,* Satoko Shojiiri,−a,b Kazuto Hoshi,c Tatsuya Furuichi,d Ryo Fukuyama,a,d Carolina A. Yoshida,a,b Naoko Kanatani,a Reiko Nakamura,a,b Atsuko Mizuno,a,f Akira Zanma,a,g Kazuki Yano,e,h Hisataka Yasuda,i Kanji Higashio,c,j Kenji Takada,b and Toshihisa Komori,b,k,l

From the aDepartment of Molecular Medicine, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, bDepartment of Orthodontics and Dentofacial Orthopedics, Osaka University Faculty of Dentistry, Suita, Osaka 565-0871, cDepartment of Orthopaedic Surgery Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, dDepartment of Pharmacology, Faculty of Pharmaceutical Science, Setsunan University, Hirakata, Osaka 573-0101, eInstitute for Biomedical Research, Teijin Ltd., Hino, Tokyo 191-8512, fResearch Institute of Life Science, Snow Brand Milk Products, Co., Ltd., Shimotsuga-gun, Tochigi 329-0512, and gJapan Science and Technology Corporation, Kauaguchi City, Saitama Prefecture 332-0012, Japan

Receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and macrophage-colony stimulating factor play essential roles in the regulation of osteoclastogenesis. Runx2-deficient (Runx2−/−) mice showed a complete lack of bone formation because of maturational arrest of osteoblasts and disturbed chondrocyte maturation. Further, osteoclasts were absent in these mice, in which OPG and macrophage-colony stimulating factor were normally expressed, but RANKL expression was severely diminished. We investigated the function of Runx2 in osteoclast differentiation. A Runx2−/− calvaria-derived cell line (CA120–4), which expressed OPG strongly but RANKL barely, severely suppressed osteoclast differentiation from normal bone marrow cells in co-cultures. Adenoviral introduction of Runx2 into CA120–4 cells induced RANKL expression, suppressed OPG expression, and restored osteoclast differentiation from normal bone marrow cells, whereas the addition of OPG abolished the osteoclast differentiation induced by Runx2. Addition of soluble RANKL (sRANKL) also restored osteoclast differentiation in co-cultures. Forced expression of sRANKL in Runx2−/− livers increased the number and size of osteoclast-like cells around calcified cartilage, although vascular invasion into the cartilage was superficial because of incomplete osteoclast differentiation. These findings indicate that Runx2 promotes osteoclast differentiation by inducing RANKL and inhibiting OPG. As the introduction of sRANKL was insufficient for osteoclast differentiation in Runx2−/− mice, however, our findings also suggest that additional factor(s) or matrix protein(s), which are induced in terminally differentiated chondrocytes or osteoblasts by Runx2, are required for osteoclastogenesis in early skeletal development.

In the process of endochondral ossification, chondrocytes mature to hypertrophic chondrocytes, matrix around terminally differentiated chondrocytes (terminal hypertrophic chondrocytes) is mineralized, blood vessels invade into the calcified cartilage, and cartilage is replaced by bone (1). Osteoclasts accelerate these processes by resorption of the calcified matrix leading to bone marrow formation. Osteoclasts differentiate from hematopoietic precursor cells through direct contact with osteoblastic/stromal cells (2). Recently, osteoprotegerin (OPG) binds osteoclastogenesis inhibitory factor, which is an inhibitor of osteoclast differentiation (3, 4), and receptor activator of NF-κB (RANK) ligand (RANKL)/tumor necrosis factor-related activation-induced cytokine/OPG ligand/osteoclast differentiation factor, which is an inducer of osteoclast differentiation (5–8), were identified. RANKL, which is expressed on the surface of osteoblastic/stromal cells or released as a soluble factor, binds to its receptor RANK (9, 10), which is expressed on the surface of osteoclast precursors and osteoclasts, and induces osteoclast differentiation and activation. OPG, which binds RANKL with higher affinity than RANK, acts as a decoy receptor for RANKL and inhibits osteoclast differentiation and activation. Further, macrophage-colony stimulating factor (M-CSF), which is secreted by osteoblastic/stromal cells, is also required for osteoclast differentiation and activation (11–13), and the presence of M-CSF and RANKL was shown to be sufficient for osteoclast differentiation from spleen cells in vitro (8). RANK, RANKL, OPG, and M-CSF are key regulators in osteoclast development, bone formation, and bone remodeling (14–18).

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†The abbreviations used are: OPG, osteoprotegerin; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; sRANKL, soluble RANKL; EGFP, enhanced green fluorescence protein; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage-colony stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tg, transgenic; E, embryonic day.
Runx2 (runt-related transcription factor 2/Cbfa1) is a transcription factor that belongs to the runt domain gene family (19) and functions by forming a heterodimer with Cbfβ (core binding factor β) (20–22). Runx2−/− mice completely lack bone formation because of the maturational arrest of osteoblasts, indicating that Runx2 is an essential factor for osteoblast differentiation (23, 24). In addition, chondrocyte maturation is also disturbed in Runx2−/− mice (25, 26), and Runx2 has been shown to be an important factor for chondrocyte maturation (27–29). Although chondrocytes had matured, and the chondrocytes were visualized in restricted parts of the skeleton of Runx2−/− mice, osteoclasts were completely absent, and no vascular invasion into the calcified cartilage occurs (23). Therefore, Runx2 plays important roles in multiple processes of endochondral ossification, including chondrocyte maturation, vascular invasion into the cartilage, osteoclast differentiation, and osteoblast differentiation (30).

We have shown that Runx2−/− calvaria-derived cells have less ability to support osteoblast differentiation from normal spleen cells and, and RANKL expression is severely diminished in Runx2−/− mice, suggesting that Runx2 is involved in osteoclastogenesis through the regulation of RANKL expression in osteoblastic/stromal cells (31). However, Runx2 binding elements are also present in the promoter region of OPG, and Runx2 increased the activity of the OPG promoter, suggesting that Runx2 inhibits osteoclast differentiation and activation through OPG induction (32). Further, Runx2 failed to stimulate the transcriptional activity of the 0.7-kb 5′-flanking region of the RANKL gene (33). Therefore, the role of Runx2 in osteoclast differentiation remains to be clarified.

In the present study, we investigated the involvement of Runx2 in RANKL and OPG expression and osteoclast differentiation in vitro and in vivo. Runx2 induced RANKL expression and suppressed OPG expression in vitro, leading to the promotion of osteoclast differentiation. Further, overexpression of soluble RANKL (sRANKL) partially rescued the blockage of osteoclast differentiation in Runx2−/− mice, indicating the involvement of Runx2 in osteoclastogenesis by regulating RANKL-RANKL signaling.

MATERIALS AND METHODS

Establishment of Calvaria-derived Runx2−/− Cell Lines—Runx2−/− mice (23) were mated with p53+/+ mice (34) to generate Runx2−/−p53−/− mice. Runx2+/−p53−/− mice were generated by mating Runx2+/−p53−/− mice. Calvarial cells derived from E18.5 Runx2−/−p53−/− embryos were prepared and cultured as described previously (35). Colonies of the calvarial cells were isolated by digestion with trypsin/EDTA for 5 min at 37°C within stainless steel cloning rings. Isolated cells were then expanded and reseeded by limiting dilution. Prior to the study, all experiments were reviewed and approved by Osaka University Medical School Animal Care and Use Committee.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from cellular samples using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Aliquots of 20 μg of total RNA were separated by electrophoresis and transferred onto nylon membrane filters. A 1.5-kb fragment of mouse RANKL cDNA (8), a 1.5-kb fragment of OPG cDNA (4), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (36) were labeled with [α-32P]dCTP using a Megaprime DNA labeling kit (Amersham Biosciences). The intensities of RANKL, OPG, and GAPDH bands were quantitated by densitometry using FMBIO analysis software (Hitachi Software Engineering Co. Ltd., Tokyo, Japan).

Construction of Adenovirus—A mouse cDNA containing the entire open reading frame of Runx2 (37) was inserted into the BamHI site of pIRE52-EGFP (Clontech), and a DNA fragment containing Runx2, internal ribosome entry site, and enhanced green fluorescence protein (EGFP) was inserted into the BamHI-XhoI sites of pACCMV.pLpA shuttle vector (38). The constructed vector was co-transfected with the adenovirus cloned plasmid pJM17 (39) into human kidney 293 cells by the SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. A recombinant adenovirus was generated by homologous recombination events between the two plasmids. Amplified crude viral stocks were purified by CsCl gradient ultracentrifugation and used for the infection. The recombinant adenovirus carrying only internal ribosome entry site-EGFP was also generated and used for the control infection.

Adenoviral Introduction of Runx2 into CA120–4 Cells—CA120–4 cells were plated at a density of 1 × 105 cells/dish in collagen-coated 60-mm plates (Iwaki Glass, Chiba, Japan). On the following day, cells were infected with either EGFP-expressing (control) or Runx2- and EGFP-expressing virus. After the viral infection, cells were cultured with or without 1α,25(OH)2D3 and harvested for RNA extraction.

Osteoclast Differentiation in Vitro—CA120–4 cells were inoculated in 24-well plates at a density of 2 × 103 cells/well. Twenty-four h after plating, cells were infected by either EGFP-expressing (control) or Runx2- and EGFP-expressing virus for 2 h. Thereafter, cells were rinsed twice with phosphate-buffered saline to eliminate adenovirus and subsequently cultured with bone marrow cells (2 × 105 cells/well) in α-minimum Eagle’s medium (0.5 ml/well) containing 10% fetal calf serum and 10−8 M 1α,25(OH)2D3. Cultures were fed every 3 days by replacing 0.4 ml of old medium with fresh medium (40). Recombinant human OPG and sRANKL proteins (R&D Systems, Minneapolis, MN) were administrated at a final concentration of 100 and 30 ng/ml, respectively. On the seventh day of the co-culture, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline, treated with ethanol-acetone (50:50), and stained for tartrate-resistant acid phosphatase (TRAP) using the TRAP kit (Sigma–Aldrich) according to the manufacturer’s protocol.

Western Blot Analysis—Equal amounts of proteins (20 μg) were separated on a 10% gel by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking the membrane with 5% skimmed milk, the membrane was incubated with a mouse monoclonal antibody against Runx2 (41) at a 1:500 dilution and then with peroxidase-conjugated goat anti-mouse IgG antibody. Bound antibody was visualized by an ECL detection system (Amersham Biosciences).

Generation of Runx2−/−tg Mice—sRANKL transgenic mice were generated using the hSAP (human serum amyloid P component) gene promoter (42) and a sRANKL DNA fragment containing immunoglobulin κ-chain leader sequence and the extracellular domain sequence of RANKL (murine RANKL residue 71–316) (43). The sRANKL transgenic mice were mated with Runx2−/− mice, Runx2−/−p53−/− mice, Runx2+/−sRANKL transgenic mice (Runx2+/−tg mice) were generated by mating Runx2−/−sRANKL transgenic mice with Runx2−/−p53−/− mice. Genotypes were determined by Southern blot analysis for Runx2 and PCR analysis for sRANKL, as described previously (23, 43). Serum sRANKL level was measured by enzyme-linked immunosorbent assay using a rabbit polyclonal antibody raised against mouse RANKL (44).

Histological Examination—Tissue preparation was performed as described previously (45). Briefly, E10.5 embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for paraffin sections, and a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.067 M (pH 7.4) cacodylate buffer for epoxyresin sections. Decalcified 3–5-μm paraffin sections of tibiae were used for hematoxylin and eosin staining or TRAP activity. For histomorphometric analysis, bone volumes were measured at the diaphyses of tibiae (from 345 μm below the proximal growth plates to 460 μm beyond the distal growth plates), using a semiautomated system (Bone Histomorphometric System; System Supply, Nagano, Japan). Nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (46). For electron-microscope examination, following postfixation with 1% OsO4 in 0.1 M cacodylate buffer (pH 7.4), the tibiae were embedded in PolyBed 812 resin and sliced into 60–80-nm sections. These sections were observed under a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) after staining with tannic acid, uranylacetate, and lead citrate.

RESULTS

Establishment of Runx2−/− Cell Line, CA120–4—We showed previously (35) that Runx2−/− calvarial cells are able to differentiate into adipocytes spontaneously and into chondrocytes with BMP-2. We isolated 93 clones from the calvarial cells of Runx2−/−p53−/− mice. Each cell line had its own characteristics with regard to capacity to differentiate into either chondrocytes or adipocytes. In all of the Runx2−/− cell lines, RANKL...
expression was barely detectable, but OPG expression was clearly detected by Northern blot analysis, and one of the cell lines (CA120–4) was used for the following experiments (Fig. IA) (data not shown). This cell line lacked the ability to differentiate into chondrocytes or adipocytes, but alkaline phosphatase activity was induced by BMP-2 treatment (data not shown). Consistent with the expression levels of RANKL and OPG in CA120–4 cells, osteoclast differentiation was severely inhibited in the co-culture of CA120–4 cells and normal bone marrow cells (Fig. 1B).

Runx2 Induces RANKL Expression and Promotes Osteoclast Differentiation—To clarify the role of Runx2 in the regulation of RANKL expression and osteoclastogenesis, we introduced the Runx2 gene into CA120–4 cells by adenoviral gene transfer. We first confirmed that the infected cells produced Runx2 protein by immunoblotting. As shown in Fig 2A, a band of about 57 kDa detected in the Runx2 virus-infected cells corresponded to that in endogenous Runx2 in ATDC5 cells, which were used as a positive control.

Adenoviral introduction of the Runx2 gene clearly induced RANKL expression in the presence of 1α,25(OH)₂D₃, and the induction was already apparent at 24 h after infection and continued for at least 6 days, whereas the induction was never observed in the control virus-infected cultures even in the presence of 1α,25(OH)₂D₃ (Fig. 2, B and C). In the absence of 1α,25(OH)₂D₃, however, Runx2 failed to induce RANKL expression. 1α,25(OH)₂D₃ suppressed OPG expression as described previously (31), but the introduction of Runx2 gene suppressed OPG expression at 24 h after infection without 1α,25(OH)₂D₃ (Fig. 2, B and D). The 1α,25(OH)₂D₃-independent suppression of OPG by Runx2 was not apparent at 3 days after infection, but Runx2 and 1α,25(OH)₂D₃ synergistically reduced OPG expression for at least 6 days. Although the presence of CA120–4 cells completely suppressed osteoclast differentiation of normal bone marrow cells (Fig. 1B), the introduction of Runx2 into CA120–4 cells restored the formation of TRAP-positive osteoclast-like cells from the bone marrow cells (Fig. 3, B and F). However, the addition of OPG abolished...
In the present study, we have shown for the first time that Runx2 induces RANKL expression and suppresses OPG expression, leading to the induction of TRAP-positive osteoclast-like cells by the introduction of Runx2 (Fig. 3, C and F). These data indicate that Runx2 induces osteoclast differentiation through RANK-RANKL signaling.

To confirm the functional involvement of RANKL in this co-culture system, we administered sRANKL and examined the effects on the formation of TRAP-positive osteoclast-like cells (Fig. 3, D and E). In the presence of CA120–4 cells, sRANKL also restored the formation of TRAP-positive osteoclast-like cells from normal bone marrow cells (Fig. 3E), indicating that RANKL can overcome the inhibitory effect of CA120–4 cells on osteoclast differentiation. Thus, these findings indicate that Runx2 promotes osteoclast differentiation by inducing RANKL expression and inhibiting OPG expression.

Generation of Runx2–/– Mice with sRANKL Transgene—Our in vitro studies indicated that RANKL was a downstream gene of Runx2 and RANKL mediated osteoclast differentiation in the absence of Runx2. Thus, we next asked whether RANKL could induce osteoclast differentiation in Runx2–/– mice.

Overexpression of sRANKL under the control of the chicken β-actin promoter and cytomegalovirus immediate early enhancer, which direct the expression from an early developmental stage, resulted in mortality at the perinatal stage (43). Therefore, we generated another transgenic mouse, which overexpressed sRANKL under the control of the hSAP promoter (42). The transgene under the control of this promoter was expressed in the liver, and its expression increased at the post-natal stage (47), resulting in an osteoporoic phenotype in adulthood (43). The sRANKL transgenic mice were mated with Runx2−/− mice, and then Runx2−/−tg mice were generated. Because the activity of the hSAP promoter at the embryonic stage was lower than in adults, we injected CCl4 into pregnant mice at embryonic day E14.5–15.5 to induce acute inflammation and activate the promoter during the embryonic stage. The treatment enhanced the expression of sRANKL in the livers of transgenic embryos but not of wild-type embryos (Fig. 4A), and the serum sRANKL levels in the transgenic embryos were equivalent to the concentrations we used for in vitro culture (Table 1). The trabecular bones of sRANKL transgenic mice were decreased, and the number of TRAP-positive cells was increased with large multinucleated cells (Fig. 4, B–G). Histomorphometric analysis using E18.5 tibias showed that the induction of sRANKL in fetal liver resulted in 40% decrease of bone mass and more than one-third increase of osteoclast surface and number (Fig. 4, H–J). These findings indicate that sRANKL expression in the embryos was enough for the promotion of osteoclast differentiation and activation.

**Forced Expression of sRANKL Promoted Osteoclast Differentiation but Failed to Induce Fully Differentiated Osteoclasts in Runx2−/− Mice—**At E18.5, a few TRAP-positive mononuclear cells were observed at the perichondrial regions of calcified cartilages, including tibia, fibula, radius, and ulna of Runx2−/− embryos, without invasion into the cartilage (Fig. 5, A and C) (45), whereas the number of TRAP-positive cells was increased in the same regions, and they were multinucleated, and superficial invasion into the calcified cartilage was observed in Runx2−/−tg embryos (Fig. 5, B and D). As the invasion of osteoclasts into the cartilage was not prominent in Runx2−/−tg embryos, the morphology of the osteoclasts was examined by transmission electron microscope images. In Runx2−/− embryo, development of a ruffled border was barely detectable on the border between osteoclasts and the cartilage matrix. Even in Runx2−/−tg embryos, the development of the osteoclast ruffled border was still poor, and intracellular polarization, including clear zone formation or mitochondrial accumulation into the basolateral cytoplasm, was difficult to ascertain (Fig. 6). These findings indicate that forced expression of sRANKL in Runx2−/− mice increased TRAP-positive cells and induced their multinucleation but failed to induce functionally and morphologically differentiated osteoclasts.

**DISCUSSION**

In the present study, we have shown for the first time that Runx2 induces RANKL expression and suppresses OPG ex-
Trabecular bones are decreased (Bars mice. sRANKL expression in fetal livers. CCl4 was injected into pregnant development.

terminally differentiated chondrocytes or osteoblasts by additional factor(s) or matrix protein(s), which are induced in with a previous report (31) that OPG was detected, but RANKL internal control. sRANKL is strongly expressed in the CCl4-treated regions in hematoxylin and eosin (D) transgenic fetal livers. B transgenic and wild-type (H9251 mice with embryos at E14.5. Total RNA was extracted from livers of B–G, histological analysis of sRANKL transgenic fevers at E18.5. Sections from wild-type (B, D, and F) and sRANKL transgenic (C, E, and G) fevers at E18.5 were stained with hematoxylin and eosin (B and C) or TRAP and methyl green (D–G). The boxed regions in D and E are magnified in F and G, respectively. Trabecular bones are decreased (C), and large multinucleated TRAP-positive osteoclasts are increased (B and G) in sRANKL transgenic mice. Bars, 10 μm (B–E) and 4 μm (F and G). H–I, trabecular bone volume (BV/TV; bone volume over tissue volume) (H), osteoclast surface (Oc.S; I), and osteoclast number (N.Oc; J) are compared between wild-type (white bars) and sRANKL transgenic (black bars) tibiae at E18.5. Data represent mean ± S.E. of four wild-type and six RANKL transgenic mice. **p = 0.0001 and *, p < 0.05 as determined by one-way analysis of variance.

pression and that forced expression of sRANKL in Runx2−/− mice induced multinuclear osteoclast-like cells, indicating that Runx2 promotes osteoclast differentiation through RANK-RANKL signaling. However, forced expression of sRANKL in Runx2−/− mice was not sufficient for the complete rescue of osteoclast differentiation in Runx2−/− mice, suggesting that additional factor(s) or matrix protein(s), which are induced in terminally differentiated chondrocytes or osteoblasts by Runx2, are necessary for osteoclastogenesis in early skeletal development.

We isolated Runx2−/− calvarial cell lines, most of which expressed OPG strongly but RANKL barely. It is consistent with a previous report (31) that OPG was detected, but RANKL was undetectable in Runx2−/− mice. Interestingly, the induction of RANKL in CA120–4 cells by Runx2 was dependent on the presence of 1α,25(OH)2D3 (Fig. 2). Neither Runx2 nor 1α,25(OH)2D3 alone could induce RANKL expression. Therefore, Runx2 is required for the induction of RANKL by 1α,25(OH)2D3, indicating a cooperative action of Runx2 and 1α,25(OH)2D3. Further, Runx2 and 1α,25(OH)2D3 synergistically reduced OPG expression. The mechanisms of their cooperation remain to be clarified.

It has been controversial whether Runx2 regulates RANKL expression. It has been shown that Runx2 does not stimulate the 0.7-kb 5′-flanking region of the RANKL gene, which contains two putative Runx2 binding sites, and neither Runx2 nor the dominant negative form of Runx2 expression has an effect on RANKL expression in a stromal/osteoblastic cell line, UAMS-32 (33). Therefore, the regulatory region for Runx2 in RANKL gene may be outside of the 0.7-kb 5′-flanking region. It is also possible that Runx2 indirectly induces RANKL expression, although Runx2 induced RANKL expression within 24 h (Fig. 2). As UAMS-32 cells express Runx2 strongly in a steady state (33), it may be difficult to induce or inhibit RANKL expression by the introduction of Runx2 or its dominant negative form. Further, Runx2 interacts with other transcription factors, transcriptional cofactors, and transcriptional repressors, and the interactions greatly influence Runx2 function (48–53). Therefore, it is possible that Runx2 regulates RANKL expression in cooperation with other factors, which also determine the level of RANKL expression. Indeed, the retinoid X receptor-vitamin D receptor complex is one of the candidates, because 1α,25(OH)2D3 was required for the induction of RANKL by Runx2 (Fig. 2). It is suggested that osteoprogenitor cells have more potential to support osteoclast development than more differentiated cells (54). It may explain a discrepancy of the RANKL expression in Runx2 transgenic mice using Runx2 isoforms with different N termini under the control of type I collagen promoter (37, 55). RANKL expression was decreased in type II Runx2 transgenic mice (37), whereas it was increased in type III Runx2 transgenic mice (55). In contrast to type II Runx2, which is a major isoform of Runx2 in osteoblastic cells, type III Runx2 has no ability to induce alkaline phosphatase (56), suggesting that type II Runx2 but not type III Runx2 has an ability to induce osteoblast differentiation at an early stage. Therefore, Runx2 seems to induce osteoclast differentiation by inducing RANKL in osteoprogenitor cells and simultaneously induce osteoblast differentiation, which results in a decrease of RANKL expression. Thus, Runx2-dependent induction of RANKL in the Runx2−/−/immature mesenchymal cell line is likely to mimic normal RANKL regulation in osteoprogenitor cells.

OPG has putative Runx2 binding elements in the promoter region, and Runx2 activated the OPG promoter (32). Further, the mutations of Runx2 and Smads binding elements in the OPG promoter region synergistically diminished OPG promoter activity induced by TGF-β (57), suggesting the involvement of Runx2 in transcriptional activation of OPG. However, our findings showed that Runx2 suppressed OPG expression. Therefore, additional Runx2 binding elements that negatively regulate transcription may be present outside of the 5.9-kb promoter region that was analyzed previously (32). Indeed, the possibility that Runx2 suppresses OPG indirectly cannot be excluded, although OPG was suppressed within 24 h after the introduction of Runx2 (Fig. 2). In the Runx2 transgenic mice

![Image](https://example.com/image.png)

**Table 1**

| Genotype | n, number of mice analyzed | sRANKL concentration (mean ± S.D.) |
|----------|--------------------------|-----------------------------------|
| sRANKLtg  | 16                       | 45.0 ± 6.9                        |
| Runx2−/−tg | 5                        | 35.3 ± 8.9                        |
| Wild-type | 8                        | ND                                |
| Runx2−/− | 4                        | ND                                |

*ng/ml.*
but not in Runx2/H11002 and morphologically differentiated osteoclasts in Runx2/H11002 and OPG, in the absence of osteoblasts. Further, the introduction of Runx2 transgene under the control of type II collagen promoter into Runx2−/− mice resulted in chondrocyte maturation and calcification of the cartilage, in which functionally active osteoclasts appeared in the absence of osteoblasts (29). Therefore, osteoblast differentiation is not absolutely required for osteoclast differentiation, but terminal differentiation of chondrocytes was a prerequisite for the appearance of differentiated osteoclasts. Thus, in addition to Runx2-dependent chondrocyte maturation, it is likely that additional factor(s) or matrix protein(s), which are induced in terminally differentiated chondrocytes by Runx2, play an important role in the processes of osteoclast differentiation at an early stage of skeletal development. The existence of these factor(s) or matrix protein(s) for osteoclast differentiation will also be the case in bone, and it may explain why osteoclasts are located specifically in calcified cartilage and bone, despite the relatively wide distribution of RANKL and M-CSF (58–60). In agreement with this idea, the appearance of TRAP-positive cells was restricted to the calcified cartilage in the skeletons of Runx2−/− mice, resulting in chondrocyte maturation and calcification of the cartilage, in which functionally active osteoclasts appeared in the absence of osteoblasts (29). Therefore, osteoblast differentiation is not absolutely required for osteoclast differentiation, but terminal differentiation of chondrocytes was a prerequisite for the appearance of differentiated osteoclasts. Thus, in addition to Runx2-dependent chondrocyte maturation, it is likely that additional factor(s) or matrix protein(s), which are induced in terminally differentiated chondrocytes by Runx2, play an important role in the processes of osteoclast differentiation at an early stage of skeletal development. The existence of these factor(s) or matrix protein(s) for osteoclast differentiation will also be the case in bone, and it may explain why osteoclasts are located specifically in calcified cartilage and bone, despite the relatively wide distribution of RANKL and M-CSF (58–60). In agreement with this idea, the appearance of TRAP-positive cells was restricted to the calcified cartilage in the skeletons of Runx2−/− mice, in which sRANKL was secreted from the livers (Fig. 5) (data not shown). However, we still need to determine the factors or extracellular matrix proteins induced by Runx2 in the calcified cartilage, which are really required for osteoclast differentiation.

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